Regulation and Functional Analysis of EphA2 in Normal and Ras-Transformed Mammary Epithelium

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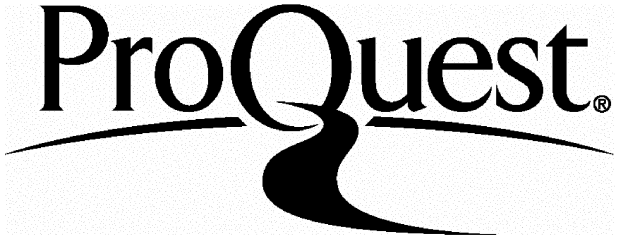

Abstract

Members of the Erythropoietin-producing hepatoma (Eph) family of receptor tyrosine kinases are dynamically expressed during embryogenesis, with roles in topographic mapping, hindbrain segmentation and neural crest cell migration thought to be mediated by repulsive/anti-adhesive cues induced by interaction with their membrane-bound ligands, known as ephrins.

Formerly known as epithelial cell kinase (eck), EphA2 is expressed primarily in cells of epithelial origin in the adult, including the mammary gland. Results presented in this thesis demonstrate that elevated steady-state levels of catalytically active EphA2 protein were characteristic of mammary tumours arising in WAP*ras* transgenic mice. These mice express v-Ha-Ras under the control of the mammary-specific whey acidic protein (WAP) promoter. Furthermore, a mouse mammary epithelial cell line expressing v-Ha-Ras also displayed elevated levels of EphA2 mRNA and protein. This implies a potential role for EphA2 in tumourigenesis, and provided a highly relevant *in vitro* cell culture model in which further investigation revealed downregulation of EphA2 in response to mitogen-activated protein kinase (MAPK) pathway inhibition.

Analysis of mice bearing an insertional mutation at the EphA2 locus crossed with WAP*ras* transgenic mice revealed continued upregulation of EphA2 protein in mammary tumours, in contrast to the significant deficiency in EphA2 protein in other adult tissues of these double mutant mice. This suggests a potentially complex mechanism for the transcriptional regulation of EphA2.

Overexpression of recombinant EphA2 in a non-transformed mammary epithelial cell line resulted in a 'rounded' cellular morphology, implicating EphA2 in the regulation of cell-extracellular matrix interactions. Furthermore, recombinant ephrinA1, cloned and expressed as a source of cognate ligand for EphA2, also influenced the cytoskeleton.

Finally, receptor activation by ephrinA1 induced transient inhibition of MAPK activity, suggesting the presence of an EphA2-MAPK regulatory feedback loop, which may be overcome by oncogenic Ras to upregulate EphA2 and thus confer anti-adhesive properties on tumourigenic cells.

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Abbreviations

AER	apical ectodermal ridge
AF-6	afadin
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5' monophosphate
cDNA	complementary DNA
cfu	colony forming unit
CREB	cAMP response element binding protein
CSF	colony-stimulating factor
DAPI	4, 6-diamidino-2-phenylindole
DEAE	diethylaminoethyl ether
DEPC	diethyl pyrocarbonate
DH	Dbl-homology
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpc	days post coitum
DTT	dithiothreitol
Eck	epithelial cell kinase
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis(β-aminoethylether)-N, N, N, N'-tetraacetic acid
eIF-4	eukaryotic translation initiation factor-4E
EMF	embryonic fibroblast
Eph	erythropoietin-producing hepatoma
ERK	extracellular signal-regulated kinase
ES	embryonic stem
FAK	focal adhesion kinase
Fc	fragment that crystallizes
FCS	foetal calf serum

FGF	fibroblast growth factor
FLAG™	refers to the amino acid sequence DYKDDDDK
G418	geneticin
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
gpt	guanine phosphoribosyltransferase
Grb2	growth factor receptor-bound protein 2
Grb10	growth factor receptor-bound protein 10
GRIP	glutamate receptor-interacting protein
GSK	glycogen synthase kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HGF/SF	hepatocyte growth factor/scatter factor
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin
IL	interleukin
JNK	c-Jun N-terminal kinase
kb	kilobase
K_d	dissociation constant
kDa	kiloDalton
LB	Luria Bertani
LMW-PTP	low molecular weight protein tyrosine phosphatase
LOH	loss of heterozygosity
LTR	long terminal repeat
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MAX	mycophenolic acid, adenosine, xanthine
MBP	myelin basic protein
MEK	MAPK/ERK kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
MMP	matrix-metalloproteinase

MMTV	mouse mammary tumour virus
MNK	MAPK signal-integrating kinase
mRNA	messenger RNA
MSK	mitogen and stress-activated protein kinase
NF	Neurofibromin
OD	optical density
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDZ	postsynaptic density protein, discs large, zona occludens
PH	pleckstrin homology
PI3K	phosphoinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulphonylfluoride
PTB	phosphotyrosine binding
PtdIns	phosphatidylinositol
PtdIns(3)P	phosphatidylinositol-3-phosphate
PtdIns(3,4)P_2	phosphatidylinositol-3,4-bisphosphate
PtdIns(3,5)P_2	phosphatidylinositol-3,5-bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol-3,4,5-bisphosphate
PTK	protein tyrosine kinase
PY	phosphotyrosine
RalGDS	Ral guanine nucleotide dissociation stimulator
RBD	Ras binding domain
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rpm	revolutions per minute
Rsk	Ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR

RXR	retinoid X receptor
SAM	sterile alpha motif
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology and collagen homology protein
SLAP	src-like adaptor protein
Sos	Son of sevenless
SRE	serum response element
SV40	Simian virus 40
TTBS	Tris-buffered saline containing 0.1% Tween 20
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
WAP	whey acidic protein
WASP	Wiscott-Aldrich syndrome protein
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranosidase

Chapter 1

Introduction

The phenotype of a cell is influenced by its ability to respond to external stimuli. Although the environment of cells within tissues of multicellular organisms is more stable than that of a unicellular organism, these cells must nevertheless co-ordinate their behaviour by means of inter- and intra-cellular signalling. Membrane-spanning receptor tyrosine kinases have evolved as allosteric enzymes that are able to perceive a diverse array of extracellular signals and transduce them into the cell. Signal transduction by tyrosine kinases is achieved by means of the rapid addition of a phosphate moiety from the adenosine triphosphate (ATP) donor onto a tyrosine residue in the substrate. Such phosphorylation can modify target proteins, resulting in the rapid relay of a positive or negative signal, which ultimately evokes a cellular response. However, the extent of the response can be influenced by, among other things, the activity of protein tyrosine phosphatases, thus the reversible process of protein phosphorylation acts as a simple, yet effective, regulatable means of transduction.

Over the last ten years, Eph receptors have emerged as the largest group of receptor tyrosine kinases. There are currently 14 Eph receptors, which are subdivided into EphA and EphB receptors, depending on whether they interact with ephrinA or ephrinB ligands. Like their receptors, ephrins are generally present as membrane-bound proteins. This is achieved either through a glycosylphosphatidylinositol (GPI) linkage in the case of ephrinA ligands, or by the presence of a transmembrane domain, as is true of ephrinBs. Interactions between ephrins and Eph receptors therefore predominantly require cell-cell contact. Ephrins are also unusual in being able to mediate "reverse" signalling, such that signals can be transduced within the ephrin-expressing cell upon the engagement of an Eph receptor extracellular domain. Roles for Eph and ephrin signalling in mediating cell positioning have been proposed.

1.1. Eph Receptors and Ephrins

1.1.1. Introduction to Eph Receptors

The Eph family of tyrosine kinase receptors takes its name from the first member, Eph, which was cloned from an erythropoietin-producing hepatoma cell line following a

search for sequences homologous to the v-*fps* viral oncogene tyrosine kinase domain (Hirai *et al.*, 1987). Thirteen Eph receptors have been identified subsequently using polymerase chain reactions (PCR) with oligonucleotide primers designed to anneal to regions of high homology in kinase domains (Wilks, 1989) (e.g. EphA4 (Lai and Lemke, 1991)), by screening expression libraries with antibodies that recognise tyrosine phosphorylated proteins (e.g. EphB2 (Pasquale, 1991)) and by the use of probes/primers to identify clones with homology to previously identified Eph receptors (e.g. EphB1 (Sajjadi and Pasquale, 1993)). In 1997, subsequent to the discovery of a multitude of independently named Eph homologues and orthologues, the Eph Nomenclature Committee established a uniform nomenclature for Eph receptors and ephrins, thus Eph, as the founder member, was named EphA1 (Eph Nomenclature Committee, 1997). Different species can be distinguished on the basis of a small-case letter prefix, thus 'h' represents human, 'm' is used for mouse, 'r' for rat, 'c' for chicken, etc. Please refer to Appendix 2 for details of previous names.

1.1.1.1. The Discovery of EphA2

The discovery of human EphA2 (hEphA2) cDNA was first reported in 1990 by Lindberg and Hunter (Lindberg and Hunter, 1990) using a degenerate oligonucleotide probe designed to anneal to the HRDLAAR amino acid sequence, which is highly conserved within the catalytic domain of protein tyrosine kinases (PTKs). Two clones with PTK homology were identified following screening of a HeLa cell cDNA library, one of which was used to re-screen the library, resulting in the isolation of a 3555 bp sequence corresponding to the 3' end of hEphA2. The remaining 5' end of hEphA2 was cloned from a human keratinocyte library using sequence from the 5' end of the HeLa sequence as a probe.

Subsequently, mouse EphA2 has been cloned independently by four different groups, either by a PCR approach using degenerate oligonucleotides corresponding to highly conserved sequences in the catalytic domain of kinases (Becker *et al.*, 1994; Ganju *et al.*, 1994; Ruiz and Robertson, 1994) or by screening using a degenerate oligonucleotide probe corresponding to the conserved amino acid sequence in subdomains VI and VII of the Eph receptor kinase domain (Lai and Lemke, 1991; Mori *et al.*, 1995).

1.1.1.2. The Protein Structure of the Eph Receptor Family

As receptor tyrosine kinases (RTKs), Eph receptors possess an extracellular, a transmembrane and an intracellular domain (Figure 1.1). Comparison of the amino acid sequences of all Eph members shows that the receptors share a number of structural features and sequence motifs. In the extracellular domain this includes an amino-terminal ligand-binding domain, a cysteine-rich region and two fibronectin type III repeats. In the intracellular domain there is a highly conserved juxtamembrane motif and an uninterrupted catalytic domain, in addition to a PDZ (postsynaptic density protein, discs large, zona occludens) binding sequence and a SAM (sterile alpha motif), which are present at the carboxy terminus.

1.1.1.2.1. The Extracellular Domain

The amino terminus of Eph receptors was originally proposed to resemble an immunoglobulin domain (O'Bryan *et al.*, 1991), which, in a number of other RTKs, can contribute to ligand binding. However, subsequent structural analysis has revealed that this N-terminal globular domain consists of 11 antiparallel β-strands forming a compact jellyroll β-sandwich which is important for ligand binding (Himanen *et al.*, 1998). Mutational analysis revealed that a long loop between two such β-strands may confer a degree of specificity between the ephrinA and ephrinB ligands (Himanen *et al.*, 1998), in agreement with a previous report in which the globular domain was shown to confer subclass-specific ligand binding specificity on the Eph receptor (Labrador *et al.*, 1997).

Carboxy terminal to the globular domain is a cysteine-rich region, consisting of two C*n*C*X*C*n*C repeats (with C representing cysteine, *n* a stretch of 12-15 amino acids, and *X* any amino acid). The spacing of the cysteine residues within this region differs from that found in the cysteine-rich regions of other RTKs such as the epidermal growth factor (EGF) receptor and the insulin receptor, and the repeats share most similarity with the EGF repeats of extracellular matrix (ECM) proteins e.g. fibrillin, tenascin and thrombospondin (Connor and Pasquale, 1995). These residues have an, as yet, unknown function in Eph receptors, although they may play a role in some structural aspects of ligand binding rather than contributing to ligand binding specificity. A three- to four- fold reduction in the dissociation constant of ephrinB1 binding to a mutant EphB2 receptor lacking the cysteine-rich domain was observed by Labrador (Labrador

et al., 1997), and studies by Lackmann and colleagues (Lackmann *et al.*, 1998) also implicate this region in ligand binding (see Section 1.1.4.1).

Between the cysteine-rich region and the transmembrane domain are two fibronectin type III repeats, a repeated series of predominantly hydrophobic amino acids similar to those found in fibronectin (Skorstengaard *et al.*, 1986). Such repeats are common in many cell adhesion molecules, which led to speculation that Eph receptors may be involved in cell-cell contact. However, these repeats also feature in the ectodomains of many other proteins, including RTKs, receptor tyrosine phosphatases, and cytokine receptors, where they are postulated to mediate receptor dimerisation (Somers *et al.*, 1994). Indeed, recent studies by Lackmann and colleagues have shown that a region of EphA3, incorporating the fibronectin type III repeats, is able to induce ligand-independent transphosphorylation of endogenous full length EphA3 molecules, implying the presence of a dimerisation motif within this region (Lackmann *et al.*, 1998) (also see Section 1.1.4.1).

The extracellular domain of EphA2 is subject to N-linked glycosylation, which is likely to account for the migration of EphA2 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as a 125-130 kDa doublet (Lindberg and Hunter, 1990). Ganju and colleagues (Ganju *et al.*, 1994) demonstrated that N-linked glycosylation accounted for ~ 20 kDa of the size of the 130 kDa protein detected by EphA2 antisera. There are three potential glycosylation sites in hEphA2 and two sites in mEphA2 (see Appendix 1).

1.1.1.2.2. The Intracellular Domain

Carboxy terminal to the transmembrane domain, which in EphA2 consists of 24 hydrophobic amino acids, is the cytoplasmic domain of the receptor. The first region of homology shared by all Eph receptors is the juxtamembrane region, a short stretch of ~ 10 amino acids, beginning approximately 25-30 residues downstream of the transmembrane domain, that contains two invariant tyrosine residues (tyrosines 589 and 595 in mEphA2) (Lhotak and Pawson, 1993) (Figure 1.2). This region is important for both intracellular signalling (Ellis *et al.*, 1996; Holland *et al.*, 1997; Hock *et al.*, 1998) and for the catalytic activity of the receptor (Zisch *et al.*, 1998; Binns *et al.*, 2000; see also Section 1.1.4.2).

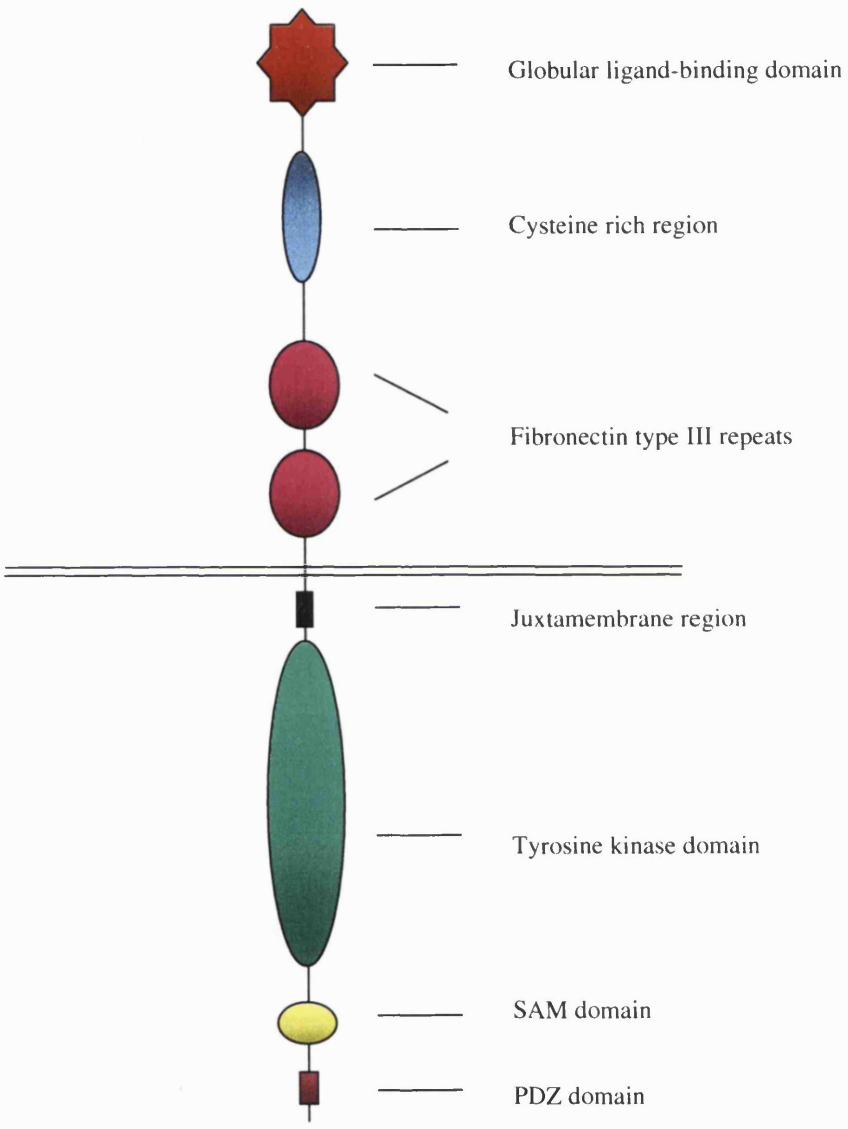


Figure 1.1. Diagram of the Protein Structure of Eph Receptors
The Eph family of receptor tyrosine kinases are type I transmembrane proteins, and share a number of common features, as depicted. For more details, please refer to the text.

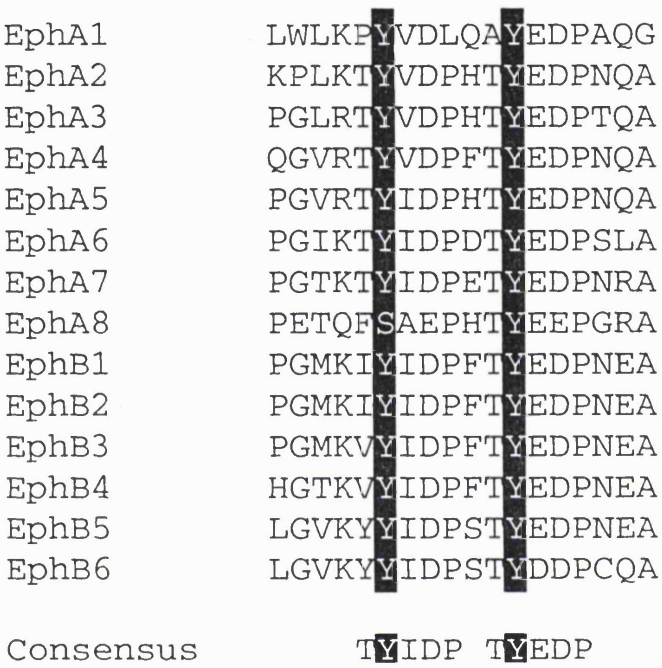


Figure 1.2. Two Tyrosine Residues in the Juxtamembrane Domain of Eph Receptors are Highly Conserved

The predicted amino acid sequence of the juxtamembrane domain of mouse Eph receptors was aligned in relation to the tyrosines (Y) at residues 600 and 606 of mouse EphA1. Sequences were aligned by eye. Adapted from Ellis *et al.*, 1996.

In PTKs, catalysis of the transfer of the γ-phosphate of ATP to tyrosine residues of the substrate occurs in a cleft created by two lobes in the kinase domain. The conserved catalytic domain of Eph receptors spans approximately 250 amino acids and contains all of the 11 major subdomains described by Hanks and colleagues (Hanks *et al.*, 1988), displaying a particularly high degree of conservation in subdomains VI to IX. Subdomain VI, which forms the catalytic loop, contains the HRDLAAR sequence that was used to clone hEphA2 (Lindberg and Hunter, 1990). A conserved tyrosine residue in the activation loop (tyrosine 773 in mEphA2) is thought to be necessary for stimulating catalytic activity (Section 1.1.4.2; Kalo and Pasquale, 1999a; Binns *et al.*, 2000). Another protein tyrosine kinase consensus motif, PI/VK/RWT/MAPE, is present in subdomain VIII of EphA2 as PIRWTAPE. Interestingly, whereas all other RTK families have a methionine in this sequence, the corresponding residue in Eph receptors is a threonine (van der Geer *et al.*, 1994). Furthermore, according to studies by Songyang and Cantley, this residue influences the substrate specificity of the kinase domain (Songyang and Cantley, 1995), which implies potential differences in signalling between Eph receptors and other RTKs, an implication which would be consistent with differences in the functions of the Eph receptor family compared to more conventional growth factors of the receptor tyrosine family.

The X-ray crystal structures for the SAM domains of EphA4 and EphB2 have recently been elucidated (Stapleton *et al.*, 1999; Thanos *et al.*, 1999). SAM domains comprise 60-70 residue conserved regions which are often present in proteins involved in developmental regulation. In Eph receptors, the SAM domain is present C-terminal to the kinase domain (Schultz *et al.*, 1997) and forms a five helix-containing 'lobster claw' (Stapleton *et al.*, 1999). Two such structures are believed to be able to homo- or hetero-dimerise with other SAM domain-containing proteins, implying a role for these domains in mediating protein-protein interactions. Although the function of Eph SAM domains is unclear, EphA4 SAM domain homodimerisation is an attractive mechanism by which Eph receptor dimerisation may occur (Stapleton *et al.*, 1999). Furthermore, Thanos and colleagues (Thanos *et al.*, 1999) have provided a mechanism, albeit theoretical, by which Eph receptor oligomerisation could occur via multiple SAM domain interactions at high receptor concentrations, which may thus be dependent on the ligand aggregation state. This high order clustering, in turn may create novel binding surfaces which could account for the differences in EphB1 and EphB2 substrate recruitment observed when using either dimerised and oligomerised ephrinB1 (Stein *et*

al., 1998a) (see Section 1.1.3.2). Alternatively, it is possible that SAM domains may negatively influence the formation of Eph receptor dimers by forming repressive intra- or inter- molecular interactions, and that, as described above (Section 1.1.1.2.1), dimerisation via the fibronectin type III repeats (Lackmann *et al.*, 1998) may serve to release such an inhibition. In addition to proposed receptor-receptor interactions, Eph receptor SAM domains may mediate substrate binding.

Eph receptors contain a PDZ domain binding motif at their extreme carboxy terminus, comprising a sequence of four or more amino acids, which is recognised by PDZ domain-containing proteins (PDZ proteins). Different PDZ proteins reportedly have different carboxy-terminal binding specificities (Saras and Heldin, 1996; Songyang *et al.*, 1997), implying that the repertoire of Eph receptor-PDZ protein interactions is likely to be extensive. PDZ proteins have been reported to mediate the correct subcellular localisation of proteins, the aggregation of PDZ domain binding proteins into multimers, and the assembly of proteins containing PDZ binding motifs into signalling complexes, all of which are likely to be of significance in Eph-mediated signalling.

1.1.1.3. Chromosomal Location of EphA2

EphA2 was mapped to the distal region of chromosome 4 in the mouse (Becker *et al.*, 1994; Ganju *et al.*, 1994) and p36.3-p36.1 region of human chromosome 1 (Becker *et al.*, 1994). hEphA2 has subsequently been mapped more precisely to 1p36.1 (Sulman *et al.*, 1997), in close proximity to the EphB2 gene (Kiyokawa *et al.*, 1994; Ikegaki *et al.*, 1995). Interestingly, EphB1 and EphB3 are closely associated on chromosome 3 (Bohme *et al.*, 1993; Tang *et al.*, 1995) and EphA1 and EphB4 are both found on chromosome 7 (Yoshida *et al.*, 1989; Bennett *et al.*, 1994), suggesting the existence of Eph receptor gene clusters.

1.1.1.4. The Gene Structure of Eph Receptors

Analysis of the genomic organisation of the chicken EphB2 (cEphB2) gene has revealed that the exon-intron structure correlates with the highly conserved protein domain structure of Eph receptors (Connor and Pasquale, 1995) as described in Section 1.1.1.2. This strongly implies that the Eph receptor exon structure is remarkably conserved, and the genomic structure of EphA2 is therefore likely to show strong similarity to that of

EphB2. The cEphB2 gene is comprised of at least 16 exons, spanning over 35 kb (Connor and Pasquale, 1995). A number of Eph receptor isoforms have been described that can be explained on the basis of insertion, deletion or substitution within the exon-intron structure identified for cEphB2 (Connor and Pasquale, 1995). The function of the different Eph receptor isoforms is unclear, but based on both *in vitro* and *in vivo* observations, they are likely to play an important role in the precise regulation of Eph receptor signalling. For example, receptors lacking a functional kinase domain may act either in a dominant negative form by sequestering ligand (Xu *et al.*, 1995; Durbin *et al.*, 1998) or, as described in Section 1.1.3.3, may function as ligands in "reverse signalling". Recently, a function for kinase deficient truncated forms of the EphA7 receptor in promoting adhesion in the developing neural tube has been proposed (Holmberg *et al.*, 2000).

None of the splice variants described in the literature thus far for other Eph receptors has been found to occur in EphA2 transcript processing. However, a novel exon (exon 5.2) located ~ 10 kb upstream of the 5' end of the published cDNA sequence was identified following a gene trap-mediated insertion event into the EphA2 locus (Chen *et al.*, 1996). The function of this exon is unclear, as it comprises nucleotide sequence that does not contribute to functional protein, but transcripts have been isolated in which exon 5.2 sequence is spliced to nucleotide 179 of the published EphA2 cDNA sequence (Ganju *et al.*, 1994; Ruiz and Robertson, 1994). Such transcripts would have the potential to encode a truncated protein beginning at nucleotide 202 of the EphA2 cDNA sequence (Chen *et al.*, 1996). However, such a protein would lack the signal peptide sequence and therefore would be expected to reside intracellularly (see Section 1.1.8).

1.1.1.5. Eph Receptor Evolution

With the exon-intron structure of the vertebrate Eph receptor genes being largely conserved in those regions studied so far (Maru *et al.*, 1988; Chan and Watt, 1991; Sajjadi *et al.*, 1991; Connor and Pasquale, 1995; Lackmann *et al.*, 1998), it is quite possible that the Eph receptors have evolved from one ancestral gene by a process of gene duplication and subsequent mutations. So far, the EphA2 gene has been isolated from human, mouse and *Xenopus,* with a putative zebrafish homologue, based on a partial sequence. An alignment of the amino acid sequences of these four homologues is shown in Figure 1.3.

Figure 1.3. Species Sequence Alignment of the EphA2 Receptor

Alignment of the predicted amino acid sequence of EphA2 from human (accession number (#)M59371), mouse (#X78339), *Xenopus* (#AJ002493) and a putative zebrafish homologue (#AJ005027). Boxes represent conserved residues. Alignment was performed by the MegAlign program (DNAStar) using the Jotun Hein method.

Sequence analysis reveals a 92% overall identity at the amino acid level between human and mouse EphA2 (Ganju *et al.*, 1994). The *Xenopus* and zebrafish sequences show most divergence from the human and mouse sequences in the fibronectin type III repeats, the cysteine rich region, the transmembrane domain and the carboxy terminus.

Other Eph receptors have been widely studied in rat, chicken, quail, *Xenopus* and zebrafish, with the identification, more recently, of Eph receptors in *Drosophila* (Scully *et al.*, 1999) and *C. elegans* (George *et al.*, 1998). Interestingly, the *C. elegans* and *Drosophila* Eph receptor proteins display all the conserved domains of vertebrate Eph receptors, but both genes consist of only 10 exons (George *et al.*, 1998; Scully *et al.*, 1999), implying that intron gain may have been a feature in the evolutionary process of Eph receptors. Furthermore, the sequences of both *C. elegans* and *Drosophila* Eph receptor proteins show equal similarity to both vertebrate EphA and EphB receptors (George *et al.*, 1998; Scully *et al.*, 1999), implying that vertebrate EphA and EphB receptors have diverged from a common ancestor.

Subdivision of the Eph family into the EphA and EphB subfamilies is based both on sequence homology of their extracellular domains, and their binding and activation specificities towards the two subclasses of ephrin ligands, ephrinAs and ephrinBs (Gale *et al.*, 1996a).

1.1.2. Introduction to Ephrins

Ligands for the Eph receptors are known as ephrins, Eph family receptor interacting proteins (Eph Nomenclature Committee, 1997). Alternatively, the name can also be derived from the Greek word "ephoros" meaning 'overseer' or 'controller' (Eph Nomenclature Committee, 1997). Eph receptors were for some time known as 'orphan' receptors, as a long period of time elapsed before the identification of the ephrin ligands. Although it is usually the case that RTKs are cloned before their ligands, the delay in cloning ephrins was partly due to the lack of a suitable bioassay by which they could be identified and partly due to the ligands being predominantly membrane bound. Ephrins are subdivided into ephrinAs and ephrinBs on the basis of whether they are attached to the membrane via a GPI linkage or exist as transmembrane proteins, respectively. Similar to the subdivision of Eph receptors, ephrinA and ephrinB subclasses also reflect the degree of homology shared between their extracellular

domains and their binding properties towards the EphA and EphB receptors (Gale *et al.*, 1996a).

1.1.2.1. The Discovery and Cloning of EphrinA1

The first ephrin to be identified was ephrinA1, which, on the basis of its binding affinity and expression pattern, is now widely accepted as the cognate ligand for the EphA2 receptor, although other ephrins are also able to bind EphA2. Using the extracellular domain of EphA2 attached to a BIAcore sensorchip, concentrated cell culture supernatants were screened for binding activity (Bartley *et al.*, 1994). Following further purification and N-terminal sequencing, ephrinA1, which had previously been cloned as B61, a tumour necrosis factor α (TNFα)-inducible early response gene, was identified as a binding partner (Holzman *et al.*, 1990). Purified soluble ephrinA1 was demonstrated to bind to EphA2-expressing cells and to induce a dose-dependent increase in tyrosine phosphorylation of the EphA2 receptor (Bartley *et al.*, 1994). However, a membrane-bound form of ephrinA1 was also observed (Bartley *et al.*, 1994), and subsequent analysis revealed that ephrinA1 is attached to the cell membrane through a GPI linkage which can be cleaved by phospholipase C (PLC) (Shao *et al.*, 1995). Furthermore, membrane-bound ephrinA1 is also able to induce EphA2 receptor phosphorylation (Shao *et al.*, 1995).

Other ephrins have been cloned following similar receptor affinity chromatography approaches (Lackmann *et al.*, 1996; Sakano *et al.*, 1996). However, a number of ephrins (both A and B) have been cloned using a soluble receptor affinity approach, in which so-called receptor-bodies (fusion proteins comprising the extracellular domain of Eph receptors coupled to the Fc region of IgG (Aruffo *et al.*, 1990) or alkaline phosphatase tags (Flanagan and Leder, 1990) are used to screen expression libraries for positive binding activity. The ligand ephrinA5 was identified following a screen for GPI-linked proteins with repellent activity (Drescher *et al.*, 1995). It is likely that other ephrins remain to be cloned, as no binding partner for EphB5 has been reported, and ephrinA1 only binds modestly to EphA1 (Gale *et al.*, 1996a).

1.1.2.2. The Protein Structure of Ephrins

As membrane proteins, both ephrinA and ephrinB ligands possess a short signal peptide at their amino-terminus, followed by a relatively conserved sequence of 125 amino

acids which is thought to represent the receptor binding domain, a spacer region and a hydrophobic region (Kozlosky *et al.*, 1995) (see Figure 1.4).

The predicted open reading frame for mouse ephrinA1 encodes a 205 amino acid polypeptide of approximately 25 kDa. The first stretch of hydrophobic amino acids in all ephrins (18 residues in ephrinA1) comprises the signal peptide which is subject to cleavage. There are a number of conserved residues in the putative receptor-binding domain (Zhou, 1998), including four invariant cysteine residues that are present in all ephrins (Kozlosky *et al.*, 1995). The extracellular domain of ephrinA1 contains a single site for potential N-linked glycosylation, the occurrence of which has been demonstrated by N-terminal sequence analysis (Bartley *et al.*, 1994). The carboxy terminus of ephrinA1 is thought to function as a signal for GPI linkage to the membrane. The 23 hydrophobic amino acids are cleaved and a GPI moiety is attached via ethanolamine to the carboxy terminus. Consistent with the identification of ephrinA1 as a soluble protein (Bartley *et al.*, 1994), the GPI linkage of ephrinA ligands can be cleaved specifically by phospholipases, as has been demonstrated for ephrinA1 (Shao *et al.*, 1995) and ephrinA2 (Cheng and Flanagan, 1994).

Attachment of ephrinB ligands to the cell membrane is achieved by a stretch of ~ 25 hydrophobic amino acids comprising a transmembrane domain, followed by an 80-90 amino acid cytoplasmic stretch which contains 5 conserved tyrosine residues of potential importance in bidirectional signalling (see Section 1.1.3.3). Interestingly, a valine is present as the last carboxy terminal residue of all ephrinB ligands. A carboxy-terminal valine residue is also present in transforming growth factor α (TGFα), colony-stimulating factor-1 (CSF-1) and stem cell factor (SCF), membrane-bound proteins that can also exist as soluble factors, and it has previously been demonstrated that extracellular cleavage of membrane-bound TGFα into its soluble form requires this valine residue (Bosenberg *et al.*, 1992). The carboxy terminus of ephrinB ligands also contains a PDZ domain binding motif (-YKV) (Bergemann *et al.*, 1998; Torres *et al.*, 1998; Lin *et al.*, 1999), similar to that found in the carboxy terminus of Eph receptors (see Section 1.1.1.2.2). This may provide a mechanism through which recruitment of signalling components to ephrinB ligands can occur for reverse signalling (see Section 1.1.3.3) or by which ephrinB clustering may occur in order to facilitate EphB receptor activation (see Section 1.1.3.2).

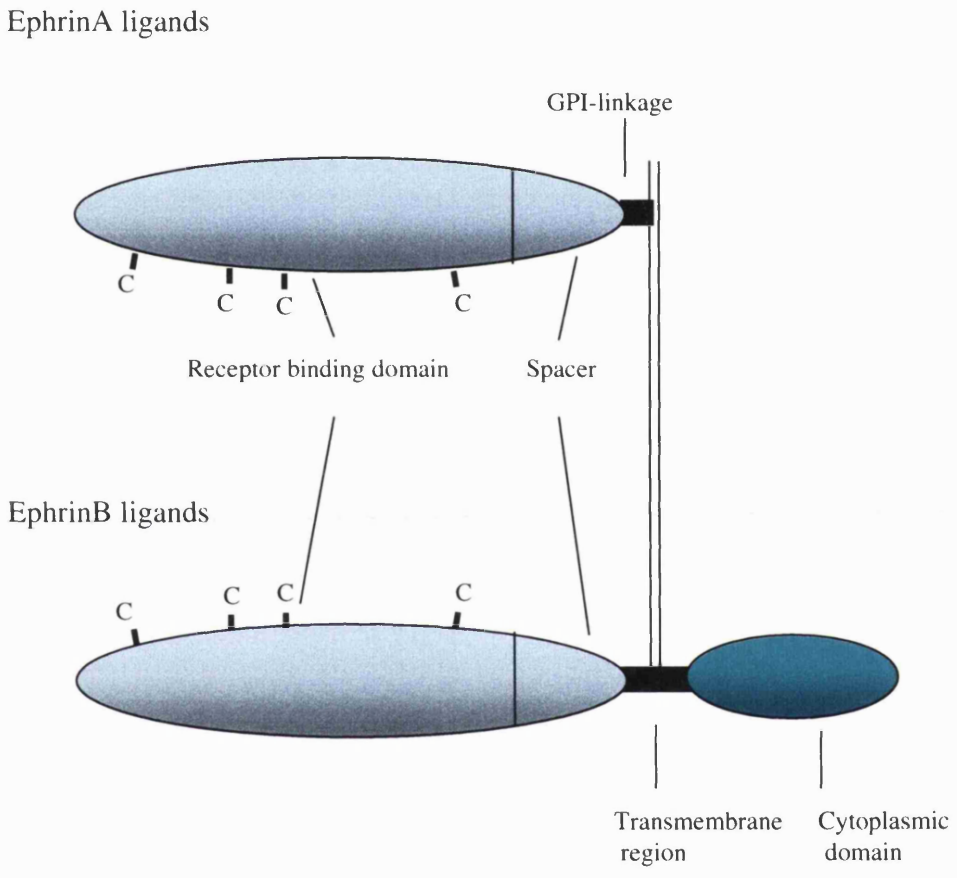


Figure 1.4. Diagram of the Protein Structure of Ephrin Ligands
Ligands for Eph receptors are divided into ephrinAs or ephrinBs, depending on whether they are attached to the cell membrane via a GPI linkage (ephrinAs) or whether they exist as transmembrane proteins (ephrinBs). Four cysteine residues within the receptor-binding domain are conserved in all ephrins. The carboxy terminus of the cytoplasmic domain of ephrinB ligands shows a very high degree of homology.

1.1.2.3. Chromosomal Location and Gene Structure of EphrinA1

EphrinA1 is encoded by a single copy gene (Holzman *et al.*, 1990) that resides on mouse chromosome 3 (Shao *et al.*, 1995; Cerretti *et al.*, 1996) and human chromosome 1q21-22 (Cerretti *et al.*, 1996), regions which are syntenic. Interestingly, as is observed for the Eph receptor genes, several of the ephrinA ligands are clustered in the same chromosomal region (Cerretti *et al.*, 1996).

Although studies on the genomic organisation of vertebrate ephrin genes have been limited, the exon-intron structure of mouse ephrinA3 and ephrinA4, and that of human ephrinA2 and ephrinA4, have been elucidated (Cerretti and Nelson, 1998; Aasheim *et al.*, 2000). Mouse ephrinA3 and ephrinA4 genes contain five and four exons, respectively, whilst human ephrinA2 and ephrinA4 genes both contain four exons. Unlike the situation in Eph receptors where the intron-exon structure correlates with the domain structure (Connor and Pasquale, 1995), the gene structure of ephrinAs does not strictly correspond to the four domains characterised by the signal peptide, receptor-binding region, spacer region and hydrophobic GPI linkage region proposed by Kozlosky and colleagues (Kozlosky *et al.*, 1995). The first three exons of the ephrinA ligands studied by Cerretti and Nelson (Cerretti and Nelson, 1998) contain sequence from the 5' untranslated region to the beginning of the spacer domain, with three intervening introns. The presence of an additional intron in the spacer region determines whether the remainder of the protein and the 3' noncoding region is contained in sequences derived from one or two more exons. However, for the first three exons, the location of the introns within the predicted sequence is conserved (Cerretti and Nelson, 1998).

Far fewer splice variants have been documented for ephrins than the number of isoforms reported for Eph receptors (Flenniken *et al.*, 1996; Weinstein *et al.*, 1996; Aasheim *et al.*, 2000). Several of these isoforms give rise to soluble ligands, the reason for which is unclear, since soluble forms of ephrins can be created by mechanical processes outlined in Section 1.1.2.1.

1.1.2.4. Ephrin Ligand Evolution

The ephrinA1 gene has been cloned from human, mouse and rat sources, and putative homologues have also been identified in *Xenopus* and zebrafish.

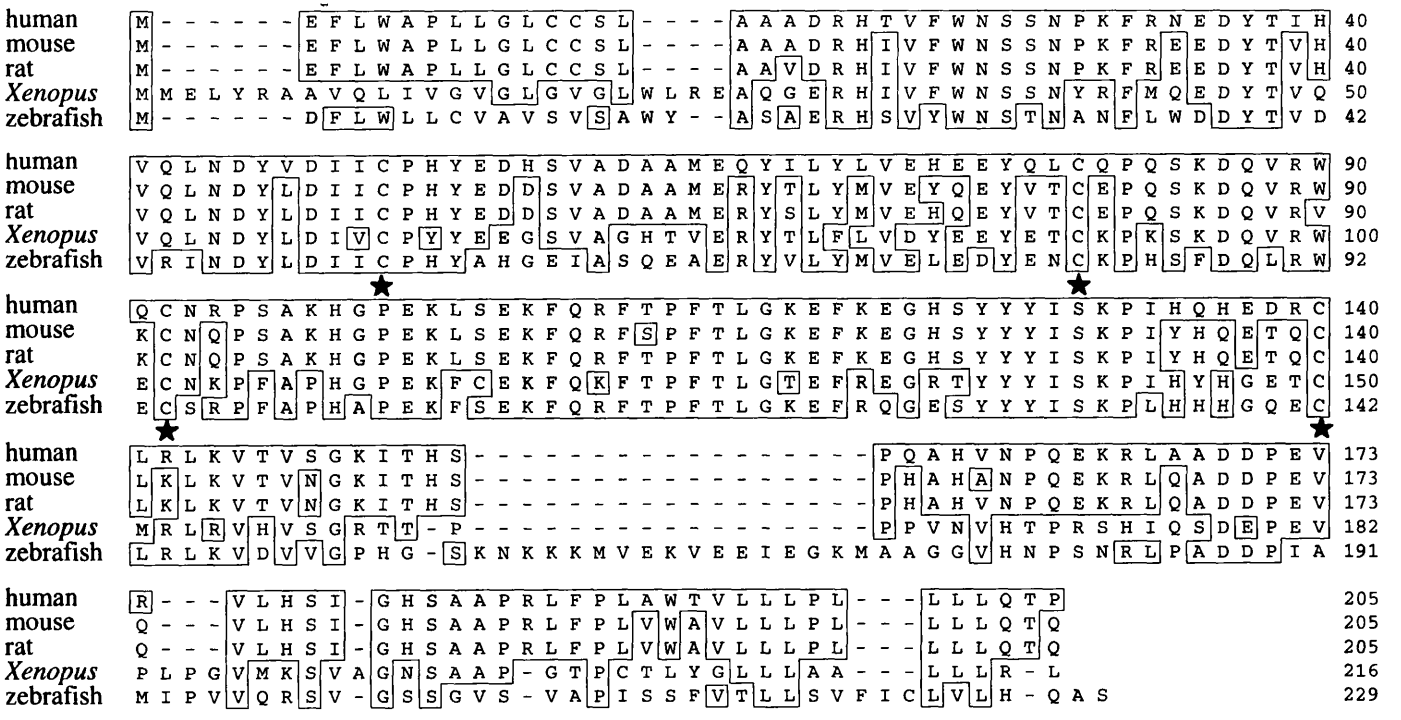


Figure 1.5. Species Sequence Alignment of EphrinA1
Alignment of the predicted amino acid sequence of ephrinA1 from human (accession number (#) M57730), mouse (#U26188), rat (#D38056) and putative *Xenopus* (#AAA74485) and zebrafish (#CAA07264) homologues. Boxes represent conserved residues. Asterisks denote conserved cysteine residues. Alignment was performed by the MegAlign program (DNAStar) using the Jotun Hein method.

An alignment of the amino acid sequences of the ephrinA1 gene from these species is shown in Figure 1.5. Strikingly, the four conserved cysteine residues are present in all species. The discovery of ephrins in *Drosophila* is imminent following the identification of a *Drosophila* Eph receptor (Scully *et al.*, 1999). Four putative ephrin-encoding genes, *efn-1*, *efn-2*, *efn-3* and *efn-4*, have been identified in *C. elegans* (Wang *et al.*, 1999). The *C. elegans* ephrins contain a putative amino-terminal signal peptide and a carboxy-terminal GPI attachment signal, but they share more homology with the conserved region of vertebrate ephrinB ligands than ephrinAs (Chin-Sang *et al.*, 1999; Wang *et al.*, 1999). The gene structure of *efn-1/vab-2*, *efn-2* and *efn-3* consists of 4, 5 and 6 exons, respectively (Chin-Sang *et al.*, 1999; Wang *et al.*, 1999).

1.1.3. Interactions Between Eph Receptors and Ephrins

1.1.3.1. Promiscuity of Ligand-Receptor Binding Interactions

As described in Section 1.1.2.1, many of the Eph receptor ligands were isolated from expression libraries using the soluble extracellular domain of the receptor coupled to a suitable tag such as IgG or alkaline phosphatase to aid detection (so-called receptor bodies). At an early stage in the process of identifying ephrins, a number of groups using this technique independently identified the same ligand using the extracellular domain of different Eph receptors (e.g. ephrinA1 was found to bind EphA2, EphA3 and EphA5 (Bartley *et al.*, 1994; Beckmann *et al.*, 1994; Davis *et al.*, 1994)) or different ligands using the same receptor-body (e.g. ephrinA1 and ephrinB1 were bound by EphA3 (Kozlosky *et al.*, 1995)), resulting in the accumulation of a number of different names for the same ephrin or Eph isolated from different species. For clarity, a new nomenclature was adopted (Eph Nomenclature Committee, 1997). However, it was apparent from these observations that a great deal of promiscuity existed in Eph receptor-ligand interactions. *In vitro* binding studies carried out by Gale and colleagues (Gale *et al.*, 1996a) revealed that the ephrins can be functionally separated into two subclasses, depending on their ability to bind to EphA or EphB receptors. Significantly, this subdivision tends to correspond to the structural separation of the ligands into GPI-linked or transmembrane ephrins, such that ephrinA ligands bind to EphA receptors, and ephrinB ligands display preferential binding activity to EphB receptors, although EphA4 has been reported to be bound by ephrinB2 and ephrinB3 (Gale *et al.*, 1996a; Gale *et al.*, 1996b) (see Figure 1.6).

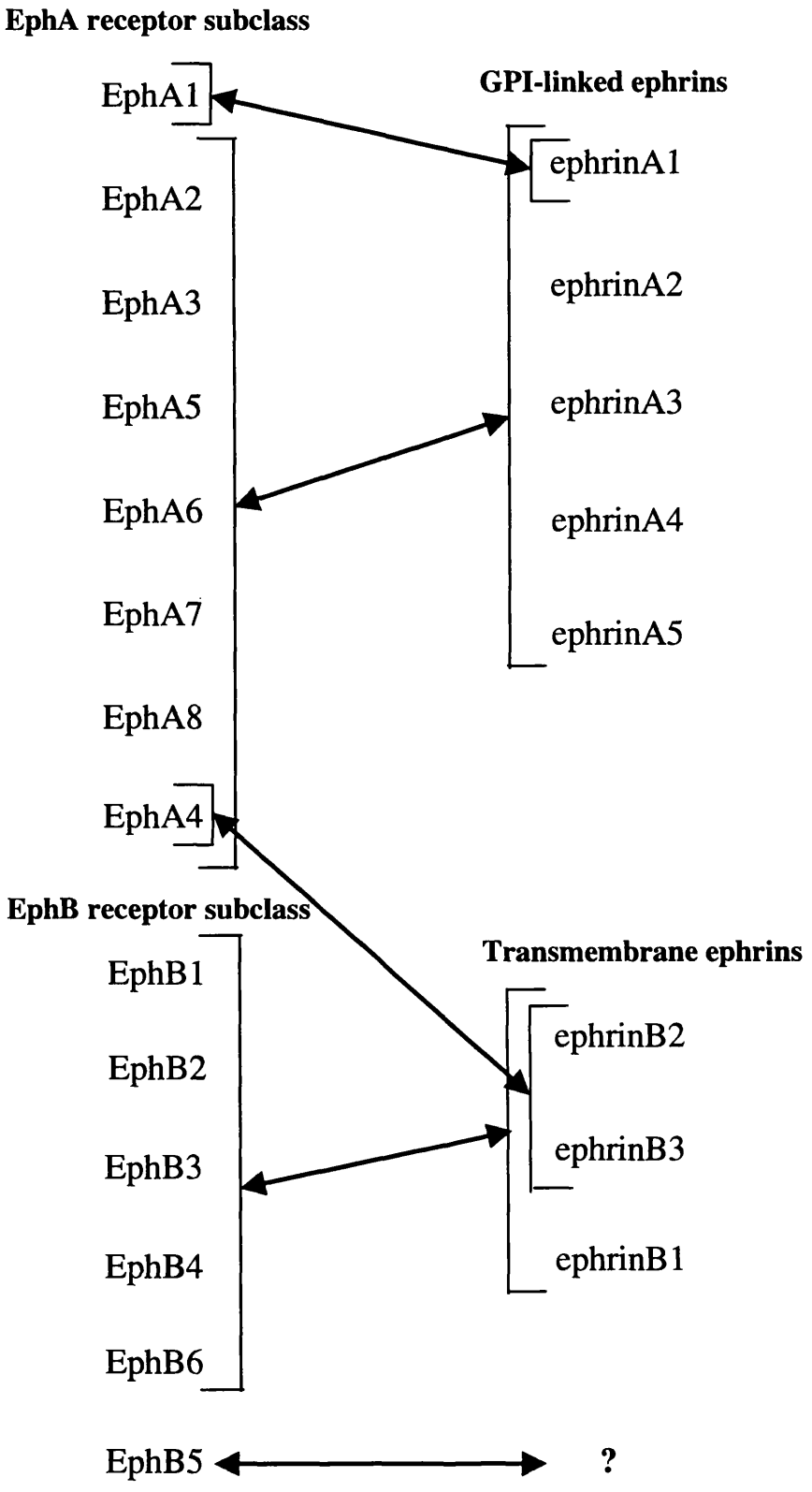


Figure 1.6. Summary of Binding Specificities of Eph Receptors and Ephrins
Eph receptors are divided into two subgroups based on sequence homology and ligand subclass binding specificity. The EphA subfamily interacts primarily with GPI-linked ephrinA ligands, whilst EphB receptors bind transmembrane ephrinB ligands. Only EphA4 shows significant binding activity towards both ephrinA and ephrinB ligands. However, within each subclass, there is a great deal of variation in the binding affinities of individual Eph-ephrin interactions, which may be highly significant *in vivo*. In contrast, EphA1 only appears to be bound by ephrinA1, whereas no ligand has yet been identified for EphB5.

However, within each subgroup there is a great deal of variation in the binding specificities of ligand-receptor interactions, for example, ephrinA2 exhibits a 20-fold difference between its strongest affinity interaction (K_d = 1.03 nM for EphA7) and its weakest (K_d = 20.1 nM for EphA2) (Gale *et al.*, 1996a). Such differences in receptor binding have been shown to be of functional significance *in vivo* where, for example, the different affinity of the EphA3 receptor towards ephrinA2 and ephrinA5 has been shown to be important in retinotectal mapping (Monschau *et al.*, 1997) (see Section 1.1.7.1). Furthermore, consistent with the *in vitro* binding studies, members from one ephrin subclass are only capable of inducing tyrosine phosphorylation in members of the respective Eph receptor subclass (Gale *et al.*, 1996a). However, in some cases, high affinity binding interactions are not indicative of induction of receptor phosphorylation (Davis *et al.*, 1994; Lackmann *et al.*, 1997) and discrepancies in binding affinities obtained using different receptor-body tags have been reported (e.g.. Gale *et al.*, 1996a; Lackmann *et al.*, 1997; Bergemann *et al.*, 1998). The reason for such inconsistencies is likely due to the use of Fc fusions of receptor- or ligand- bodies in many of the studies of Eph-ephrin interactions. A detailed biochemical study carried out by Lackmann and colleagues (Lackmann *et al.*, 1997) demonstrated that divalent Fc-tagged ephrinA2 and ephrinA5 ligand bodies both had similar binding affinities and kinetics for the EphA3 receptor, whereas monovalent FLAG™-tagged ephrinA5 showed a much lower dissociation rate compared to FLAG™-tagged ephrinA2. Cross-linking the monovalent ligand bodies with an anti-FLAG™ antibody resulted in high affinity binding of both ligands to EphA3, implying that only at high local concentrations would ephrinA2 be able to interact with EphA3, whereas a stable interaction between ephrinA5 and EphA3 could occur at much lower concentrations of ligand (Lackmann *et al.*, 1997). Thus, many of the ephrin-Eph interactions that have been identified using Fc-fusion proteins may not be representative of a physiologically relevant ligand-receptor interaction.

1.1.3.2. A Requirement for Ephrin Clustering in Eph Receptor Activation?

Fc-tagged ligand bodies have also been used in the study of Eph-ephrin interactions to mimic the membrane-bound state of ephrins. This followed reports that, although able to bind their receptors, ephrins were unable to induce tyrosine phosphorylation of Eph receptors in their soluble form, but could be induced to do so by artificial clustering (Davis *et al.*, 1994). The existence of the ephrins as membrane-bound ligands was therefore highly significant and positive Eph-ephrin interactions were only thought possible upon cell-cell contact, perhaps reflecting the need for Eph receptors themselves

to dimerise or oligomerise before downstream signalling can occur (Lemmon and Schlessinger, 1994). However, the fact remained that ephrinA1 had been cloned as a soluble protein and, in this form, was capable of inducing tyrosine phosphorylation of the EphA2 receptor (Bartley *et al.*, 1994). Paradoxically, the soluble, myc-tagged ephrinA1 used in studies by Davis and colleagues (Davis *et al.*, 1994) was not capable of inducing EphA5 tyrosine phosphorylation until artificially dimerised, which led to speculation that soluble forms of the ephrins may actually function as antagonists of Eph receptor activation. Since then, there have been conflicting reports regarding the requirement of the ephrins to be artificially clustered in order to induce Eph receptor activation and elicit downstream signalling events. EphrinA1, as previously mentioned, was identified as a soluble protein and demonstrated to induce EphA2 phosphorylation in colon carcinoma cells (Bartley *et al.*, 1994). However, ephrinA1 has also been shown to be biologically active as a membrane-bound ligand (Shao *et al.*, 1995). Subsequent studies have used dimeric ephrinA1 as an Fc fusion protein to activate EphA2, leading to the identification of receptor binding partners (see Section 1.1.4.4.1).

Thus, ephrinA1 has been shown to activate EphA2 as a soluble, dimeric or membrane-bound ligand. EphrinA1Fc also binds to and activates EphA8 in the absence of further clustering (Choi *et al.*, 1999). However, despite displaying a high affinity *in vitro* interaction as an Fc fusion protein, ephrinA1 is only able to activate EphA7 in its membrane bound form (Ciossek and Ullrich, 1997). Further variations have also been seen with other ephrin-Eph interactions (e.g. Bennett *et al.*, 1995; Sakano *et al.*, 1996; Park and Sanchez, 1997; Ohta *et al.*, 1997), with a number of reports that soluble ligands are antagonistic (e.g. Winslow *et al.*, 1995; Durbin *et al.*, 1998). The requirement for ephrins to be dimerised, clustered or membrane-bound therefore seems to be highly variable depending on the particular ligand-receptor interaction being studied, and may also reflect the cell type and conditions under which the experiment is performed. Interestingly, ephrinB ligands appear to have more of a requirement to be clustered into oligomers than do ephrinA ligands for efficient Eph receptor activation (Davis *et al.*, 1994; Gale and Yancopoulos, 1997). Furthermore, it can be envisaged that the same ephrin-Eph interaction may vary in its affinity and consequent ability to induce receptor phosphorylation depending on the cell type studied, which even then is not representative of an *in vivo* situation where it is likely that many other influences, such as co-operativity or inhibition from other cell-cell interactions will occur. Thus, *in vitro* studies alone cannot be used to predict whether an *in vitro* ligand-receptor

interaction will be physiologically relevant *in vivo*. A *bona fide* ephrin-Eph receptor interaction is likely to occur if both ligand and receptor are demonstrated to co-localise to the same or neighbouring cells in addition to being shown to have a high affinity interaction *in vitro*. EphA2 and ephrinA1 have been shown to co-localise in a number of different tissues (see Sections 1.1.6.1 and 1.1.6.2).

In addition to influencing whether or not the interaction will occur, ephrin clustering may also be an important factor in determining the cellular response. Both ephrinB1Fc dimers and tetramers were shown to induce EphB1 and EphB2 receptor phosphorylation to the same extent in endothelial and P19 cells, but only ephrinB1 tetramers recruited the low molecular weight-protein tyrosine phosphatase, LMW-PTP, to the phosphorylated receptors (Stein *et al.*, 1998a). This distinction between different oligomeric forms of ligand may be of fundamental importance in the response of cells to different gradients of ephrin in aspects of development.

The mechanism by which ephrins are clustered at the cell membrane is not yet clear, but recent studies have proposed that the presence of a PDZ domain binding motif at the carboxy terminus of ephrinB ligands may be highly significant (Torres *et al.*, 1998; Lin *et al.*, 1999). Torres and colleagues (Torres *et al.*, 1998) demonstrated that co-expression of ephrinB1 and the PDZ protein PICK1 (protein interacting with Cα kinase 1) resulted in significant co-clustering. Other PDZ proteins with which ephrinB ligands have been reported to interact include syntenin (Lin *et al.*, 1999), glutamate receptor-interacting protein (GRIP) (Bruckner *et al.*, 1999; Lin *et al.*, 1999), a novel ephrin-interacting protein, PHIP (Lin *et al.*, 1999), and the tyrosine phosphatase FAP-1 (Fas-associated phosphatase) (Lin *et al.*, 1999). At least some of these proteins may be involved in the localisation and consequent clustering of ephrins at the cell membrane, while others may have a role in ephrinB-directed signalling. An additional method of clustering relevant to ephrinA ligands may be mediated by the concentration in lipid-rich caveolae of GPI-linked proteins (Simons and Ikonen, 1997), as has been reported for ephrinA5 (Davy *et al.*, 1999).

1.1.3.3. Reverse Signalling – Eph Receptors as Ligands

Both genetic and biochemical evidence has demonstrated that ephrins are unusual in being able to act both as conventional ligands for Eph receptors and themselves initiating intracellular signalling pathways that are independent of the catalytic activity

of Eph receptors. The concept that the cytoplasmic domain of ephrinB ligands may feature in some aspect of signalling was initially proposed upon aligning the amino acid sequences of the three ephrinB ligands (Gale *et al.*, 1996b). The carboxy terminus shows 91% homology over the last 33 amino acids, and contains 5 conserved tyrosine residues, some of which have the potential to become phosphorylated and thus act as docking sites for molecules involved in signalling (Pawson, 1995). Evidence supporting the occurrence of ephrinB phosphorylation was provided by cell culture studies (Holland *et al.*, 1996; Bruckner *et al.*, 1997). Presentation of EphB2 as a clustered Fc receptor body to cells expressing full length ephrinB1 or ephrinB2 resulted in their tyrosine phosphorylation, whereas cells expressing an ephrinB1 mutant lacking the cytoplasmic domain failed to become phosphorylated. Furthermore, co-culture of EphB2-expressing cells with cells expressing either ephrinB1 or ephrinB2 resulted in the phosphorylation of tyrosine residues in the EphB2 receptor and the ephrinB ligands, implying that signalling could occur in both the Eph- and ephrin- expressing cells (Holland *et al.*, 1996), a concept termed bi-directional signalling. Evidence that phosphorylation of ephrinB ligands can also occur in tissues was provided by both groups who were able to immunoprecipitate tyrosine phosphorylated ephrinB ligands from mouse embryos (Holland *et al.*, 1996; Bruckner *et al.*, 1997). Since the cytoplasmic domain of ephrins does not possess intrinsic kinase activity, the kinase(s) responsible for the tyrosine phosphorylation is unknown. Although able to phosphorylate ephrinB ligands *in vitro* (Holland *et al.*, 1996; Bruckner *et al.*, 1997), a role for Src family kinases in *in vivo* phosphorylation of ephrinB ligands is unclear due to their slower rate of activation compared with that of ephrinBs (Bruckner *et al.*, 1997). Tyrosine phosphorylation of ephrinB1 was also observed following the addition of platelet-derived growth factor (PDGF) (Bruckner *et al.*, 1997), and following *in vitro* incubation with glutathione S-transferase (GST)-Tie2 (Adams *et al.*, 1999), but the significance of these observations is also unclear. A recent study demonstrated that presentation of the ectodomain of EphB2 to ephrinB1 resulted in the recruitment of a serine/threonine kinase to the ephrinB1 ligand via its interaction with the PDZ protein GRIP (Bruckner *et al.*, 1999), implying that PDZ proteins may be involved in the phenomenon of ephrinB-mediated reverse signalling.

Genetic evidence in support of reverse signalling was provided by studies on EphB2 deficient mice, which show defective anterior commissure formation (Henkemeyer *et al.*, 1996). However, mice expressing mutant EphB2 receptors lacking the kinase

domain displayed correct guidance of anterior commissure axons, which, together with the localisation of ephrinB1 to the migrating axons implied that signalling pathways regulating proper commissure formation were being activated in the ephrinB1-expressing axons by EphB2 extracellular domain-expressing cells in the adjacent territory (Henkemeyer *et al.*, 1996). Additional evidence for kinase-independent signalling comes from recent studies in *C. elegans*. Since mutations affecting the kinase domain of the *C. elegans* Eph receptor VAB-1 caused weaker phenotypes compared to *vab-1* null mutations (George *et al.*, 1998), it was proposed that VAB-1 may have both kinase-dependent and -independent functions (George *et al.*, 1998), similar to the scenario in EphB2 mutant mice (Henkemeyer *et al.*, 1996). Mutations of one of the four *C. elegans* ephrin genes, *efn-1*, synergised strongly with *vab-1* kinase domain mutations, again implying a kinase-independent role for VAB-1. This is somewhat surprising, as all four of the *C. elegans* ephrins, being GPI-linked (Wang *et al.*, 1999) lack any cytoplasmic residues. Moreover, recent reports suggest that ephrinA5 is also capable of eliciting intracellular signalling following stimulation with EphA5-Fc, resulting in an increase in cell adhesion concomitant with an increase in tyrosine phosphorylation within lipid-rich caveolae (Davy *et al.*, 1999; Davy and Robbins, 2000). Stimulation of ephrinA2 or ephrinA5 by EphA3 has also been demonstrated to result in the tyrosine phosphorylation of a 120 kDa membrane raft protein (Huai and Drescher, 2001). Indeed, the 10 minute lag phase preceding the increased tyrosine phosphorylation of a 120 kDa protein following EphA3 treatment may well be the result of changes in the structure or content of lipid rafts leading to activation of signalling components (Huai and Drescher, 2001). Signal transduction via GPI-linked ephrins may occur in conjunction with other transmembrane proteins or co-receptors, as has been postulated to occur for the GPI-linked ciliary neurotrophic factor (CNTF) receptor (Davis *et al.*, 1991) and the glial cell-derived neurotrophic factor (GDNF) receptor (Jing *et al.*, 1996; Treanor *et al.*, 1996).

1.1.4. Signalling Through Eph Receptors

1.1.4.1. Ligand-Receptor Binding

Eph receptor activation, whether by cell-cell juxtaposition or engagement of soluble ligand, is proposed to involve a two-step process whereby ligand-receptor interactions facilitate localised Eph receptor clustering and, only on reaching a critical threshold of Eph receptor concentration is oligomerisation (putatively mediated by the extracellular

membrane-proximal region), and subsequent autophosphorylation, able to occur (Lackmann *et al.*, 1998). In accordance with this, truncated or kinase-deficient receptors act in a dominant negative manner by dimerising with full length receptors and thus impairing the ability of dimers to autophosphorylate (Xu *et al.*, 1995; Xu *et al.*, 1996; Smith *et al.*, 1997; Durbin *et al.*, 1998).

Biochemical analysis by Lackmann and colleagues (Lackmann *et al.*, 1997) has demonstrated that the stoichiometry of the ephrin:Eph interaction is 1:1, implying that additional factors may be involved in the formation of a stable Eph receptor dimer. Similar to ephrinB ligands, Eph receptors may also be held in pre-assembled complexes via interactions with PDZ proteins. Such complexes occur in neuronal synapses where, for example, the NR2 subunits of the N-methyl-D-aspartate (NMDA) receptor are thought to be clustered via interactions with the synapse-associated protein SAP90 (Kim *et al.*, 1995a). More recently, the PDZ protein afadin (AF-6) has been demonstrated to interact and co-cluster with a number of Eph receptors in transfected cells, and to co-localise in hippocampal postsynaptic membranes (Buchert *et al.*, 1999). However, although such pre-assembly may occur, a mechanism must also exist for Eph receptor activation to occur as a consequence of ephrin-induced receptor oligomerisation. A delayed time course for the activation of EphB2 by ephrinB1 has been proposed to be a function of the assembly of receptor oligomers (Gale and Yancopoulos, 1997), although this may vary according to the cell type and the ligand-receptor interaction being studied.

1.1.4.2. Activation of Eph Receptor Tyrosine Kinase Activity

Stimulation of the catalytic activity of receptor tyrosine kinases is generally initiated by ligand-induced receptor oligomerisation or re-positioning to facilitate the juxtaposition of the cytoplasmic domains of the receptors. Such positioning is thought to enable autophosphorylation to occur in *trans* between receptors, resulting in the phosphorylation of tyrosine residues in the activation segment of the kinase domain and within the cytoplasmic domain. These phosphorylation events lead to both stimulation of catalytic activity and the creation of substrate docking sites (Ullrich and Schlessinger, 1990).

Recently, Binns and colleagues (Binns *et al.*, 2000) have employed site-directed mutagenesis in both *in vitro* and cellular assays to investigate the involvement of

phosphotyrosine residues in Eph receptor activation. Similar to most other RTKs, mutation of a conserved tyrosine residue within the putative activation loop of the kinase domain significantly decreased kinase activity. However, phosphorylation of the highly conserved juxtamembrane tyrosine residues is also apparently necessary for full Eph receptor activation, as previously observed by Zisch and colleagues (Zisch *et al.*, 1998). Furthermore, additional sites of autophosphorylation within the kinase domain of Eph receptors have also been reported (Choi and Park, 1999; Kalo and Pasquale, 1999a), suggesting a complex mechanism for the regulation of Eph receptor kinase activation.

Many proteins have been identified that are capable of binding to the cytoplasmic domain of Eph receptors. Such substrates may bind only when the receptors are activated, i.e. are dependent upon Eph receptor phosphorylation, whereas others may be able to associate via phosphorylation-independent means, for instance, through PDZ or SAM domain interactions.

1.1.4.3. Phosphorylation-Independent Binding Partners of Eph Receptors

Thus far, a number of Eph receptor-PDZ protein associations have been reported (see Section 1.1.4.1) and, although there have been no reports of any PDZ proteins interacting with EphA2, it is likely that such interactions will be identified. As previously discussed (Section 1.1.4.1), interactions with PDZ proteins may serve to hold Eph receptors in a pre-assembled complex to facilitate receptor oligomerisation. An alternative or additional function for Eph receptor-PDZ protein interactions may be to target Eph receptors to specific signalling complexes where many proteins can be held together in what is sometimes referred to as a 'signalosome'. Within such complexes, signalling components are in close proximity to each other and may associate or dissociate depending on their phosphorylation status (Pawson and Scott, 1997). Thus, a constitutive Eph receptor-PDZ protein interaction may occur in the absence of receptor activation, but ephrin-induced receptor tyrosine kinase activity may modify the components in a phosphorylation-dependent manner, as described in Section 1.1.4.4. Furthermore, different Eph receptor-PDZ interactions in different subcellular locations may play additional roles in regulating the interactions of Eph receptors with their substrates. In *C. elegans*, mislocalisation of the RTK Let-23 by mutation of any one of three PDZ proteins results in defective signalling (Torres *et al.*, 1998), emphasising the importance of localised signalling.

1.1.4.4. Phosphorylation-Dependent Binding Partners of Eph Receptors

Autophosphorylation of residues within the cytoplasmic domain of activated RTKs mediates subsequent phosphorylation-dependent intracellular signalling. Many cytoplasmic substrates are able to bind to such phosphorylated tyrosine residues and in turn recruit or modify other components. This results in propagation of the initial signal via protein-protein interactions in a signalling cascade (reviewed in Pawson, 1995). The cytoplasmic domain of Eph receptors contains 12 conserved tyrosine residues (Zhou, 1998) which, if phosphorylated, may serve as binding/docking sites for signalling components that contain Src-Homology 2 (SH2) or phosphotyrosine binding (PTB)/phosphotyrosine interacting domains (PID). Such modules recognise not only the phosphorylated tyrosine residue but also short amino acid sequences (up to 8 residues) either amino- or carboxy- terminal to it (Pawson and Scott, 1997), which may therefore function to maintain specificity between the 14 different Eph receptors. Substrates interacting with phosphorylated Eph receptors include enzymes, adaptor proteins and structural proteins (reviewed in van der Geer *et al.*, 1994). Additionally a number of novel proteins have also been identified in the pursuit of Eph binding partners. Several of these substrates have the potential to influence the behaviour of the cytoskeleton, and may therefore impinge on aspects of cell adhesion and migration, as has been reported in many cases (see Section 1.1.5). Substrates that interact directly with Eph receptors are listed in Table 1.1, whilst EphA2 binding partners are described below in Section 1.1.4.4.1.

Additionally, Eph receptor-mediated tyrosine phosphorylation of proteins that are implicated in cell morphology, such as R-ras (Zou *et al.*, 1999) and L1 (Zisch *et al.*, 1997) has been demonstrated, although it is unclear whether these proteins bind directly to the activated receptors. A number of Eph receptors have also been reported to influence the activity of components of the so-called mitogen-activated protein kinase (MAPK) modules, most notably causing activation of c-Jun N-terminal kinase (JNK) (Stein *et al.*, 1998b; Becker *et al.*, 2000) and extracellular signal-regulated kinase (ERK)/MAPK (Zisch *et al.*, 2000), and, more recently, reverse-signalling through ephrinA5 has been demonstrated to activate p42 MAPK and p44 MAPK (Davy and Robbins, 2000), suggesting that Eph- and ephrin- mediated signalling might be involved in the regulation of pathways that control transcriptional changes.

Substrate	Eph receptor	Reference	Significance/potential function
Fyn	EphA3 EphA4 EphA8 EphB3	(Zisch *et al.*, 1998) (Ellis *et al.*, 1996) (Choi and Park, 1999) (Hock *et al.*, 1998)	Can interact with WASP. May regulate cell shape, adhesion and migration (similar to Src and Yes) (Parsons and Parsons, 1997)
Src	EphA3 EphA4 EphB2 EphB5	(Zisch *et al.*, 1998) (Holland *et al.*, 1997)	Interacts with, and is able to phosphorylate, components of focal adhesions. Negatively regulates the ability of cortactin to cross-link actin.
Yes	EphA3 EphA4 EphB2 EphB5	(Zisch *et al.*, 1998)	Similar to Src and Fyn.
PLCγ	EphA4 EphB2	(Kalo and Pasquale, 1999b) (Holland *et al.*, 1997)	May effect cytoskeletal organisation via increased intracellular calcium concentration.
p120 GAP	EphB2 EphB3	(Holland *et al.*, 1997) (Hock *et al.*, 1998)	Stimulates hydrolysis of GTP-bound Ras. Constitutively associated with RhoGAP, and may regulate its activity (McGlade *et al.*, 1993). Mediates complex formation between Nck and $p62^{dok}$
Crk	EphB3	(Hock *et al.*, 1998)	Binds to RasGEFs. Capable of interacting with CAS to promote Rac-dependent migration and invasion (Klemke *et al.*, 1998).
Nck	EphB1	(Stein *et al.*, 1998b)	Required for axonal pathfinding in *Drosophila* (Garrity *et al.*, 1996) Interacts with PAK and WASP.
Grb2	EphB1	(Stein *et al.*, 1996)	Recruits Sos to plasma membrane to bring into proximity of Ras. Sos also stimulates activity of Rac (Nimnual *et al.*, 1998)
Grb10	EphB1	(Stein *et al.*, 1996)	Shares a region of homology with the *C. elegans* Mig-10 protein, which is required for migration during embryogenesis (Manser *et al.*, 1997).
LMW-PTP	EphB1 EphB2	(Stein *et al.*, 1998a)	Negatively regulates mitogenesis? (Chiarugi *et al.*, 1998)
R-Ras	EphB2	(Zou *et al.*, 1999)	R-Ras activity promotes integrin function (Zhang *et al.*, 1996a). Phosphorylation of R-Ras inhibits its activity, thus compromising cell-ECM interactions.
SHEP (SH2 domain-containing Eph receptor-binding protein 1)	EphB2	(Dodelet *et al.*, 1999)	Contains an N-terminal SH2 and a C-terminal GEF domain. Binds the GTPases R-Ras and Rap1A, thus may mediate integrin function and endo/exocytosis, respectively (Bos, 1998)

Table 1.1. Summary of Phosphorylation-Dependent Eph Receptor Substrates
Adapted from Kalo and Pasquale, 1999b

1.1.4.4.1. Binding Partners of EphA2

The first Eph receptor substrate to be identified was the β isoform of the p85 subunit of phosphoinositol 3-kinase (PI3K) (Pandey *et al.*, 1994). The interaction was detected using the cytoplasmic domain of EphA2 as bait in a yeast two-hybrid screen and is thought to occur via the carboxy-terminal SH2 domain of p85. Although the tyrosine residue mediating this interaction has not been mapped, it is likely to reside in the kinase domain of EphA2, consistent with the presence of two YXXM motifs (Songyang *et al.*, 1993) at sites 686 and 848 (corresponding to the mouse sequence) in the kinase domain of EphA2. Moreover, stimulation of smooth muscle cells with ephrinA1Fc resulted in an increase in PI3K activity (Pandey *et al.*, 1994), which may have implications in cytoskeletal rearrangement, in particular, Rac-induced membrane ruffling (Ridley *et al.*, 1992).

Characterisation of other positive clones from the same yeast two hybrid screen that identified the EphA2-PI3K interaction also led to the discovery of a novel protein which contains SH2 and Src homology 3 (SH3 motifs) bearing strong homology to those of Src family kinases, but lacks a catalytic domain, and thus was named Src-like adaptor protein, SLAP (Pandey *et al.*, 1995a). Like p85, SLAP also binds EphA2 in an ephrinA1Fc-dependent manner. SLAP has subsequently been found to act as a negative regulator of PDGF-induced mitogenesis in fibroblasts (Roche *et al.*, 1998) and of T-cell receptor signalling (Sosinowski *et al.*, 2000).

EphA2 has been reported to constitutively associate with focal adhesion kinase (FAK), a non-receptor tyrosine kinase present at regions known as focal adhesions where cells are closely associated with the extracellular matrix through integrins (Miao *et al.*, 2000). Upon stimulation with ephrinA1Fc, FAK became dephosphorylated and the association with EphA2 was lost. This was demonstrated to negatively affect cell-ECM interactions, cell spreading and cell migration (Miao *et al.*, 2000) (see Section 1.1.5.2). Prior to FAK dephosphorylation, the protein tyrosine phosphatase Shp2 was recruited to EphA2, presumably via one of its two SH2 domains. Interestingly, another protein tyrosine phosphatase, LMW-PTP, is also found to coimmunoprecipitate with the EphA2 receptor, but the association appears to be constitutive, as it occurs irrespective of ephrinA1Fc-induced EphA2 phosphorylation (Miao *et al.*, 2000). The mechanism by which EphA2 and phosphorylated FAK associate is unclear and it is quite feasible that FAK is indirectly associated with EphA2 via a component of a larger protein complex.

1.1.5. Cellular Studies - Consequences of Signalling Through Eph Receptors

Roles for Eph receptors in several cellular functions, as outlined below, have been demonstrated.

1.1.5.1. The Involvement of Eph Receptors in Cell-Cell Interactions

The first strong evidence that Eph-ephrin interactions were able to influence cell-cell interactions came to light when ephrinA5 was identified as a repellent molecule in the *in vitro* stripe assay and hence was originally called RAGS (repulsive axon guidance signal) (Drescher *et al.*, 1995). Membranes from mock- or ephrinA5- transfected COS cells were used to coat a laminin substrate in alternating stripes. Retinal axons grew preferentially on the membranes prepared from the mock-transfected COS cells as a result of being repelled away from the ephrinA5-prepared membranes (Drescher *et al.*, 1995). In a similar experiment, retinal axons were demonstrated to avoid ephrinA2-containing membranes (Nakamoto *et al.*, 1996). Likewise, stripe assays using immobilised ephrinB1Fc or ephrinB2Fc alternating with an Fc control were used to demonstrate the repulsion of both neural crest cells and motor axons by these ephrins (Wang and Anderson, 1997).

Activation of an EGFR/EphA4 receptor chimera in *Xenopus* embryos resulted in loss of cell adhesion at the blastula stage (Winning *et al.*, 1996). Co-expression of a kinase dead chimeric receptor prevented this dissociation, as did co-expression of C-cadherin, suggesting that cadherins may be a target of Eph receptors (Winning *et al.*, 1996). Conversely, co-incubation of EphB3-expressing and ephrinB1-expressing 32D myeloid cells resulted in the formation of cell aggregates (Bohme *et al.*, 1996), which could be inhibited by the addition of soluble ephrinB1Fc. This difference in outcome, i.e. dissociation versus aggregation, may be due to cell-type specific effects, such that some cell types recruit different substrates to the activated receptors, or it may be due to contact occurring between cells that facilitates ligand-receptor interaction but not receptor activation (see Section 1.1.5.2 on mechanical tethering).

A recent elegant study has demonstrated that cell mingling between neighbouring populations is prevented by Eph-ephrin-mediated bidirectional signalling, whereas unidirectional signalling serves only to repel one cell population, whilst allowing the

other to encroach into the adjacent cells (Mellitzer *et al.*, 1999). Evidence from the same study suggests that interactions between ephrins and their receptors are also capable of restricting cell-cell communication through gap junctions, most likely due to repulsive/de-adhesive effects preventing the assembly of connexins into intercellular channels (Mellitzer *et al.*, 1999). Among the potential consequences of impaired gap junction formation are effects on cell proliferation, differentiation and tissue patterning (Simon and Goodenough, 1998). Interestingly, rhombomeres of the hindbrain possess their own segmental identity which may correlate with the absence of gap junctions at rhombomere boundaries that are established at the interface of Eph-ephrin interactions (see Section 1.1.7.2).

1.1.5.2. The Involvement of Eph Receptors in Cell-ECM Interactions

In addition to influencing cell-cell behaviour, Eph-ephrin interactions are also thought to play a role in regulating the interaction of cells with their surrounding ECM environment. There are, however, conflicting reports concerning the ability of ephrins to either promote or inhibit adhesion and/or spreading of Eph receptor-expressing cells on their substrates. EphA2-expressing prostate epithelial cells were inhibited in their migration towards fibronectin by ephrinA1Fc (Miao *et al.*, 2000). This result is consistent with the induction of cell rounding in adherent cells treated with ephrinA1Fc, and with the inhibition of integrin-mediated adhesion and spreading of cells plated onto fibronectin containing an immobilised source of ephrinA1Fc (Miao *et al.*, 2000). Furthermore, decreased cell-ECM attachment upon treatment with ephrinA1Fc has also been demonstrated in mammary epithelial cells (Dodge-Zantek *et al.*, 1999). Many more recent studies have also implicated Eph signalling in the disruption of cell-ECM attachment. Activation of EphB2 in 293T cells resulted in partial substrate detachment and a rounded cell morphology compared with 293T cells transfected with a kinase-dead form of EphB2, and treatment of EphB2-transfected cells with ephrinB1Fc caused a reduction of cell adhesion (Zou *et al.*, 1999). Similar observations were made by Zisch and colleagues, and Binns and colleagues, using 293 and NG108-15 cells, respectively (Binns *et al.*, 2000; Zisch *et al.*, 2000), and overexpression of EphA8 was also shown to attenuate cell attachment responses 293 cells (Choi and Park, 1999).

However, a number of groups have reported an increase in cell-substrate adhesion in response to Eph receptor activation (Stein *et al.*, 1998a; Stein *et al.*, 1998b; Huynh-Do *et al.*, 1999; Becker *et al.*, 2000; Zisch *et al.*, 2000), and more recently, reverse

signalling through ephrinA5 has been demonstrated to increase adhesion by promoting integrin function (Davy *et al.*, 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). At least in some cases, the response may depend on the specific ephrin-Eph interaction being studied, the cell types being used, and the method used to detach the cells before assessing their adhesion. Recent data by Miao and colleagues (Miao *et al.*, 2000) demonstrated that the method of ephrin presentation to the cells may also influence the response. Immobilised ephrinB1Fc and ephrinA1Fc were found to artefactually promote adhesion of cells expressing EphB1 and EphA2, respectively, by mechanical tethering caused simply by the interaction of Ephs and ephrins. Although this may explain some of the contradictory findings, a study published by Stein and colleagues found a dramatic difference in the attachment response of EphB1-expressing endothelial cells depending on whether the ephrinB1 ligand was presented as a dimer or a multimer (Stein *et al.*, 1998a). Only tetramers were able to induce cell attachment, and also to recruit LMW-PTP to phosphorylated receptor complexes. An attractive theory has recently been proposed to explain such differences (Wilkinson, 2000a). For a repulsive signal to occur, activation of the Eph receptor must be above a certain threshold, but the nature of the repulsive response does not facilitate the assembly of multimers. However, in situations where the threshold cannot be reached, the interaction between the ephrin and its receptor mediates cell-cell adhesion and enables higher order Eph oligomers to form. This theory might also explain the recent findings of Holmberg and colleagues in which kinase-deficient splice variants of the EphA7 receptor were found to promote closure of the neural tube by adhering to ephrinA5-expressing cells (Holmberg *et al.*, 2000).

1.1.5.3. The Involvement of Eph Receptors in Cytoskeletal Rearrangement

Changes in the cytoskeleton are brought about by the actions of the small GTPases Rho, Rac and Cdc42, the activation of which brings about the formation of stress fibres, lamellipodia and filopodia, respectively (Nobes and Hall, 1995; Hall, 1998). Since the demonstration that many different ephrin ligands were capable of inducing growth cone collapse in a variety of neuronal cell types (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Meima *et al.*, 1997; Wang and Anderson, 1997) Eph-mediated signalling has been proposed to affect pathways controlling cytoskeletal regulation that are therefore able to bring about rapid changes in cell morphology. Indeed, studies by Meima and colleagues (Meima *et al.*, 1997), using fluorophore-conjugated phalloidin to detect filamentous actin, showed that immediately prior to growth cone collapse, there is a

rapid redistribution of actin away from filopodia and lamellipodia concentrated at the tips of the growth cone into the centre of the growth cone. Studies by Wahl and colleagues revealed that activation of the small GTPase Rho and its downstream effector Rho kinase, concomitant with downregulation of Rac activity, was responsible for the ephrinA5-mediated collapse of retinal ganglion cells (Wahl *et al.*, 2000). Changes in the actin cytoskeleton have also been reported in response to receptor activation in 293 cells transiently transfected with EphB2 (Zisch *et al.*, 2000), and neuronal NG108-15 stably expressing EphB2 (Binns *et al.*, 2000). The signalling pathways responsible for mediating Eph receptor-induced changes in the activity of the small GTPases are not yet known, although a number of downstream substrates found to associate with Eph receptors in a ligand-dependent manner are likely candidates (see Table 1.1).

1.1.5.4. The Involvement of Eph Receptors in Cell Proliferation

A role for Eph receptor activation in mitosis was initially postulated on the basis that the first Eph receptor to be cloned, EphA1, was found to be expressed in a number of human carcinomas (Hirai *et al.*, 1987). Subsequent studies by Maru and colleagues (Maru *et al.*, 1990) implicated EphA1 in cell transformation due to its ability to confer anchorage independent growth when overexpressed in NIH3T3 cells. However, no evidence for a role in mitosis was provided and despite the fact that a large number of Eph receptors are found to be overexpressed in a variety of different tumours (see Section 1.1.7.6), activation of Eph receptors is not thought to be mitogenic. This is supported by evidence from studies by Lhotak and Pawson using an EGF receptor/EphB1 receptor chimera which, upon activation with EGF, caused no significant change in the growth properties of transfected NIH3T3 cells (Lhotak and Pawson, 1993). A mitogenic response was not observed when EphA2-expressing cells were treated with ephrinA1 (Bartley *et al.*, 1994). Similarly, ligands failed to induce focus formation in EGF/EphA3 or TrkB/EphA3 chimeric assays (Brambilla *et al.*, 1995). Stimulation of a human glioblastoma cell line overexpressing EphA5 with ephrinA1Fc induced receptor phosphorylation, but again failed to induce cell proliferation (Bruce *et al.*, 1999). In the majority of cases where Eph receptors have been artificially overexpressed to levels where ligand-independent tyrosine phosphorylation is induced, changes in cell morphology have been the predominant observation.

However, in some cases, increased proliferation has been reported upon ligand-induced receptor activation, most likely depending on the cell type studied. EphrinA1 was able to stimulate the growth of two melanoma cell lines which overexpressed the EphA2 receptor (Easty *et al.*, 1995). EphrinA1 was also reported to stimulate the growth of a colonic adenocarcinoma cell line (Rosenberg *et al.*, 1997). EphrinB2 has also been reported to induce the proliferation of EphB4-expressing umbilical cord blood mononuclear cells and to increase DNA synthesis in a haematopoietic cell line (Sakano *et al.*, 1996).

1.1.6. Expression Patterns of Eph Receptors and Ephrins

A combination of all Eph receptors and their ephrin ligands is expressed in most, if not all, tissues during embryogenesis (in the mesoderm, endoderm and ectoderm) and in a number of adult tissues. A study of the expression of Eph receptors and ephrins during mouse development was carried out by Gale and colleagues (Gale *et al.*, 1996a), who made use of the ability of tagged fusion proteins of ligand- and receptor- bodies to bind to their respective Eph receptors and ephrin ligands. This revealed that EphA receptors and ephrinA ligands are expressed reciprocally, as are EphB receptors and their ephrinB ligands, giving the impression that Eph-ephrin interactions only occurred at distinct regions where the site of expression of one encountered that of the other. However, further conventional studies have revealed that Eph receptors and their ligands can be co-expressed within the same tissue (e.g. within the mammary gland (Nikolova *et al.*, 1998)) and even within the same cell type e.g. endothelial cells (Pandey *et al.*, 1995b), melanoma cells (Easty *et al.*, 1995), Caco-2 cells (Rosenberg *et al.*, 1997) and mammary tumour cells (Ogawa *et al.*, 2000). Such co-expression, which is most likely undetected by receptor- or ligand- bodies due to ligand or receptor binding sites being "masked" by endogenous interactions (Sobieszczuk and Wilkinson, 1999), may lead to receptor desensitisation, with potentially important consequences in cell positioning (Hornberger *et al.*, 1999).

Within many tissues, there can be a gradient of Eph or ephrin expression. For instance, ephrinA5 and ephrinA2 are expressed in an increasing anterior to posterior gradient in the developing tectum (Drescher *et al.*, 1995; Monschau *et al.*, 1997). Such gradients are known to play a critical role in the precise positioning of cells, such as occurs during the establishment of retinotectal connections (reviewed in Wilkinson, 2000b; see also Section 1.1.7.1).

Many of the Eph receptors and their ephrins are expressed predominantly in the developing and adult brain, where they are known to play a role in the development of the nervous system. However, Eph and ephrin expression is not exclusive to the brain, and more recently an important role for Eph signalling in the development of the vasculature has also been discovered (see Section 1.1.7.5). During embryogenesis expression of Eph receptors and ephrins can also be observed in a number of tissues, including limb buds and somites. For a detailed account of the precise expression patterns of Ephs and ephrins other than those of EphA2 and ephrinA1, please refer to a recent detailed review by Zhou (Zhou, 1998).

1.1.6.1. Localisation of EphA2

The distribution of EphA2 protein in the developing mouse embryo has been studied by immunohistochemical staining using anti-EphA2 antibodies (Ganju *et al.*, 1994). EphA2 protein is first detected in the primitive streak of 7.5 dpc embryos undergoing gastrulation, with particularly strong EphA2 staining in notochordal cells aggregating in the midline. EphA2 protein is also present in increasing levels along the rostrocaudal axis of both the notochord and developing neural tube. In the developing hindbrain, EphA2 protein is detectable in the neuroepithelia, both before and after segmentation of rhombomere 4, implying that it may function in some aspect of rhombomere specification. Subsequent to segmentation, EphA2 protein is transiently expressed in neural crest cells of the second and third branchial arches, with barely detectable levels in these regions by 10.5 dpc. High levels of EphA2 protein are also observed in presumptive trunk neural crest cells in 9.5 dpc embryos. Expression of EphA2 protein is also found in the distal mesenchyme of both fore- and hind- limb buds at 10.5 dpc, with EphA2 protein localising to hand and foot plate regions in 11.5 dpc embryos. Also at 10.5 dpc, expression of EphA2 protein is observed in foetal tissue epithelia of the lung. Embryonic epithelia of the limb, tongue, thymus and submandibular gland have also been reported to express EphA2 protein.

Analysis of EphA2 transcripts in the developing mouse and adult by northern blotting and *in situ* hybridisation has revealed that EphA2 gene expression is largely consistent with the localisation of EphA2 protein as detected by immunohistochemistry (Lindberg and Hunter, 1990; Becker *et al.*, 1994; Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Mori *et al.*, 1995). Similarly, β-galactosidase staining patterns observed following

targeting of the EphA2 gene with a functional lacZ gene (Chen *et al.*, 1996; Michael *et al.*, 1999) also appear to reflect protein expression and localisation.

In the adult mouse, EphA2 mRNA is detected at high levels in the lung, skin, small intestine and ovary, and at lower levels in the kidney, brain, spleen and submaxillary gland (Lindberg and Hunter, 1990; Ganju *et al.*, 1994). EphA2 expression has also been reported in the mammary gland (Andres *et al.*, 1994). A number of other tissues contain very low levels of EphA2 mRNA (Lindberg and Hunter, 1990; Ganju *et al.*, 1994). Consistent with the original naming of EphA2 as epithelial cell kinase, EphA2 protein expression has been observed in several cell lines of epithelial origin, such as HeLa cells, keratinocytes, prostate cells, colon carcinoma cells and normal and transformed mammary cells (Lindberg and Hunter, 1990; Andres *et al.*, 1994; Rosenberg *et al.*, 1997; Dodge-Zantek *et al.*, 1999; Walker-Daniels *et al.*, 1999; Miao *et al.*, 2000; Ogawa *et al.*, 2000). EphA2 mRNA is also expressed in embryonic stem (ES) cells (Ganju *et al.*, 1994), and EphA2 protein has been detected in melanomas (Easty *et al.*, 1995; Easty *et al.*, 1999) and endothelial cells (Pandey *et al.*, 1995b).

1.1.6.2. Localisation of ephrinA1

For the occurrence of a true *in vivo* interaction, an Eph receptor and its ephrin ligand are expected to be expressed within the same regions due to the membrane-bound nature of both the receptor and ligand. The expression pattern of ephrinA1 in developing mouse and rat embryos is very similar to that of EphA2 in the mouse, with ephrinA1 transcripts being detected in the lung, kidney, intestine and skin in the later stages of organogenesis (Shao *et al.*, 1995; Takahashi and Ikeda, 1995). Additional expression is also seen in the salivary gland, cells in the developing tooth, hypertrophic chondrocytes, osteoblasts and in the dorsal root ganglia (Shao *et al.*, 1995; Takahashi and Ikeda, 1995). Interestingly, during the early part of development, ephrinA1 transcripts are found in mesoderm derived from the primitive streak and endothelial cells of the developing vasculature (McBride and Ruiz, 1998), whereas expression switches to epithelial cells in the later stages (Takahashi and Ikeda, 1995).

EphrinA1 protein has been detected in melanocytes and melanoma cells (Easty *et al.*, 1995), and increased levels of ephrinA1 and EphA2 protein have been reported during melanoma progression (Easty *et al.*, 1999). EphrinA1 is also co-expressed with EphA2

in endothelial cells (Pandey *et al.*, 1995b), in colon carcinoma cells (Rosenberg *et al.*, 1997), and in mammary tumours (Ogawa *et al.*, 2000).

1.1.6.3. Regulation of EphA2 Expression

The expression of EphA2 in the primitive streak and in prospective r4 coincides with that of Hoxa1 and Hoxb1, members of the Hox family of transcription factors. A 0.9 kb sequence of EphA2 genomic DNA was found to contain a rhombomere-specific enhancer to which Hoxa1 and Hoxb1, in combination with the Pbx cofactor, were able to bind *in vitro* and induce expression of a reporter gene in a cell based assay (Chen and Ruley, 1998). Furthermore, mice with null mutations in both *hoxa1* and *hoxb1* express markedly decreased levels of EphA2 (Gavalas *et al.*, 1998; Studer *et al.*, 1998), suggesting that these transcription factors regulate aspects of EphA2 expression in r4. However, additional *cis*-regulatory elements must also exist, as the 0.9 kb genomic sequence was insufficient to drive EphA2 expression in the node (Chen and Ruley, 1998).

In E-cadherin$^{-/-}$ ES cells, levels of EphA2 mRNA and protein are downregulated, whereas ectopic expression of E-cadherin in NIH3T3 cells increased the normally low levels of EphA2 protein (Orsulic and Kemler, 2000). Moreover, EphA2 in E-cadherin$^{-/-}$ ES cells is localised to the perinuclear and Golgi regions, rather than at the cell periphery as observed in wild-type ES cells (Orsulic and Kemler, 2000). Differences in EphA2 localisation have also been observed between normal and neoplastic mammary epithelial cells, with EphA2 being localised to cell-cell contacts in normal cells, in contrast to a diffuse distribution in neoplastic cells (Dodge-Zantek *et al.*, 1999). Also in mammary tissue, upregulation of EphA2 mRNA expression in response to the *ras* oncogene has been reported (Andres *et al.*, 1994). EphA2 mRNA expression was also reported to be positively regulated by cytokines and EGF in Caco-2 colon carcinoma cells, although the increase in EphA2 mRNA was only transient (Rosenberg *et al.*, 1997). Factors that have been reported to regulate the expression of other Eph receptors include Hoxa2 (Taneja *et al.*, 1996), Krox-20 (Theil *et al.*, 1998), retinoic acid and bone morphogenetic protein 2 (Patel *et al.*, 1996), and Valentino (a zebrafish homologue of Kreisler) (Cooke *et al.*, 2001).

1.1.6.4. Regulation of EphrinA1 Expression

EphrinA1 was cloned as an early response gene on the basis of its upregulation by the cytokines TNFα and IL-1 (Holzman *et al.*, 1990). Other ephrins are reported to be regulated by retinoic acid (Bouillet *et al.*, 1995) and *en-1* and *en-2*, homologues of the *Drosophila engrailed* segment polarity gene (Logan *et al.*, 1996; Shigetani *et al.*, 1997).

1.1.7. *In vivo* Studies –Consequences of Signalling Through Eph Receptors

The proposed roles of Eph receptor- and ephrin- mediated signalling have been mainly inferred from the detailed study of their *in vivo* expression patterns in conjunction with knockout studies and supporting *in vitro* data. The widespread expression of Eph and ephrin genes during embryogenesis, and subsequently in restricted tissues of the adult, implies a role in development and tissue patterning, achieved by means of dynamic changes in cell-cell and cell-substrate interactions. A number of systems in which Eph receptor signalling is known to function, although not necessarily involving EphA2, will be briefly described below.

1.1.7.1. Retinotectal Mapping

In searching for molecules involved in the formation of neuronal connections between the retina and the optic tectum, ephrinA2 and ephrinA5 were identified from the chick tectum on the basis of their ability to repel retinal axons in the membrane stripe assay and to induce growth cone collapse in such axons (Drescher *et al.*, 1995; Monschau *et al.*, 1997). Both ephrinA2 and ephrinA5 proteins are expressed in an increasing anterior to posterior gradient in the chick tectum, but ephrinA5 has a much steeper gradient than that of ephrinA2 and is restricted to the more posterior area of the tectum (Drescher *et al.*, 1995; Monschau *et al.*, 1997). In retinal axons, there is also an increasing anterior (nasal) to posterior (temporal) gradient of EphA3 expression (Cheng *et al.*, 1995). The retinotectal topographic map is thought to be established by the differential repulsion of retinal axons as they enter the tectum. Temporal retinal axons expressing high levels of EphA3 can migrate only a short distance before they are repelled by the low levels of ephrinA2 at the anterior of the tectum. Conversely, nasal retinal axons expressing low levels of EphA3 are only repelled upon encountering a combination of high concentrations of ephrinA2 and ephrinA5 at the posterior of the tectum. Doubtless, this is a gross oversimplification of a highly intricate system, and it is known that there are

additional Eph receptors and ephrins present both in gradients and evenly distributed throughout the retina (Hornberger *et al.*, 1999), the effects of which must be finely counterbalanced by the existence of attractive cues to establish precise axonal mapping. Gradients of ephrinB ligands and EphB2 are also expressed in a dorsoventral manner in the retina and tectum, suggesting that a similar scheme may also operate in establishing the dorsoventral axis, although the precise mechanism by which this occurs is unclear (reviewed in Wilkinson, 2000a). Furthermore, gradients of Eph receptors and ephrin ligands may underlie the establishment of additional topographic maps such as the projection of retinal axons into the lateral geniculate nucleus (Feldheim *et al.*, 1998), projections from the thalamus to the cortex (Gao *et al.*, 1998), from the septum to the hippocampus (Zhang *et al.*, 1996b), and from the substantia nigra to the striatum (Yue *et al.*, 1999).

1.1.7.2. Hindbrain Segmentation

The hindbrain is one of two regions of the vertebrate body axis that shows clear segmentation, giving rise to 7 or 8 repeated morphological units called rhombomeres upon the completion of gastrulation. Within each rhombomere, the cells are free to move, but cannot do so into neighbouring rhombomeres due to the formation of morphological boundaries that restrict cell movement. However, cells from rhombomeres 2, 4 and 6 (r2, r4 and r6) are able to intermingle, as can cells from r3 and r5. Interestingly, ephrinB1, B2 and B3 are expressed in r2, r4 and r6 (Bergemann *et al.*, 1995; Flenniken *et al.*, 1996; Gale *et al.*, 1996b) and EphA4, EphB2 and EphB3 are expressed predominantly in r3 and r5 (Nieto *et al.*, 1992; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994). EphA2 protein is transiently expressed in r4 (Ganju *et al.*, 1994), but as yet, no ephrinA ligands have been identified in the hindbrain.

It is widely thought that the morphological rhombomere boundaries are established at the sites of interaction between regions of alternate Eph and ephrin expressions. The first evidence implicating Eph receptors and ephrin ligands in the establishment of such boundaries came from studies in which expression of a dominant negative truncated EphA4 receptor into zebrafish embryos disrupted rhombomere formation (Xu *et al.*, 1995). Recent studies by Xu and colleagues demonstrated that this was a result of the impaired ability to restrict cell movement, rather than due to any effects on cell identity (Xu *et al.*, 1999a). Overexpression of full length or truncated EphA4 or ephrinB2 in r2, r4, r6 or r3, r5, respectively, resulted in the activation and subsequent sorting of cells to

their appropriate boundaries, sometimes from within the middle of the rhombomere. Whether or not bidirectional signalling is involved in boundary formation is unclear, as another recent study (Mellitzer *et al.*, 1999) concluded that activation of both full length receptor and ligand were required to prevent cell mixing, whilst activation of ligand or receptor alone resulted in the invasion of one cell population by the other. However, in both cases, communication through gap junctions was prevented (Mellitzer *et al.*, 1999). This is most likely due to the Eph-mediated repulsive or anti-adhesive effects preventing the formation of stable cell-cell contacts. Indeed, enlarged intercellular spaces are present between regions of Eph and ephrin expression in rhombomeres (Lumsden, 1999). Such restricted cell-cell communication between different rhombomeres may well provide a means for establishing segment identity within the rhombomeres.

The second region of the vertebrate body axis in which segmentation occurs is during the formation of the somites from the paraxial mesoderm. Similar to the alternating patterns of Eph and ephrin expression in the rhombomeres of the developing hindbrain, a number of Eph receptors and ephrins are expressed in an alternating pattern in the anterior and posterior halves of developing somites (Nieto *et al.*, 1992; Bergemann *et al.*, 1995; Irving *et al.*, 1996; Krull *et al.*, 1997; Wang and Anderson, 1997). Furthermore, injection of dominant negative receptor or ligand constructs into zebrafish embryos results in the formation of abnormal somite boundaries (Durbin *et al.*, 1998).

1.1.7.3. Neural Crest Cell Migration

Neural crest cells are derived from the most dorsal point of the neural tube and migrate to several regions in the body before they differentiate to form a number of cell types. The migration of cephalic/cranial neural crest cells to form the skeletal and muscular components of the vertebrate head occurs according to their rhombomeric origin. In the developing mouse hindbrain, EphA2 expression is transiently observed in r4 and in neural crest cells migrating from this region (Ganju *et al.*, 1994; Ruiz and Robertson, 1994). Similarly, EphA2 mRNA expression in *Xenopus* is observed in r4 and in migrating neural crest cells (Brandli and Kirschner, 1995; Weinstein *et al.*, 1996; Helbling *et al.*, 1998). In *Xenopus* embryos, branchial/visceral neural crest cells migrate in four adjacent, but separate, streams originating from r2-r6 to populate 4 branchial/visceral arches. It is important that the adjacent populations of migrating crest cells are prevented from mixing, as their rostrocaudal identity is already established.

Eph receptors and ephrins have been implicated in maintaining separation between the adjacent populations, similar to the situation that occurs during the formation of rhombomere boundaries in the hindbrain (see Section 1.1.7.2), most likely by means of repulsive cues. In *Xenopus* embryos, interaction between ephrinB2-expressing second arch neural crest cells and third arch EphA4 and EphB1-expressing neural crest cells has been demonstrated to be necessary in preventing third and second arch neural crest cells from intermingling (Smith *et al.*, 1997). Interestingly, EphA2 is expressed in migrating cells of the second branchial arch (Helbling *et al.*, 1998), whereas ephrinA ligand expression has been detected in all four branchial/visceral arch neural crest cell populations (Helbling *et al.*, 1998). However, overexpression of a truncated form of EphA2 resulted in the abnormal separation of third and fourth arch neural crest cells rather than any defects involving EphA2-expressing second arch cells (Helbling *et al.*, 1998). The reason for this is unclear, but it may be due to the sequestration by EphA2 of ephrinA ligands that signal to other Eph receptors involved in the separation of these two regions of arch neural crest cells.

Eph receptors and ephrins are also involved in the ventrolateral migration of trunk neural crest cells through the anterior half of each sclerotome. The expression of ephrinB ligands in the posterior half of the somites/sclerotome is thought to be responsible for repelling EphB3-expressing trunk neural crest cells, thus 'guiding' them through the anterior half (Krull *et al.*, 1997; Wang and Anderson, 1997), forming dorsal root and sympathetic ganglia. *In vitro* studies have demonstrated that ephrinB ligands inhibit the migration of trunk neural crest cells (Krull *et al.*, 1997; Wang and Anderson, 1997).

1.1.7.4. Limb Development

A potential role for EphA2 in limb development is suggested by its expression in the distal mesenchyme of fore- and hind- limb buds (Ganju *et al.*, 1994) following formation of the apical ectodermal ridge (AER). EphrinA1 expression also occurs in the developing limb, both in the distal mesenchyme (Flenniken *et al.*, 1996) and in the developing vasculature (McBride and Ruiz, 1998). Mesenchyme underlying the AER, known as the 'progress zone', is induced to proliferate by signals from the AER, thus enabling growth along the proximal-distal axis. Similarly, signals from the mesenchyme cells are responsible for sustaining the AER and determining the type of

limb to be formed. Thus, epithelial-mesenchymal interactions are important in the development of the proximal-distal axis of the limb.

EphA4 and EphA7 are also expressed in the limb bud mesenchyme (Patel *et al.*, 1996; Araujo *et al.*, 1998). The expression of EphA7 has been implicated in innervating distinct regions of the developing limb (Araujo *et al.*, 1998). The role of EphA4 in limb development is unclear, since, although targeted disruption of the EphA4 gene resulted in defective co-ordination of limb movement in homozygous mutant mice, this was attributed to defects in the corticospinal tract (Dottori *et al.*, 1998). Similarly, EphA2 mutant mice do not show any detectable defects in limb development (Chen *et al.*, 1996). It is possible that EphA2 may have a role, in conjunction with that of ephrinA1, in the development of the limb vasculature (see Section 1.1.7.5).

1.1.7.5. Vasculogenesis and Angiogenesis

An initial primitive vascular network arises during the process of vasculogenesis, in which angioblasts assemble to form endothelial cells, which, in turn, form a series of interconnected vessels. Additionally during vasculogenesis, the primordia of the heart and the major trunk vessels are formed. The subsequent process of angiogenesis then serves to extensively remodel this network, which involves processes of sprouting, branching and proliferation (Risau, 1997), resulting in the vascularisation of developing organs and the distinction between arteries, veins and capillaries.

Following its isolation as a gene upregulated in human umbilical vein endothelial cells (HUVECs) in response to cytokines (Holzman *et al.*, 1990), ephrinA1 has been implicated in the process of vasculogenesis. In early embryogenesis, ephrinA1 is expressed in the developing vasculature, including the dorsal aorta, primary head vein, intersomitic vessels and branchial arch arteries (Takahashi and Ikeda, 1995). Studies by Pandey and colleagues (Pandey *et al.*, 1995b) demonstrated that ephrinA1Fc was able to act as a chemoattractant for EphA2-expressing HUVECs, and to induce neovascularisation in normally avascular rat corneas. A subsequent study showed that ephrinA1 was able to induce HUVECs to assemble into capillary-like structures (Daniel *et al.*, 1996). More recently, the assembly of such capillary-like structures was shown to require elongation of endothelial cells, a process that was inhibited by the introduction of a dominant-negative form of EphA2 into HUVECs (Ogawa *et al.*, 2000). In the same study, a detailed analysis of the localisation of ephrinA1 and EphA2 in tumours

revealed that receptor and ligand were expressed in tumour cells and the surrounding endothelial cells, implicating both in tumour vascularisation, and suggesting that Eph-ephrin interactions between the tumour and its surrounding endothelium may be important in initiating angiogenesis (Ogawa *et al.*, 2000).

Direct evidence for the involvement of Eph receptors and ephrins in the development of the vasculature was originally provided by the lethal phenotype of ephrinB2 null mice (Wang *et al.*, 1998). Homozygous null mice died at E11 due to the failure of the primitive vascular network to be remodelled. EphrinB2 expression was detected in arterial endothelial cells, whereas the EphB4 receptor was expressed in endothelial cells of embryonic veins, leading to speculation that correct development of the vasculature required reciprocal signalling between both components, as neither veins nor arteries developed in the mutant mice (Wang *et al.*, 1998). However, although a subsequent independent study, also using ephrinB2 mutant mice, confirmed the lethal phenotype, the expression of additional Eph receptors and ephrins was also reported (Adams *et al.*, 1999), ephrinB1 and EphB3 being expressed in both embryonic arteries and veins, and ephrinB2 and EphB2 expression occurring in the adjacent mesenchyme. Interestingly, further analysis of EphB2/EphB3 double mutants (Orioli *et al.*, 1996) revealed a non-viable phenotype similar to that occurring in ephrinB2 null mice with defective angiogenesis (Adams *et al.*, 1999), implying a complex role, with possible Eph-ephrin interactions occurring both in *cis* and *trans* between arteries, veins and the surrounding mesenchyme (Gale and Yancopoulos, 1999). *In vitro* analysis demonstrated that ephrinB1 and ephrinB2 were able to induce sprouting of adrenal cortex-derived microvascular endothelial cells (Adams *et al.*, 1999).

1.1.7.6. Tumourigenesis

The overexpression in several different tumour types and cell lines derived from tumours of many Eph receptors strongly implicates them in some aspect of cellular transformation. The first Eph receptor, EphA1, was isolated from an erythropoietin-producing human hepatocellular carcinoma cell line on the basis of its homology to the tyrosine kinase domain of the v-*fps* viral oncogene, and found to be overexpressed in several human carcinomas including lung adenocarcinoma and colon, mammary and hepatocellular carcinomas (Hirai *et al.*, 1987). Subsequently, overexpression of several Eph receptors has been reported in tumours derived from a wide range of tissues (Maru *et al.*, 1988; Boyd *et al.*, 1992; Wicks *et al.*, 1992; Cance *et al.*, 1993; Iwase *et al.*,

1993; Kiyokawa *et al.*, 1994; Soans *et al.*, 1994; Ikegaki *et al.*, 1995; Robinson *et al.*, 1996). In particular, EphA2 is overexpressed in tumours from mammary gland (Andres *et al.*, 1994; Dodge-Zantek *et al.*, 1999; Ogawa *et al.*, 2000) and prostate (Walker-Daniels *et al.*, 1999), and in melanomas (Easty *et al.*, 1995). Overexpression of ephrinA1 also occurs in metastatic melanomas (Easty *et al.*, 1999), where it is reported to stimulate mitosis (Easty *et al.*, 1995), and more recently in breast, lung, gastric and kidney tumour and endothelial cells (Ogawa *et al.*, 2000). However, in most cases, Eph receptor activation does not stimulate proliferation (Lhotak and Pawson, 1993; Brambilla *et al.*, 1995; Bruce *et al.*, 1999; see also Section 1.1.5.4), implying that Eph receptor overexpression is likely to perform an alternative function in tumour progression. In view of the roles attributed to Eph receptors in the regulation of cell movement occurring, for example, in axonal guidance, hindbrain segmentation and neural crest cell migration, a role for Eph receptors in tumour cell invasion and metastasis is highly plausible.

Indeed, the level of overexpression of EphA2 correlates positively in many cases with the metastatic potential of the tumours in which it is expressed. Mammary tumours arising in transgenic mice in which oncogenic *ras* is overexpressed from a mammary-specific promoter are characterised by being metastatic, and these tumours display markedly upregulated levels of EphA2 mRNA. This is in contrast to highly differentiated, but poorly metastatic, mammary tumours derived from transgenic animals expressing the *myc* oncogene from the same promoter, in which EphA2 upregulation was not observed (Andres *et al.*, 1994). Similar observations were made in invasive mammary and prostatic tumours derived from non-transgenic mice (Dodge-Zantek *et al.*, 1999; Walker-Daniels *et al.*, 1999), and greater levels of EphA2 were also observed in metastatic melanomas than primary melanomas (Easty *et al.*, 1995).

Although the precise mechanism by which EphA2 activation may positively influence tumour cell metastasis is unclear, *in vitro* studies have implicated EphA2 in the negative regulation of cell-ECM attachment (Dodge-Zantek *et al.*, 1999; Miao *et al.*, 2000). EphA2 has also been reported to stimulate PI3K activity in smooth muscle cells, which may have implications on the activity of Rac (Ridley *et al.*, 1992), a small GTPase involved in the formation of membrane ruffles and lamellipodia. Induction of tumour vascularisation, in which both EphA2 and ephrinA1 have recently been implicated (Ogawa *et al.*, 2000), is also critical in metastasis.

The recent finding that Eph receptor signalling can prevent gap junction mediated cell-cell communication (Mellitzer *et al.*, 1999) may also be significant in tumourigenesis, as various disorders, including cancer, often display altered gap junctional intercellular communication (Simon and Goodenough, 1998).

1.1.8. Targeted Disruption of the EphA2 Gene

The EphA2 locus has been disrupted by the insertion of a gene trap retrovirus (Chen *et al.*, 1996). The mutation, designated *eck*^{i}, was identified by screening for genes that were developmentally regulated following *in vitro* differentiation of ES cells into which a β-galactosidase-encoding retrovirus had integrated. Being promoterless, these so-called 'gene trap' retroviral insertions usually occur in or near 5' expressed exons, thus disrupting expression of an endogenous gene. Indeed, following transmission of the mutant *eck*^{i} allele through the germ-line, its expression was evident in the primitive streak and in the hindbrain of homozygous mutant (eck$^{i/i}$) embryos (Chen *et al.*, 1996), thus reproducing the pattern of EphA2 expression seen by *in situ* hybridisation and immunohistochemical staining in wild-type mice (Becker *et al.*, 1994; Ganju *et al.*, 1994; Ruiz and Robertson, 1994). eck$^{i/i}$ mice were born to the correct Mendelian ratio, developed normally and were fertile, despite a severe deficiency in EphA2 protein (Chen *et al.*, 1996). A detailed examination of the derivatives of the branchial arch revealed no abnormalities, as did analysis of a number of adult tissues including the lung, kidney, intestines and liver (Chen *et al.*, 1996). Closer inspection of the site into which the provirus had integrated revealed the presence of an unidentified functional exon, designated exon 5.2, 1.8 kb upstream of the integration site. Further analysis indicated that exon 5.2 was located ~ 9.8 kb upstream of the previously published EphA2 cDNA. Moreover, cDNA and mRNA transcripts containing both exon 5.2 and EphA2 sequence were isolated. Further analysis of the cDNAs revealed that the 5' splice site of exon 5.2 was joined to nucleotide 179 of the previously published EphA2 cDNA sequence (Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Chen *et al.*, 1996). Thus, the first 82 bp of the coding sequence, including sequence encoding the signal peptide, would be spliced out. According to studies by Connor and Pasquale (Connor and Pasquale, 1995), and Lackmann and colleagues (Lackmann *et al.*, 1998), this sequence, together with 5' untranslated sequence, is likely to reside on exon 1. Transcripts comprising the upstream exon 5.2 and sequence downstream of nucleotide 179 could potentially encode a truncated protein, beginning at the ATG codon at

nucleotide 202 of the EphA2 sequence, although, in the absence of the signal peptide, the function of such a protein is unclear (Chen *et al.*, 1996).

Null mutations of EphA4, EphA8, EphB2 and EphB3 have resulted in either anatomical or behavioural defects, although all the mutant mice are viable (Henkemeyer *et al.*, 1996; Orioli *et al.*, 1996; Park *et al.*, 1997; Dottori *et al.*, 1998; Cowan *et al.*, 2000). The absence of a readily detectable phenotype in $eck^{i/i}$ mice may be due to residual activity from full length EphA2 protein, a compensatory activity of the putative truncated EphA2 protein, or functional redundancy occurring within the EphA subfamily, several members of which have been demonstrated to interact with ephrinA1 (Gale *et al.*, 1996a). Thus, as appears to be the case in EphB2/EphB3 double mutant mice, stronger phenotypes may only be manifest in the absence of more than one receptor. The reason for the existence of 14 different Eph receptors is unclear, but based on their distinct and overlapping temporal and spatial expressions and their promiscuous ligand binding affinities, it is likely that they have evolved to perform finely tuned functions which require both individual and co-operative roles.

1.2. The Small GTPase Ras

The Ras proteins Ha-Ras, Ki-Ras4A, Ki-Ras4B and N-Ras are ubiquitously expressed 21 kDa proteins that, in addition to four Rap proteins, two R-Ras-like proteins, two Ral proteins and Rheb, are members of the Ras superfamily of small GTPases. The four Ras proteins are encoded by three *ras* genes Ha-*ras*, Ki-*ras* and N-*ras*, the two Ki-Ras isoforms arising from alternate exon usage. The *ras* genes were first discovered in their oncogenic capacity as the transforming sequence in the Harvey and Kirsten murine sarcoma retroviruses, and only subsequently were their cellular proto-oncogenic counterparts further characterised. The Ras GTPase proteins function as molecular switches, cycling between a guanosine triphosphate (GTP)-bound 'on' and a guanosine diphosphate (GDP)-bound 'off' state, to control a wide range of cellular responses.

1.2.1. Protein Structure and Localisation

Ras is synthesised as a cytoplasmic protein and is targeted to the inner surface of the plasma membrane by a series of post-translational modifications. The carboxy terminus of the protein contains a CAAX motif, where C is a cysteine residue, A represents an aliphatic amino acid, and X corresponds to a serine or a methionine. This motif is

recognised by a farnesyl protein transferase, which catalyses the addition of a 15-carbon farnesyl isoprenoid moiety to the cysteine residue. Following the removal of the three C-terminal amino acids, the exposed cysteine is then methylated. Immediately upstream of the CAAX motif is the hypervariable region, also required for membrane localisation, which comprises either a cysteine residue which undergoes palmitoylation, or a region of lysine residues, which confers a net positive charge.

In contrast to the variable nature of the carboxy terminus, the amino terminus of Ras is highly conserved. This region contains the guanine nucleotide binding site and the GTPase catalytic domain. Two regions of Ras differ conformationally between the GDP- and GTP- bound state and are referred to as Switch I and Switch II (Milburn *et al.*, 1990). Switch I (comprising residues 30-38) contains the so-called effector domain (residues 32-40), which is identical among the four Ras proteins and is involved in the binding of Ras to its effectors.

1.2.2. Regulation of Ras Activity

For Ras to function efficiently as a molecular switch, cycling between the active GTP- and the inactive GDP- bound forms must be rapid and reversible. Although Ras proteins do possess intrinsic GTPase and GDP/GTP exchange activities, these are too slow (John *et al.*, 1988; Neal *et al.*, 1988) to facilitate precisely controlled signalling, therefore Ras activity is regulated by the opposing actions of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs enhance the rate of GDP-GTP exchange, thereby positively regulating Ras, whereas GAPs confer negative regulation by increasing the intrinsic rate of GTP hydrolysis. In turn, these GEFs and GAPs are subject to regulation by the activity of cell surface receptors, thus providing a mechanism by which Ras activity is influenced by extracellular cues.

1.2.2.1. GEFs

The principal action of GEFs is to promote the release of GDP from the GTPase, enabling it to bind free GTP to become active again. Dominant negative mutations of Ras result in its existence in a permanent nucleotide-free, or GDP-bound, state. In an attempt to revert to a GTP-bound state, GEFs are bound to N17 Ras but are unable to be released, thus sequestering free GEFs and ultimately resulting in the inactivation of wild-type Ras (see Quilliam *et al.*, 1995).

The first Ras GEF, CDC25, was identified in *S. cerevisiae* (Camonis *et al.*, 1986). The domain responsible for mediating exchange factor activity, termed the 'exchange factor domain' is common to all GEFs that have been subsequently identified in yeast, *Drosophila* and mammals (Boguski and McCormick, 1993). Additionally, Ras GEFs possess an amino terminal protein-protein interaction domain such as an SH3 or a pleckstrin homology (PH) domain, which may facilitate their localisation to membrane-bound targets (Boguski and McCormick, 1993)

The mouse counterpart of yeast CDC25, RasGRF (Ras guanine nucleotide releasing factor) was partially cloned using a complementation approach to restore function to a yeast strain lacking the CDC25 gene (Martegani *et al.*, 1992), which, in conjunction with other reports (Bollag and McCormick, 1991a), indicates functional homology between species. Following the cloning and characterisation of further Ras GEFs, it is apparent that Ras activation can occur through RTK- dependent and independent pathways (Crespo *et al.*, 1994; Farnsworth *et al.*, 1995; Shou *et al.*, 1995; Mattingly and Macara, 1996; Fam *et al.*, 1997; Ebinu *et al.*, 1998).

1.2.2.1.1. The Ras GEF Sos Links RTK Activation to Ras

The best characterised Ras GEF is the son-of-sevenless (Sos) protein, which regulates RTK-mediated activation of Ras. The *sos* gene was discovered by genetic studies in *Drosophila*, where signalling from the Sevenless protein tyrosine kinase via Ras is involved in the development of the ommatidiae of the *Drosophila* eye. *Drosophila sos* (*D-sos*) was able to compensate for loss-of-function mutations in the *sevenless* gene only in the presence of Ras, and on closer inspection it was revealed that the protein showed homology to the yeast CDC25 Ras GEF (Simon *et al.*, 1991; Bonfini *et al.*, 1992). A carboxy-terminal proline-rich domain implied that Sos could interact with SH3 domain-containing proteins, which was subsequently demonstrated between D-Sos and Drk (Olivier *et al.*, 1993; Simon *et al.*, 1993), Drk being the *Drosophila* homologue of the adaptor protein Grb2 (Growth factor receptor-bound protein 2), which contains two SH3 domains (Lowenstein *et al.*, 1992) (see below).

The ubiquitously expressed Sos1 and Sos2 proteins (Bowtell *et al.*, 1992; Chardin *et al.*, 1993) both contain the exchange factor domain originally identified in CDC25 and a proline-rich carboxy terminal domain required for binding to Grb2. Interestingly, deletion of this proline-rich region results in elevated Ras GEF activity, implicating it in

the negative regulation of Sos (Aronheim *et al.*, 1994; Wang *et al.*, 1995). A further study has also implicated a region N-terminal to the catalytic domain, which contains a Dbl-homology (DH) and a PH domain, in the positive regulation of Sos (McCollam *et al.*, 1995).

Another component of the signalling pathway from RTKs to Ras was discovered by genetic analysis using *C. elegans*, in which activation of Ras is involved in vulval development. Sem-5 was found to act upstream of the Let-60 Ras-like protein and downstream of the Let-23 RTK. Sequence analysis revealed that the Sem-5 protein contained one SH2 and two SH3 domains, suggesting that it acted as an adaptor protein (Clark *et al.*, 1992). In a rather fortuitous sequence of events leading to the elucidation of the RTK-Ras signalling pathway, the mammalian homologue of Sem-5, Grb2, had recently been isolated on the basis of its ability to bind to ligand-activated EGF and PDGF receptors via its SH2 domain (Lowenstein *et al.*, 1992). Together with a number of reports demonstrating complex formation between the EGF receptor and Grb2, or Grb2 and Sos, or all three components (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993), this suggested that Grb2 binds to activated growth factor receptors in an SH2-dependent manner, thereby recruiting Sos to the plasma membrane via SH3 domain interactions to bring it in proximity to membrane-bound Ras. Buday and Downward argued that this was sufficient to promote Ras GDP/GTP exchange after observing no increase in guanine nucleotide exchange activity of Sos following stimulation with EGF (Buday and Downward, 1993). Support for this model came from the findings of two independent groups who targeted either full length Sos or its catalytic domain to the membrane and reported Ras activation and cell transformation (Aronheim *et al.*, 1994; Quilliam *et al.*, 1994).

The carboxy terminus of Sos also contains a number of consensus sites for phosphorylation by ERK/MAPK and has been demonstrated to be phosphorylated by ERK/MAPK *in vitro* (Cherniack *et al.*, 1994; Buday *et al.*, 1995). *In vivo* evidence also suggests that phosphorylation of Sos is involved in part of a feedback inhibitory loop (Burgering *et al.*, 1993). More recent evidence suggests that a MAPK substrate, MAP Kinase Activated Protein Kinase-1b (MAPKAPK-1b)/p90 Ribosomal S6 Kinase 2 (p90 Rsk2), may also phosphorylate Sos (Douville and Downward, 1997). Interestingly, in yeast, phosphorylation of CDC25 following glucose stimulation results in its dissociation from Ras (Gross *et al.*, 1992).

The adaptor protein Shc (src homology and collagen homology) can also be involved in mediating interactions between growth factor receptors and the Grb2-Sos complex. A role for Shc in growth factor signalling was first proposed after it was demonstrated that it bound to the ligand-activated EGF receptor and caused transformation when overexpressed in NIH3T3 cells (Pelicci *et al.*, 1992). Furthermore, overexpression of Shc in PC12 cells caused neurite outgrowth (Rozakis-Adcock *et al.*, 1992). Evidence suggests that the amino-terminal PTB domain of Shc, rather than the carboxy-terminal SH2 domain, is responsible for the majority of receptor interactions between Shc and phosphorylated tyrosines on cell surface receptors (van der Geer *et al.*, 1995). Tyrosine phosphorylation of Shc subsequently creates a binding site for the Grb2-Sos complex, thus providing an alternative pathway whereby the Ras GEF, Sos, is recruited to the plasma membrane. Three isoforms of Shc exist, p46, p52 and p66 (Pelicci *et al.*, 1992). In contrast to the p46 and p52 isoforms, which are implicated in signalling via the Grb2-Sos complex to Ras, as described above, the p66 isoform is differentially expressed, and is postulated to inhibit Ras signalling (Migliaccio *et al.*, 1997).

1.2.2.2. GAPs

It is apparent from the existence of constitutively active oncogenic variants of Ras that downregulation of Ras activity is required for normal cellular function. The intrinsic GTPase activity of Ras can be enhanced by GAPs. GAP activity was first detected in *Xenopus* oocytes (Trahey and McCormick, 1987) and p120 GAP, a 120 kDa protein responsible for increased RasGTP hydrolysis, was subsequently cloned from human placenta (Trahey *et al.*, 1988).

Consistent with p120 GAP acting as a negative regulator of Ras, PDGF-stimulated p120 $GAP^{-/-}$ cells display higher levels of RasGTP and sustained MAPK activity (van der Geer *et al.*, 1997). As a predominantly cytoplasmic protein, p120 GAP, like Ras GEFs, must be brought to the membrane in order to bind Ras (Huang *et al.*, 1993). p120 GAP contains two SH2 domains and has been found to associate with activated growth factor receptors (Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990), thereby providing a mechanism for membrane localisation.

A second Ras GAP, neurofibromin (NF1), is the product of the *NF1* tumour suppressor gene (Martin *et al.*, 1990; Xu *et al.*, 1990) which is lost or mutated in patients with von Recklinghausen neurofibromatosis. Such patients display abnormal neural crest cell

growth, characterised by the occurrence of malignant neurofibrosarcomas, which may be a direct result of increased RasGTP levels (Basu *et al.*, 1992). In yeast, loss of the Ras GAP genes *IRA1* or *IRA2* results in increased levels of RasGTP and consequent accumulation of cyclic adenosine 3', 5' monophosphate (cAMP) (Wigler, 1990). Similar to p120 $GAP^{-/-}$ cells, Schwann cells from $NF1^{-/-}$ mice display elevated levels of GTP-bound Ras (Kim *et al.*, 1995b). Studies have shown that NF1 binds Ras with a higher affinity than p120GAP, but it is less active, (Bollag and McCormick, 1991b) suggesting that NF1 might be the more effective Ras GAP at low Ras concentrations. A third mammalian Ras GAP, Gap1, exists as two forms, $Gap1^{m}$ (Maekawa *et al.*, 1994) and $Gap1^{IP4B}$ (Cullen *et al.*, 1995).

1.2.3. Ras Effectors

That more than one effector pathway is responsible for mediating the biological effects of Ras is evident from studies in yeast (Wang *et al.*, 1991; Chang *et al.*, 1994; Marcus *et al.*, 1995). In mammalian cells, a multitude of effector proteins have been reported to mediate the response of Ras to a diverse range of extracellular stimuli. The putative effectors show preferential binding to GTP-bound Ras, and a study by White and colleagues (White *et al.*, 1995) was the first to demonstrate that different mutations within the effector domain could differentially modulate the binding of these effectors to Ras. A number of subsequent studies have utilised these mutants to dissect the role of individual Ras effector pathways in Ras-mediated transformation (see Section 1.2.5).

In addition to the Ras effectors described in detail below, other putative Ras effectors have also been described. These include the zeta isoform of protein kinase C (PKCξ), AF-6, Rin, MEKK 1, and Nore (reviewed in Campbell *et al.*, 1998). However, many of these interactions have not been fully characterised and their functional significance is unclear. The role of the Ras GAP, p120 GAP, as a Ras effector protein remains controversial. The amino terminus of p120 GAP contains two SH2 domains and one SH3 domain (Boguski and McCormick, 1993) and was demonstrated to complex via its SH2 domains with two proteins, p190 (RhoGAP) and p62 ($p62^{dok}$) (Ellis *et al.*, 1990). Moreover, the p120 GAP amino terminus was demonstrated to disrupt stress fibres and decrease focal contacts (McGlade *et al.*, 1993), most likely through its association with RhoGAP (Settleman *et al.*, 1992).

1.2.3.1. Ral Guanine Nucleotide Dissociation Stimulator (RalGDS)

The Ral proteins RalA and RalB are members of the Ras superfamily of GTPases, and as such, are under the regulation of specific GEFs and GAPs. The Ral GEFs Ral guanine nucleotide dissociation stimulator (RalGDS) and its closely related homologues, Rgl and Rgl-2/Rlf have been identified as Ras effectors following yeast two hybrid and *in vitro* binding studies (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994; Peterson *et al.*, 1996; Wolthuis *et al.*, 1996). All interact with the effector domain of Ras in a GTP-dependent manner through their carboxy-terminal Ras binding domain (RBD). Further evidence that Ral GEFs are effectors of Ras is provided by the demonstration of increased levels of GTP-bound Ral in response to Ras activation (Urano *et al.*, 1996; Murai *et al.*, 1997; Wolthuis *et al.*, 1997) and growth factors (Wolthuis *et al.*, 1998). Moreover, dominant negative Ras blocked the growth factor-induced activation of Ral (Wolthuis *et al.*, 1998).

A role for Ral proteins in regulating the small GTPases Rac and Cdc42 has been postulated but not extensively studied. A GAP for Cdc42 and Rac, known as Ral-binding protein 1 (Cantor *et al.*, 1995) or Ral-interacting protein 1 (Jullien-Flores *et al.*, 1995; Park and Weinberg, 1995), binds to Ral in a GTP-dependent manner. Although this GAP contains both a Ral binding domain and displays GTPase activity towards Rac and Cdc42 (Jullien-Flores *et al.*, 1995; Park and Weinberg, 1995), it has yet to be demonstrated that Ral binding influences its GTPase activity. There is also evidence that Ral may control phospholipase D (PLD) activity. Ral appears to be capable of interacting with PLD via an amino-terminal binding site irrespective of whether Ral is GDP- or GTP- bound, however, dominant negative Ral inhibited the ability of Ras to induce PLD activity, implying that Ral is involved in the Ras-induced activation of PLD (Jiang *et al.*, 1995).

1.2.3.2. Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinase enzymes (PI3Ks) are lipid kinases that phosphorylate phosphoinositides at the 3' position of the inositol ring, thereby creating 3' phosphorylated lipids such as phosphatidylinositol-3-phosphate (PtdIns(3)P), PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3. Three classes of PI3Ks have been described (reviewed in Vanhaesebroeck and Waterfield, 1999). Class I comprise the most extensively studied PI3Ks which are characterised by a 110 kDa catalytic subunit

(p110) and an associated regulatory subunit. Class I PI3Ks preferentially phosphorylate PtdIns(4,5)P_2 *in vivo*. A further subdivision into class I_A and class I_B is made on the basis of their activation by tyrosine kinases and heterotrimeric G-proteins, respectively, although both subclasses can associate with Ras (Vanhaesebroeck and Waterfield, 1999). The regulatory subunit of class I_A PI3Ks comprises an 85 kDa protein containing two SH2 domains and an SH3 domain. The SH2 domains are able to bind to phosphotyrosine residues of YXXM motifs, found in many RTKs and adaptor proteins (Songyang *et al.*, 1993). This is thought to localise the p110 catalytic subunit, via its p85 binding domain, to the membrane.

A role for Ras in the activation of PI3K was proposed following the demonstration that dominant negative Ras could inhibit growth factor-induced 3' phosphoinositide production, and that p110 and Ras could directly associate *in vitro* (Rodriguez-Viciana *et al.*, 1994). A subsequent study showed that Ras-mediated activation of PI3K acted in synergy with p85 binding to tyrosine phosphopeptides (Rodriguez-Viciana *et al.*, 1996), suggesting, in accordance with studies by Klinghoffer and colleagues (Klinghoffer *et al.*, 1996), that optimal activation of PI3K may require both RTK-mediated p85 binding and Ras-mediated p110 binding. The association of GTP-bound Ras with p110 was abrogated by a mutation within the effector domain of Ras, and addition of peptides spanning the effector domain also reduced the interaction (Rodriguez-Viciana *et al.*, 1994). Thus, strong evidence is provided that PI3K acts as an effector of Ras. In contrast, however, the findings of Hu and colleagues place Ras downstream of PI3K. In this study, it was demonstrated that c-*fos* transcription was increased in response to a constitutively activated form of PI3K (Hu *et al.*, 1995a). Moreover, levels of GTP-bound Ras were also elevated, and expression of dominant negative Ras or Raf constructs was able to block the response. Although there appears to be no explanation to reconcile these apparent differences, it is important to consider the potential of the cell to produce autocrine growth factors in response to activated signalling pathways, thus making the dissection of the role of individual signalling molecules a complex issue.

A role for PI3K as an effector molecule for Ras is particularly attractive when considering the process of transformation. Among the potential downstream candidates of PI3K are protein kinase B (PKB/Akt), Rac and $p70^{S6}$ kinase. PKB/Akt, named on the basis of its homology to protein kinases A and C and it being the cellular homologue

of the viral oncoprotein v-Akt, is implicated in the inhibition of apoptosis (Dudek *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997). Since the PI3K inhibitors wortmannin and LY294002 (Powis *et al.*, 1994; Vlahos *et al.*, 1994) induce apoptosis (Yao and Cooper, 1995), and PI3K has been implicated in PKB/Akt activation (Burgering and Coffer, 1995; Franke *et al.*, 1995; Klippel *et al.*, 1996; Franke *et al.*, 1997), PI3K appears to be a strong candidate for regulating cell survival. Initial studies suggested that PKB/Akt activity was regulated by the binding of PIP_2 to the PH domain of PKB/Akt (Klippel *et al.*, 1997). However, more recent studies have demonstrated that PtdIns(3,4,5)P_3 is able to activate 3'-phosphoinositide-dependent kinase 1 (PDK1) which can phosphorylate and contribute to PKB/Akt activation (Alessi *et al.*, 1997; Vanhaesebroeck and Waterfield, 1999). PKB/Akt activation may prevent apoptosis by phosphorylating a number of proteins such as BAD, caspase-9, forkhead and I-κB (reviewed in Vanhaesebroeck and Waterfield, 1999). More recently, inhibition of the activity of glycogen synthase kinase-3 (GSK-3), a further substrate of PKB/Akt, using selective inhibitors has been demonstrated to protect against cell death (Cross *et al.*, 2001), providing a further way in which PI3K may prevent apoptosis.

Additionally, PI3K can influence the activity of Rac, a small GTPase implicated in the formation of lamellipodia and membrane ruffles (Ridley *et al.*, 1992; Nobes and Hall, 1995), thereby providing a means by which Ras activation can be linked to the activation of Rac. Activation of the PDGF receptor results in membrane ruffling and chemotaxis, and studies by two independent groups demonstrated that PI3K activity was required (Kundra *et al.*, 1994; Wennstrom *et al.*, 1994). Subsequently, PDGF-induced production of PtdIns(3,4,5)P_3 was demonstrated to increase the levels of GTP-bound Rac (Hawkins *et al.*, 1995).

An additional target of PI3K is $p70^{S6}$ kinase, which is required for entry into S phase following mitogenic stimulation (McIlroy *et al.*, 1997), although $p70^{S6}$ kinase activation may occur indirectly through PI3K-mediated activation of Rac or PKB/Akt (Welch *et al.*, 1998).

PI3K activity may be involved in the negative regulation of Ras, as Sos activity *in vitro* was inhibited by the interaction of PtdIns(4,5)P_2 with the PH domain of Sos (Jefferson *et al.*, 1998).

1.2.3.3. Raf

The most extensively studied effector of Ras are the serine/threonine kinase Raf proteins. The mammalian Raf family comprises three distinct genes encoding the 74 kDa proteins, Raf-1 (also known as c-Raf), A-Raf and B-Raf, all displaying three highly conserved regions, CR1, CR2 and CR3. The amino terminus contains CR1 and CR2 and plays a regulatory role, whereas the catalytic domain is found at the carboxy terminus within CR3.

Early biochemical and genetic studies implicated Raf as a downstream substrate of Ras (reviewed in Dickson and Hafen, 1994). Furthermore, inhibition of Raf function interfered with Ras signalling (Kolch *et al.*, 1991; Schaap *et al.*, 1993). Raf had previously been identified as an important signalling molecule that acted downstream of Ras by the demonstration that oncogenic v-*raf* was able to overcome Ras neutralising antibodies to induce transformation in NIH3T3 cells (Smith *et al.*, 1986). A number of groups demonstrated a high affinity interaction between Raf and GTP-bound Ras; (Koide *et al.*, 1993; Moodie *et al.*, 1993; Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). Furthermore, mutations in the effector domain of Ras abrogated the interaction, as did incubation with a peptide corresponding to the effector region of Ras (Warne *et al.*, 1993).

Inactive Raf is localised in the cytoplasm, but the association with Ras results in the relocalisation of Raf to the membrane (Traverse *et al.*, 1993; Wartmann and Davis, 1994). Early reports suggested that the sole function of Ras was to recruit Raf to the membrane, and it was demonstrated that artificially targeting Raf to the plasma membrane by addition of a CAAX motif increased the activity of Raf (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). However, Ras is reported to interact with two amino-terminal Raf sites, the Ras-binding domain (RBD) (Vojtek *et al.*, 1993; Pumiglia *et al.*, 1995), and a cysteine-rich domain (CRD), which is proposed to be dependent on Ras binding to the RBD (Brtva *et al.*, 1995; Hu *et al.*, 1995b; Drugan *et al.*, 1996), and since removing the regulatory amino terminus of Raf creates a constitutively active protein (Stanton, Jr. and Cooper, 1987; Stanton, Jr. *et al.*, 1989; Heidecker *et al.*, 1990), the function of Ras may be more than simply to target Raf to the membrane. In addition, Raf-CAAX activity could be further induced in a Ras-independent manner by EGF (Leevers *et al.*, 1994), suggesting that once at the membrane, additional signals are required for optimal Raf activation. Indeed, a number of Ras-independent signals

promoting serine and tyrosine phosphorylation of Raf have been reported to modulate the activity of Raf (Marais *et al.*, 1995; Marais *et al.*, 1997; Mason *et al.*, 1999), which, in conjunction with reports that Raf interacts with other components such as molecular chaperones and phospholipids (reviewed in Campbell *et al.*, 1998) suggests that the regulation of Raf is evidently a highly complicated process in which Ras is necessary but not sufficient.

1.2.4. MAPK Cascades

In mammalian cells, five MAPK cascades have so far been identified, of which the ERK/MAPK, the JNK/stress-activated protein kinase (SAPK) and the p38 pathways represent the three most well-characterised (see Figure 1.7). The hierarchical organisation of such cascades provides a means by which extracellular signals are transduced into intracellular responses, but can be subject to positive and negative regulation at all levels, thereby facilitating amplification, divergence or attenuation of the initial stimulus.

Activation of Raf as a consequence of Ras activation results in the phosphorylation, and subsequent activation, of MAPK/ERK Kinase (MEK), which then activates ERK/MAPK. ERK/MAPK will subsequently be referred to as MAPK. In addition to phosphorylating several cytoplasmic proteins (see Section 1.2.4.2), MAPK is able to regulate cellular responses by phosphorylating a number of transcription factors (see Section 1.2.4.3). In mammalian cells, the JNK/SAPK and p38 pathways are responsive to stress, rather than mitogenic stimuli, (reviewed in Tibbles and Woodgett, 1999), but their modular organisation is strikingly similar to the MAPK pathway, and they are also able to regulate the activity of a number of transcription factors.

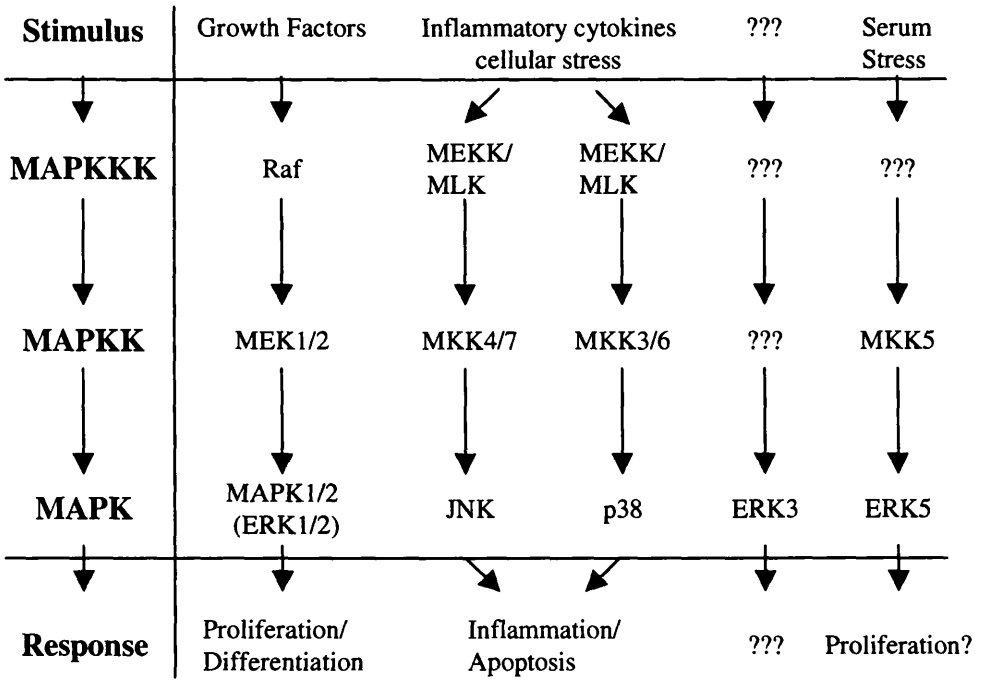


Figure 1.7. Schematic Diagram of Mammalian MAPK Cascades
Mammalian MAPK pathways regulate cell growth, differentiation and responses to stress. The cascades are organised into a hierarchical module comprising MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and MAPK (mitogen-activated protein kinase). Other abbreviations – MLK (mixed-lineage kinase), MKK (MAPKK).

1.2.4.1. Activation of MAPK via Raf and MEK

Once activated, the serine/threonine kinase Raf directly associates with, and phosphorylates, MEK on two highly conserved serine residues, thereby activating it (Dent *et al.*, 1992; Kyriakis *et al.*, 1992; Van Aelst *et al.*, 1993; Alessi *et al.*, 1994; Zheng and Guan, 1994; Papin *et al.*, 1995). Two isoforms of MEK exist, MEK1 and MEK2, which share 80% sequence identity (Crews *et al.*, 1992; Zheng and Guan, 1993). A proline-rich sequence in MEK is required for its interaction with Raf, the deletion of which severely attenuates the ability of Raf to activate MEK (Catling *et al.*, 1995).

As a dual specificity kinase, MEK activates MAPK (which also exists as two isoforms, p42 MAPK (ERK2) and p44 MAPK (ERK1)) by phosphorylating it on threonine 183 and tyrosine 185 within a TEY consensus motif (Payne *et al.*, 1991). Phosphorylation of both residues is required for maximal (3000-fold) activation of MAPK, whereas only a 5-10-fold increase in activity is attained when either site alone is phosphorylated (Cobb, 1999).

Phosphorylation of MAPK greatly increases its capacity to homodimerise and form symmetrical dimers (Khokhlatchev *et al.*, 1998), although dimerisation is not thought to influence its catalytic activity (Canagarajah *et al.*, 1997; Khokhlatchev *et al.*, 1998). It does, however, play a role in the localisation of MAPK to the nucleus (Khokhlatchev *et al.*, 1998), although the mechanism by which this occurs is not fully understood. Activated MAPK is rapidly translocated into the nucleus in response to agonist stimulation (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). However, not all activated MAPK accumulates in the nucleus. Activated MAPK is also localised to microtubules in the cytoplasm (Reszka *et al.*, 1995; Morishima-Kawashima and Kosik, 1996).

Once activated, MAPK is able to phosphorylate numerous cytoplasmic and nuclear substrates, enabling it to regulate a variety of cellular responses (see Section 1.2.4.4). MAPK is a proline-directed serine/threonine kinase, and in the majority of cases, substrate phosphorylation occurs at an optimal consensus motif of PXS/TP (Gonzalez *et al.*, 1991; Songyang *et al.*, 1996)

1.2.4.2. Cytoplasmic Substrates of MAPK

MAPK was identified initially on the basis of its ability to phosphorylate the cytoplasmic microtubule-associated protein, MAP-2 (Ray and Sturgill, 1987). Subsequently, MAPK has been reported to associate with, and phosphorylate, a number of other microtubule-associated proteins, including neurofilament proteins, Tau, synapsin and stathmin (Drewes *et al.*, 1992; Roder *et al.*, 1993; Jovanovic *et al.*, 1996; Veeranna *et al.*, 2000). Cytoplasmic MAPK also phosphorylates and activates myosin light chain kinase (MLCK), thereby enhancing its ability to phosphorylate and activate myosin light chain (MLC) (Klemke *et al.*, 1997). Activated MLC increases cell motility by influencing actin polymerisation (Klemke *et al.*, 1997).

A further kinase substrate of MAPK is MAPKAPK-1/p90 Rsk (Erikson and Maller, 1986; Dalby *et al.*, 1998). MAPKAPK-1 has been shown to be activated in the cytoplasm, but is then rapidly translocated to the nucleus (Chen *et al.*, 1992). Among the cytoplasmic substrates of the serine/threonine kinase MAPKAPK-1 are GSK-3, which is inactivated on phosphorylation (Sutherland *et al.*, 1993), and the pro-apoptotic protein BAD (Bonni *et al.*, 1999), the activity of which is also inhibited by phosphorylation. The nuclear activity of MAPKAPKs has also been demonstrated to influence gene transcription (see Section 1.2.4.3).

The recently identified MAPK signal-integrating kinase (MNK) is also a downstream substrate of MAPK, but can also be activated by p38 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Once activated, MNK (which exists as two isoforms, MNK1 and MNK2) is able to phosphorylate eukaryotic initiation factor 4E (eIF-4E) (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999), thus indicating a potential involvement in translational control.

Phosphorylation of phospholipase A_2 (PLA_2) promotes the release of arachidonic acid from membrane phospholipids. MAPK is able to phosphorylate PLA_2, thereby increasing its activity (Lin *et al.*, 1993). A further potential cytosolic target of MAPKs includes the carboxy-terminal region of the Ras GEF, Sos (Section 1.2.2.1.1), (Cherniack *et al.*, 1994; Buday *et al.*, 1995). Dissociation of the Grb2-Sos complex was reported following insulin-induced Sos phosphorylation (Cherniack *et al.*, 1995), implying that MAPK-induced Sos phosphorylation may be an important negative feedback mechanism.

1.2.4.3. Nuclear Targets of the Raf/MEK/MAPK Pathway

As a nuclear kinase, the MAPK substrate MAPKAPK-1 is able to phosphorylate the transcription factors cAMP Response Element Binding Protein (CREB) (Xing *et al.*, 1996), CREB Binding Protein (CBP) (Nakajima *et al.*, 1996) and c-Fos (Chen *et al.*, 1993).

The mitogen- and stress-activated protein kinase (MSK) proteins, MSK-1 and MSK-2, can also be activated by both MAPK and p38 pathways (Deak *et al.*, 1998) and, similar to MAPKAPK-1, they are able to phosphorylate CREB. Furthermore, MSK-1 has been reported to phosphorylate chromatin proteins, thereby implying additional potential for transcriptional regulation (Sassone-Corsi *et al.*, 1999; Thomson *et al.*, 1999). More recently, MAPK has been demonstrated to directly phosphorylate carbamoyl phosphate synthetase II, an enzyme involved in the *de novo* synthesis of pyrimidines (Graves *et al.*, 2000).

In many cases, MAPK activation results in gene transcription via the serum response element (SRE) found in the promoter of many immediate-early genes, for example *c-fos*. The SRE binds a transcription factor complex comprising serum response factor (SRF) and a member of the ternary complex factor (TCF) subfamily of Ets transcription factors, which includes Elk-1, Sap-1 and Sap-2 (reviewed in Wasylyk *et al.*, 1998). MAPK has been reported to phosphorylate Elk-1 and Sap-1 (Gille *et al.*, 1995; Strahl *et al.*, 1996).

Complexes of members of the Fos and Jun transcription factor families are able to homo- and hetero- dimerise by means of their leucine zipper domains to form the AP-1 transcription factor complex. Different dimer combinations vary in their DNA binding specificity and transactivational activity, and this can be further modified by phosphorylation (reviewed in Whitmarsh and Davis, 1996). Although JNK appears to be the main activator of the c-Jun transcription factor (Derijard *et al.*, 1994), in some cases MAPK can also phosphorylate c-Jun (Pulverer *et al.*, 1991; Peverali *et al.*, 1996; Leppa *et al.*, 1998).

AP-1 binds to a specific target sequence known as the TRE (tetradecanoyl phorbol acetate (TPA)-responsive element) (Angel *et al.*, 1987), present in promoter of many genes such as the cell cycle regulator, cyclin D1 (Albanese *et al.*, 1995). Thus, the

MAPK pathway provides a means whereby an extracellular signal influences the cellular response by modifying aspects of transcriptional regulation. The analogous p38 and JNK/SAPK kinase cascades phosphorylate c-Jun, ATF2 and Elk-1 transcription factors (reviewed in Tibbles and Woodgett, 1999), thereby evoking transcriptional responses to stress stimuli.

Recent evidence suggests that other signalling pathways can be modulated by MAPK activity. MAPK phosphorylates the oestrogen receptor, thereby enhancing its transcriptional activity (Kato *et al.*, 1995a). However, growth factor-induced activation of MAPK has been reported to phosphorylate, and thereby prevent the nuclear translocation of, Smad1, a protein involved in transforming growth factor β (TGFβ) signalling (Kretzschmar *et al.*, 1997).

1.2.4.4. Biological Effects of MAPK

In yeast, *Drosophila* and *C. elegans*, MAPK appears to mediate predominantly differentiative processes, such as the mating response, photoreceptor development, and vulval formation. In mammalian cells, however, MAPK activation has been strongly implicated in the regulation of cell growth in addition to differentiation. That MAPK can act as a central regulator in the control of cell growth is evident from its ability to co-ordinately influence pathways involved in gene transcription and RNA, DNA and protein synthesis protein (Whitmarsh and Davis, 2000). Viral *raf* has been identified as an oncogene (Rapp *et al.*, 1983) and expression of a constitutively activated form of c-Raf led to cell transformation (Smith *et al.*, 1990). Similarly, expression of constitutively activated MEK resulted in fibroblast transformation (Cowley *et al.*, 1994; Mansour *et al.*, 1994), whereas inhibition of MEK inhibits mitogen-induced DNA synthesis (Dudley *et al.*, 1995), as does constitutive expression of the MAPK phosphatase-1 (MKP-1) (Brondello *et al.*, 1995). Sustained activation of MAPK induces cell cycle entry by driving expression of cyclin D1, which correlates with the expression of a subset of AP-1 genes (Balmanno and Cook, 1999). However, it is also known that expression of $p21^{Cip1}$, a negative cell cycle regulator, occurs as a consequence of sustained MAPK activation (Sewing *et al.*, 1997). A recent study has revealed that a Ras-induced increase in Rho activity is able to suppress the levels of $p21^{Cip1}$, thereby driving proliferation (Sahai *et al.*, 2001)

Sustained MAPK activation in PC12 cells, however, in response to nerve growth factor (NGF) or fibroblast growth factor (FGF) results in the translocation of MAPK into the nucleus and subsequent neuronal differentiation, whereas a transient induction of MAPK activity in response to EGF results in cellular proliferation, and MAPK is retained in the cytoplasm (Marshall, 1995). MAPK activation also plays a role in differentiation of other cell types, including thymocytes (Alberola-Ila *et al.*, 1995; Crompton *et al.*, 1996) and adipocytes (Hu *et al.*, 1996).

MAPK activity is also required for cell migration. Inhibition of MAPK activity inhibited the migration of carcinoma cells on collagen, whilst having no apparent effect on their attachment or spreading (Klemke *et al.*, 1997). Activation of MAPK was found to phosphorylate and induce the activity of MLCK, thereby resulting in the increased phosphorylation of its substrate, MLC.

1.2.5. Transformation by Ras

The initial discovery of Ras as the product of the oncogenic Harvey and Kirsten transforming retroviruses, together with the subsequent identification, in a wide range of tumours, of naturally occurring Ras point mutations (Bos, 1989), strongly implicates aberrant Ras signalling in the process of malignant transformation. Mutations at residues 12, 13 or 61 of Ras decrease its intrinsic GTPase activity and render it insensitive to negative regulation by GAPs (reviewed in Bourne *et al.*, 1990), thereby creating a constitutively active form of the protein. The incidence of these mutations varies greatly between tumour types (Bos, 1989), and is particularly low (5%) in breast tumours (Martinez-Lacaci *et al.*, 2000). However, overexpression of Ras is frequently found in breast tumours (Miyakis *et al.*, 1998). Furthermore, Ras activity is often upregulated in response to overexpression of components of upstream signalling pathways, such as ErbB2 (reviewed in Hynes and Stern, 1994).

The consensus from a wealth of studies making use of Ras effector mutants and constitutively active and dominant negative constructs is that maximal transformation by Ras requires the activation of more than one effector pathway (White *et al.*, 1995; Oldham *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; Webb *et al.*, 1998). However, inhibition of the MAPK pathway can suppress Ras transformation (Dudley *et al.*, 1995; Khosravi-Far *et al.*, 1995), and a number of studies have shown that activating mutations in the Raf/MEK/MAPK pathway alone can induce tumourigenesis (Smith *et*

al., 1986; Cowley *et al.*, 1994; Leevers *et al.*, 1994; Mansour *et al.*, 1994), although these studies do not preclude contribution from other effector pathways by an autocrine mechanism. The significance of the Raf/MEK/MAPK pathway in Ras-mediated transformation likely reflects its key role in regulating cell growth and modulating the expression of additional genes involved both in the acquisition of the Ras-transformed phenotype and in aspects of tumour progression such as metastasis (Hernandez-Alcoceba *et al.*, 2000).

The Rho family of GTPases, which are known to regulate the actin cytoskeleton (Nobes and Hall, 1995), are also thought to be involved in mediating the changes in cell morphology and motility required for metastasis. Additionally, Rho GTPases have been implicated in Ras transformation (Ridley *et al.*, 1992; Ridley and Hall, 1992; Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995a; Qiu *et al.*, 1995b; Qiu *et al.*, 1997; Sahai *et al.*, 2001). In *S. pombe*, the Cdc42 GEF, Scd1, has been identified as an effector of Ras1, (Marcus *et al.*, 1995) and filamentous growth in *S. cerevisiae* is controlled by a Cdc42-mediated pathway downstream of Ras2 (Mösch *et al.*, 1996).

A further important downstream target of Ras may include JNK (Granger-Schnarr *et al.*, 1992; Clark *et al.*, 1997). JNK may be activated either as consequence of Ras-induced Rho GTPase activity (Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995) or via the direct interaction of MEKK with Ras (Minden *et al.*, 1994; Yan *et al.*, 1994; Russell *et al.*, 1995).

1.3. The Mammary Gland

The mammary gland is an endocrine organ that exists only in mammals and is unique in that the majority of its development occurs after birth. This makes it an extremely suitable model in which to study aspects of normal development, such as proliferation, differentiation and cell death. Similar to most organs, the mammary gland comprises both an epithelial and a mesenchymal (embryonic)/stromal (adult) component.

1.3.1. Morphology of the Mammary Gland

The mammary epithelium comprises two different cell types, the luminal epithelium and the myoepithelium, embedded in surrounding mesenchymal/stromal tissue consisting of adipocytes, fibroblasts and blood vessels. In mammary ducts, the luminal epithelial

layer is surrounded by a continuous layer of myoepithelial cells, in contrast to the terminal alveoli, where the distribution of myoepithelial cells is more discrete (see Figure 1.8).

A layer of basement membrane surrounds the epithelial layers, providing both structural support and biochemical signals. The basement layer is produced both by the epithelium and the surrounding mesenchyme/stroma and is an important factor in mammary gland development and homeostasis (see Section 1.3.4.3). It also governs the highly polarised nature of mammary epithelial cells, generating a basal-apical polarity that is highly significant for luminal epithelial cells in the function of milk secretion into the lumen of the alveoli and ducts. Within the epithelial cell layer, as is typical of epithelial cells, neighbouring cells are in very close contact with each other, thus, in addition to cell-ECM and epithelial-mesenchymal/stromal interactions, cell-cell interactions also contribute significantly to mammary gland function (see Section 1.3.4.3).

1.3.2. Development of the Mouse Mammary Gland

Mouse mammary gland development begins during embryogenesis between 10 and 11 dpc, with a localised thickening of the ectoderm into a mammary ridge or crest that extends between the fore- and hind-limbs on both sides of the ventral surface of the embryo. In 12 day old embryos, five pairs of mammary buds are visible, with a small amount of growth occurring mainly as a result of the recruitment of neighbouring ectodermal cells. Male embryos begin to produce androgen at a late stage of 13 dpc, so that by 14 dpc the sexual phenotype of the mammary gland is determined. This is accomplished by the detachment of a small group of cells from the remainder of the ectoderm following the destruction of the epithelial rudiment. In female embryos, the mammary bud continues to grow downwards quite slowly into the surrounding fibroblastic mesenchyme until the 16^{th} or 17^{th} day of gestation, at which point it begins to elongate and undergo branching morphogenesis, penetrating into a second type of mesenchyme, the fat pad precursor. By the end of gestation, a small ductal tree comprising 10-20 branches has been formed from each individual mammary bud. In the first three weeks after birth, the ductal system undergoes limited elongation and branching resulting in the ramification of a ductal network throughout the fat pad. Puberty in mice occurs 4-6 weeks after birth, at which stage the distal end of each duct proliferates to form a bulbous endbud, known as a mammary or terminal end bud,

comprising epithelial cells. The mammary gland remains in this state until the second half of pregnancy, when the number of mammary epithelial cells in the gland dramatically increases leading to the production of more ducts, and alveoli are formed from the terminal end buds. The distal epithelial cells lining these alveoli differentiate to develop secretory characteristics. At this point, the stroma dramatically decreases in volume to accommodate the expanding epithelium.

Upon parturition, milk is produced and secreted, enabling lactation to proceed. After weaning is completed, milk begins to accumulate in the ducts and alveoli, resulting in the collapse of cells inside the alveoli. Massive localised apoptosis occurs during the process of involution, after which the mammary gland is restored to its post-pubertal state, with the adipocyte stroma replacing the lost epithelium. Although now morphologically resembling a virgin mammary gland again, not until the mammary gland has undergone this process of proliferation, differentiation and death is it considered functionally developed (see Figure 1.9). Moreover, localised changes in cell proliferation, differentiation and death in the mammary alveoli also occur during the oestrus cycle, but on a much smaller scale than occurs during pregnancy, lactation and involution (Andres *et al.*, 1995).

1.3.3. Mammary Gland Neoplasia

The continuous proliferative, differentiative and apoptotic signals encountered during both the oestrus and reproductive cycles make the mammary epithelium particularly prone to the accumulation of gene mutations and hence transformation. The vast majority of human breast cancers are characterised by having luminal-like tumour cells, with less than 3% displaying myoepithelial-like characteristics (Gusterson *et al.*, 1982). In addition, there is no evidence that myoepithelial cells are proliferative (Sapino *et al.*, 1990), strongly implying that luminal epithelial cells, in addition to their role in ductal growth in normal development, are responsible for dysregulated growth in mammary tumours.

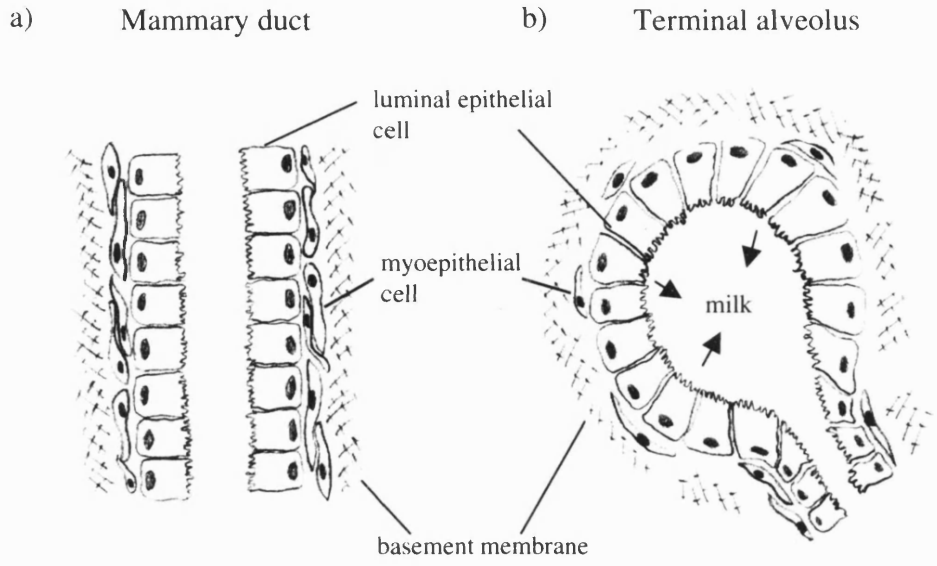


Figure 1.8. Schematic Representation of a Mammary Duct and Alveolus
Mammary ducts and alveoli comprise luminal and myoepithelial cells. In mammary ducts, luminal epithelial cells are separated from the basement membrane by a layer of myoepithelial cells (a). (b) highly polarised luminal epithelial cells in mammary alveoli are responsible for the production of milk products. The majority of these cells are in direct contact with the basement membrane, with a more discrete distribution of myoepithelial cells. Adapted from Hagios *et al.*, 1998 ; Edwards and Streuli, 1995 .

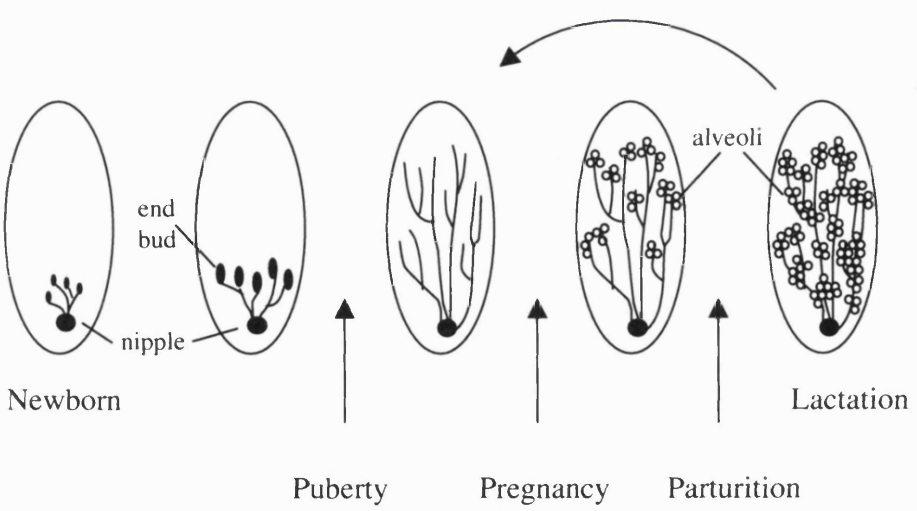


Figure 1.9. Schematic Representation of Mouse Mammary Gland Development
The rudimentary ductal system in newborn mice undergoes limited growth before the onset of puberty, during which extensive elongation and branching occurs. In the second half of pregnancy, alveoli are formed from terminal end buds. The number of alveoli reaches its maximum during lactation, after which massive localised cell death occurs during involution, and the mammary gland morphologically resembles that of a virgin mouse. Adapted from Hennighausen and Robinson, 1998.

Mammary carcinomas are classified as either non-invasive, in which case they are described as either ductal or lobular carcinomas *in situ*, or invasive, which display a variety of heterogeneous phenotypes, including infiltrating ductal NOS (not otherwise specified) carcinoma, lobular carcinoma and medullary carcinoma (Ronnov-Jessen *et al.*, 1996). A number of stages of mammary tumour formation can be identified, varying from premalignant hyperplasias to metastatic carcinomas. The most aggressive phenotype and poorest prognosis are thought to be the result of an accumulation of genetic changes which confer distinct advantages on the tumour, such as cell proliferation, cell survival and the ability of cells to invade the surrounding tissue. As with many other cancers, the development of breast cancer is a multistep process which can arise as a result of spontaneous or hereditary genetic mutations. The strongest risk factor for breast cancer appears to be a family history of the disease. Among the best characterised genes that are known to predispose women to breast cancer is *Brca1*, which is also linked to ovarian cancer (Miki *et al.*, 1994). Since loss of the wild-type allele accounts for over 90% of mammary tumours from women with *Brca1* mutations (Smith *et al.*, 1992; Neuhausen and Marshall, 1994; Cornelis *et al.*, 1995), the protein is postulated to have a tumour suppressive role. Indeed, Brca1 protein has been reported to associate with Rad51, a protein implicated in double-stranded DNA repair (Benson *et al.*, 1994). An interaction between Brca1 and p53 has also been reported (Zhang *et al.*, 1998). A second breast cancer susceptibility gene, *Brca2* (Wooster *et al.*, 1995) shows little homology to *Brca1*, but is expressed in a similar manner, being upregulated during periods of mammary epithelial proliferation and differentiation (Rajan *et al.*, 1996). The Brca2 protein has also been implicated in DNA repair (Arver *et al.*, 2000), and, since germ-line mutations and loss of heterozygosity (LOH) at the *Brca2* locus are correlated with breast tumour development, Brca2 has also been postulated to act as a tumour suppressor. In contrast to *Brca1*, a large proportion of male breast cancers is attributable to mutations in *Brca2*. Breast cancer represents 1% of all cases of male cancers, and although occurs approximately 100 times less than female breast cancer, exhibits similar histology, biology and prognosis. Male mammary tumourigenesis is commonly linked to alterations in hormonal levels (Thomas *et al.*, 1992). Female breast cancer also occurs predominantly in sexually mature women, emphasising the importance of hormonal action in mammary gland homeostasis (see Section 1.3.4.2). As such, anti-oestrogens have proved to be successful in the treatment of oestrogen-dependent mammary tumours (de Cupis and Favoni, 1997). However, treatment is critically dependent on the nature of the tumour, since treating ErbB2/Neu-

overexpressing tumours with the anti-oestrogen, Tamoxifen, has been reported to stimulate ErbB2/Neu expression (Johnston *et al.*, 1993). In the case of ErbB2/Neu-overexpressing tumours, effective treatments may be provided by antibody therapy (e.g. Herceptin) (Hynes and Stern, 1994; Weiner and Adams, 2000), the use of antisense oligonucleotides (Roh *et al.*, 2000; Yu and Hung, 2000) and small molecule inhibitors (Buchdunger *et al.*, 1994). Many of these approaches may be applied to inhibit Ras, in cases where Ras signalling is upregulated by oncogenes. Further treatments involve combinative therapy, such as the use of chemotherapy together with reductive surgery.

1.3.3.1. Mouse Models of Neoplasia

Analysis of the multistep nature of human breast cancer is facilitated by the use of transgenic mouse models bearing mutations that cause the overexpression, or deletion, of appropriate genes. Transgenic mice that developed mammary tumours were first generated in 1984 (Stewart *et al.*, 1984), when c-*myc* was expressed under the control of the long-terminal repeat (LTR) of the mouse mammary tumour virus (MMTV). Subsequent studies have demonstrated that the expression of genes amplified in human cancers, such as ErbB2/Neu and cyclin D1, is able to induce mammary tumourigenesis in transgenic mouse models. The use of transgenic mice has also enabled the consequences of overexpressing genes normally tightly regulated during mammary development to be studied, implicating a number of RTKs and their signalling pathways in mammary tumourigenesis (see Section 1.3.4.4). The role of proteins as tumour suppressors can also be assessed by the ability to specifically target genes for deletion or disruption. This is particularly relevant for the study of genes located at sites of LOH. Using a conditional knockout mouse, it has recently been demonstrated that disruption of *Brca1* in a mammary-specific manner resulted in the formation of mammary tumours (Xu *et al.*, 1999b), and has confirmed that Brca1 plays a role in DNA repair (Moynahan *et al.*, 1999). With rapid advances in the field of transgenics, it is likely that the successful use of mice as models in which to study mammary gland tumourigenesis will continue.

1.3.4. Signalling Within the Mammary Gland During Normal Development and Tumourigenesis

Within the environment of the mammary gland, the information that a cell receives can be in the form of hormones and cytokines originating several cell distances away, or local cues that it receives from cell-cell and cell-matrix interactions. Both sets of signals constitute a dynamic microenvironment in which longer-range signals can affect the interaction of cells with their immediate environment, and, reciprocally, the response to such signals may depend on the local cell environment. The signals must be tightly co-ordinated both within and between the epithelial and mesenchymal/stromal components of the mammary gland to form a functional unit which can undergo growth, morphogenesis and differentiation. Thus, it is apparent that any perturbations that upset such a unit may well contribute to deregulated growth.

1.3.4.1. The Role of Epithelial-Stromal Interactions

Development of the mammary gland requires interactions between the epithelium and its surrounding mesenchyme/stroma. Results from early studies demonstrated that epithelial-mesenchymal interactions are involved in the formation of mammary buds. Ectoderm derived from prospective mammary and non-mammary regions could be induced to form mammary buds by underlying mammary mesenchyme. Mammary epithelial bud formation is thought to be induced by signals from the underlying mesenchyme, which mediate changes in cell shape and motility rather than localised cell proliferation (Cunha, 1994).

Following formation of the epithelial bud, the sexual phenotype of the mammary gland is specified as a result of epithelium-derived signals that induce the synthesis of androgen receptors in the fibroblast mesenchyme (Heuberger *et al.*, 1982). In male mice, the presence of testosterone directly stimulates these fibroblasts to condense around the rudimentary epithelium and ultimately cause its destruction. Female mice, lacking androgen receptors, are unaffected.

Early studies also demonstrated that the mammary mesenchyme is important in directing the unique morphological development of the mammary gland (reviewed in Howlett and Bissell, 1993). When mammary epithelium is recombined with homotypic mammary mesenchyme, the epithelium develops in a mammary-specific manner.

However, the pattern of morphogenesis resembles that of a salivary gland if the mammary epithelium is recombined salivary gland mesenchyme. Conversely, if mammary mesenchyme is combined with salivary gland epithelium, the epithelium develops a mammary gland-specific morphology.

Although mammary mesenchyme contains both fibroblastic and adipose components, the adipocytes appear to predominantly specify the mammary-specific growth characteristics of the epithelium (Howlett and Bissell, 1993). The precise mechanism by which epithelial-mesenchymal interactions specify mammary-specific morphogenesis is unknown, however, it is highly likely that basement membrane- and ECM-derived signals are involved (see Section 1.3.4.3).

In the developing postnatal mouse mammary gland, continued support for mammary gland morphogenesis is provided by the adipocytes of the stroma. Although the ductal system is enveloped by basement membrane, the end buds (from which ductal elongation occurs) are surrounded only by stromal adipocytes, whereas the ductal walls (along which end buds are unable to form) are surrounded by a layer of fibroblasts before being embedded in the adipocytes of the fat pad. By 12-14 weeks after birth, expansion of the ductal system stops as the ducts reach the limits of the fat pad and make contact with the surrounding fibrous sheath (Howlett and Bissell, 1993). Thus, it appears that the fibroblastic and adipocyte components of the mammary mesenchyme have opposing roles on mammary morphogenesis.

1.3.4.2. The Role of Hormones

Embryonic mammary gland development occurs largely independently of hormonal action, in contrast to the profound effects of reproductive hormones on the development of the postnatal mammary gland in pubertal and pregnant mice. The adult mammary gland is exposed to a number of hormones following puberty, such as oestrogen, progesterone and prolactin, during the oestrus cycle and the reproductive cycle.

With the onset of puberty at approximately 4 weeks in mice, ductal growth increases significantly. Studies from oestrogen receptor mutant mice have demonstrated that oestrogen primarily affects the ductal growth of epithelial cells following binding to stromal oestrogen receptors (Korach *et al.*, 1996; Cunha *et al.*, 1997).

In contrast, the effect of progesterone on side branching and alveolar development is thought to be due to its direct action on progesterone receptor-expressing epithelial cells (Lydon *et al.*, 1995). Subsequent studies have shown that progesterone induces the expression of *Wnt-4,* which mediates the formation of ductal side branches (Brisken *et al.*, 2000).

Alveolar morphogenesis is also regulated by prolactin, as demonstrated by the formation of an extended, but undeveloped, ductal system in prolactin-deficient mice (Horseman *et al.*, 1997). However, prolactin was first identified as a lactogenic hormone by its ability to stimulate casein production in mammary organ explants (Juergens *et al.*, 1965). Thus, signalling through prolactin regulates genes involved in both differentiation and growth control.

The ability of many of the hormones expressed in the mammary gland to promote localised cell growth is highly suggestive of a putative tumourigenic role in a situation where the complex homeostatic regulation of the mammary gland is perturbed. Indeed, almost 50% of primary breast cancers depend on the growth-promoting effects of oestrogen (although these later establish oestrogen independency) (Fendrick *et al.*, 1998). Similarly, many studies have established a role for prolactin in rodent mammary tumourigenesis (reviewed in Vonderhaar, 1999).

1.3.4.3. The Role of Cell-Cell and Cell-ECM Interactions

One of the main ways in which epithelial-mesenchymal/stromal interactions exert influence over mammary gland development is by contributing different ECM constituents to the basement membrane separating the two components. The complement of ECM molecules varies considerably during mammary gland development and homeostasis (Sakakura, 1991), thus providing a variety of different ECM-derived signals to both the epithelium and the mesenchyme/stroma.

By 16 dpc, the rudimentary epithelial duct is already separated from the adipocyte mesenchyme by a continuous basement membrane (Kimata *et al.*, 1985). However, as previously mentioned (Section 1.3.4.1), mammary epithelial morphogenesis is directed by the underlying mesenchymal adipocytes. Studies have shown that salivary gland morphogenesis requires a combination of basement membrane and salivary mesenchyme (Takahashi and Nogawa, 1991), and since the mesenchymal adipocytes of

the mammary gland are able to produce components of the basement membrane (Howlett and Bissell, 1993), it is conceivable that the requirement of adipocytes for mammary epithelial morphogenesis is due to their ability to provide basement membrane signals.

ECM molecules have also been implicated in the determination of the sexual phenotype of the mammary gland. Tenascin expression is intriguingly downregulated in the developing female mammary gland (Sakakura *et al.*, 1991), in contrast to its continued expression in the fibroblast cells of the male rudimentary duct, which may destroy the surrounding epithelial rudiment through adhesion-promoting effects. There is also evidence to suggest that the negative effects of stromal fibroblasts on ductal outgrowth (see Section 1.3.4.1) may be due to an increased deposition of the ECM components collagen and chondroitin sulphate in the stroma adjacent to mammary end buds (Silberstein *et al.*, 1990). Conversely, the process of ductal branching requires degradation of ECM and basement membrane components by matrix-metalloproteinases (MMPs) (Sympson *et al.*, 1994).

The ECM also plays an important role in mammary gland differentiation. Mammary epithelial cells from mid-pregnant mice are able to synthesise milk proteins in the presence of lactogenic hormones only when cultured on floating type I collagen gels, in EHS matrix (reconstituted basement membrane derived from Engelbreth-Holm-Swarm tumour) or in co-culture with mammary adipocytes or fibroblasts, due to the presence, or induction of production, of a basement membrane (Li *et al.*, 1987; Wiens *et al.*, 1987; Reichmann *et al.*, 1989; Streuli and Bissell, 1990). Interestingly, when cultured on tissue culture plastic, mammary epithelial cells are able to produce basement membrane components but are unable to assemble them correctly (Streuli and Bissell, 1990).

Another important process in which cell-ECM interactions are of significance is involution, whereupon the differentiated milk-secreting alveolar epithelium is removed by apoptosis. Early studies reported on the loss of basement membrane around the alveoli during involution, with subsequent analyses demonstrating increased expression and activity of MMPs (Talhouk *et al.*, 1991; Talhouk *et al.*, 1992; Li *et al.*, 1994). *In vitro* studies have demonstrated that the presence of basement membrane confers protection against apoptosis (Boudreau *et al.*, 1995; Edwards and Streuli, 1995;

Boudreau *et al.*, 1996), whilst the presence of an anti-integrin antibody increased the rate of cell death (Edwards and Streuli, 1995).

Transformed cells must modify their interactions with the ECM during tumour formation and subsequent metastasis. Accordingly, many mammary carcinomas display markedly altered patterns of integrin distribution (Koukoulis *et al.*, 1991; Zutter *et al.*, 1993). The loss of cell-cell contacts is also an important factor in the acquisition of the invasive phenotype. As such, loss of the adherens junction protein, E-cadherin, correlates well with aggressive carcinomas (Behrens *et al.*, 1989; Zschiesche *et al.*, 1997). The expression of E-cadherin can be influenced by ECM components (Kato *et al.*, 1995b) and post-translational modification of E-cadherin by MMPs has been demonstrated (Lochter *et al.*, 1997).

1.3.4.4. The Role of Receptor Tyrosine Kinases

RTKs are involved in aspects of normal mammary gland development such as ductal growth and branching and accordingly, deregulation of either the receptors or their ligands is likely to impact on proliferative and invasive processes in tumourigenesis.

One of the best characterised receptor tyrosine kinases that is overexpressed in human mammary tumours is ErbB2/Neu, a member of the EGF receptor family. A mutation in the transmembrane domain of ErbB2 renders the kinase constitutively active (Weiner *et al.*, 1989) and is able to induce mammary tumour formation in mice (Muller *et al.*, 1988). An analogous mutation in human ErbB2 has not been detected, but overexpression of the wild-type receptor is frequently found in human mammary tumours (see Hynes and Stern, 1994), and is thought to confer ligand-independent receptor activation. Interestingly, tyrosine phosphorylation of ErbB3 is also observed in situations where ErbB2 is overexpressed (Alimandi *et al.*, 1995), presumably as a result of spontaneous dimerisation with ErbB2, and this heterodimer is thought to confer synergistic tumourigenic potential by activating both mitogenic and anti-apoptotic signalling pathways (Siegel *et al.*, 2000). The ErbB1 ligands EGF and TGFα elicit both proliferative and differentiative effects on normal mammary glands, causing branching and development of alveoli, and both ligands are expressed in transformed mammary tissue (Normanno *et al.*, 1994).

Members of the FGF family of RTKs and ligands are expressed during normal mouse mammary gland development (Coleman-Krnacik and Rosen, 1994; Jackson *et al.*, 1997) and are also implicated in mammary tumourigenesis. Integration of MMTV results in the expression of FGF3, FGF4 and FGF8 (Peters *et al.*, 1983; Dickson *et al.*, 1984; Peters *et al.*, 1989; MacArthur *et al.*, 1995) and mammary-specific overexpression of FGF3, FGF7 and FGF8 causes tumour formation (Muller *et al.*, 1990; Kitsberg and Leder, 1996; Daphna-Iken *et al.*, 1998).

Overexpression of the Met receptor tyrosine kinase and its ligand, hepatocyte growth factor/scatter factor (HGF/SF) is also found in human mammary tumours (Tuck *et al.*, 1996; Jin *et al.*, 1997). In the normal mammary gland, Met is involved in branching morphogenesis (Niranjan *et al.*, 1995; Soriano *et al.*, 1995; Yang *et al.*, 1995; Yant *et al.*, 1998). Transgenic expression of an activated form of Met resulted in the formation of metastatic mammary tumours (Jeffers *et al.*, 1998).

1.3.5. A Role For EphA2 During Normal Mammary Gland Development and Tumourigenesis?

Analysis of the levels of EphA2 expression revealed low levels of EphA2 mRNA expression in the mammary glands of virgin mice, with fluctuating levels of expression during the oestrus cycle (Andres *et al.*, 1994). Highest levels were observed during pro-oestrus and oestrus (characterised by epithelial growth), with levels declining during metoestrus and anoestrus following ovulation (characterised by differentiation and apoptosis) (Andres *et al.*, 1994). Levels of EphA2 mRNA also decreased during pregnancy, and only during involution could transcripts again be detected by northern blot analysis (Andres *et al.*, 1995). A similar pattern of expression was also seen for the EphB4 receptor (Andres *et al.*, 1995) and a subsequent study demonstrated that changes in EphB4 protein levels largely paralleled those of EphB4 mRNA expression during the oestrus cycle (Nikolova *et al.*, 1998). Analysis of the levels of EphA2 protein has not been undertaken.

In agreement with the initial naming of EphA2 as epithelial cell kinase (Lindberg and Hunter, 1990), mEphA2 has been isolated from the epithelial cell component of a mouse mammary co-culture system (Andres *et al.*, 1994). Significant levels of EphA2 mRNA were observed in mammary epithelial cells from both established cultures and

primary sources, compared with barely detectable levels in fibroblasts (Andres *et al.*, 1994; Andres *et al.*, 1995). However, whether the cellular source of EphA2 expression is luminal or myoepithelial was not reported. The marked upregulation of EphA2 in a number of mammary tumours (Andres *et al.*, 1994; Dodge-Zantek *et al.*, 1999; Ogawa *et al.*, 2000) strongly implies that, since the vast majority of mammary tumours comprise luminal epithelial cells, EphA2 is of luminal epithelial origin, at least in tumour tissue. However, the source of EphB4 expression was seen to change from myoepithelial to luminal with tumourigenic progression, whereas the expression of its ligand, ephrinA2, was lost altogether from luminal cells (Nikolova *et al.*, 1998). As yet, there have been no reports of ephrinA1 expression in non-transformed mammary tissue, although ephrinA1 and EphA2 are frequently co-localised in the epithelium of other tissues comprising both epithelial and stromal components, such as lung and kidney (Lindberg and Hunter, 1990; Ganju *et al.*, 1994; Shao *et al.*, 1995; Takahashi and Ikeda, 1995). A recent report has described the presence of ephrinA1 and EphA2 in both epithelial and endothelial cells in mammary gland tumours (Ogawa *et al.*, 2000).

That *in vitro* studies have suggested a role for Eph receptors in cell-cell and cell-ECM interactions (see Sections 1.1.5.1 and 1.1.5.2) is of significance given the importance of such interactions in the developing and adult mammary gland (see Section 1.3.4.3), and implicate Eph receptor signalling in cell positioning and pattern formation during mammary gland development and homeostasis. Mammary gland function may also be influenced by the ability of Eph receptor activation to perturb cell-cell communication through impaired formation of gap junctions. The expression of connexins, which form the connexon pores of gap junctions, is reported to vary during the reproductive cycle of the mammary gland, and impaired gap junction function has been implicated in tumourigenesis (reviewed in Locke, 1998). However, whether impaired gap junctions are a cause or consequence of transformation is unclear.

The ability of EphA2 to suppress integrin function (Miao *et al.*, 2000) could be very important both in normal mammary gland function and in metastasis, a process in which EphA2 has already been implicated in light of its overexpression in Ras-induced metastatic tumours (Andres *et al.*, 1994). Furthermore, the recent finding that EphA2 and ephrinA1 are both expressed in blood vessels of mammary tumours (Ogawa *et al.*, 2000) implicates EphA2 in angiogenesis, a critical step in the process of metastasis.

1.4. Thesis Aims

Tumourigenesis is a complex process in which the deregulation of a number of genes contributes to the survival and progression of the tumour. Increased expression of several Eph receptors has been reported in a number of different tumour types. In particular, EphA2 mRNA levels are increased in mammary tumours in response to mammary-specific expression of activated Ras in a transgenic mouse model. Initial experiments undertaken in this thesis were therefore designed to determine whether the Ras-induced increase in EphA2 mRNA expression corresponded to an increased level of functional EphA2 protein. Since Ras interacts with several substrates, the effector pathway(s) responsible for the upregulation of EphA2 was investigated. As EphA2 mRNA is increased in metastatic mammary tumours of WAP*ras* transgenic mice (Andres *et al.*, 1994), the consequences of expressing oncogenic *ras* in a mammary-specific manner in mice which are severely deficient for the EphA2 protein were analysed. Further experiments were also carried out to study signalling pathways downstream of activated EphA2 and to determine the consequence of EphA2 activation on the morphology of normal and Ras-transformed mammary epithelial cells.

Chapter 2

Materials and Methods

Unless otherwise specified, all chemicals were purchased from BDH or Sigma.
Water used in the preparation of all solutions was purified by treatment with activated charcoal, reverse osmosis and de-ionisation using a Purite 'Select Analyst HP' system, followed by sterilisation by autoclaving.

Radioisotopes (see below) were supplied by Amersham Pharmacia.
Redivue [γ-^{32}P]ATP, 10 µCi/µl
Redivue [γ-^{33}P]ATP, 10 µCi/µl
[α-^{32}P]dCTP, 10 µCi/µl

2.1. Nucleic Acid Manipulation

2.1.1. Stock Solutions, Buffers, Bacterial Strains and Plasmids

2.1.1.1. Media Used for Bacterial Culture

Luria-Bertani (LB) medium — 10 g bacto-tryptone (Difco), 5 g bacto-yeast extract and 5 g NaCl were dissolved in water. The pH was adjusted to pH 7.5 with 5 M NaOH and the volume adjusted to 1 l with water before autoclaving.

LB agar (Difco) — Prepared as for LB medium with the addition of 15 g/l of bacto-agar Antibiotics, when used, were added when the medium had cooled to 55°C. Plates were poured using 25-30 ml medium and stored at 4°C.

2.1.1.2. Antibiotics for Bacterial Culture

Ampicillin — Prepared as a stock solution of 50 mg/ml in water, filter sterilised using a 0.2 µm syringe filter and stored at -20°C. Used at a final concentration of 50 µg/ml.

Zeocin™ — Purchased from Invitrogen as a 100 mg/ml stock, used at 25 µg/ml final concentration. Stored in dark at -20°C

2.1.1.3. Reagents for Nucleic Acid Analysis

Caesium chloride	5.7 M caesium chloride, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8.0. Stored at room temperature.
Denaturation Solution	0.4 M NaOH, 0.6 M NaCl. Stored at room temperature.
50 x Denhardt's Solution	1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (BSA) in distilled water. Filter sterilised and stored in aliquots at -20°C.
DNA size markers	Obtained from Gibco-BRL. 50 µl of a 1 mg/ml stock was added to 100 µl of DNA loading buffer and 850 µl TE to make a 50 ng/µl solution. Stored at 4°C.
DNA loading dye (10 x)	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% (w/v) Ficoll 400. Stored at room temperature.
Ethidium bromide	Prepared as a 10 mg/ml stock in water, stored at room temperature wrapped in foil.
Genomic Lysis Buffer	100 mM Tris-HCl pH 7.9, 10 mM NaCl, 10 mM EDTA, 0.5% SDS. Stored at room temperature. Proteinase K added to 100 µg/ml immediately prior to use.
Guanidinium thiocyanate	4 M guanidinium thiocyanate, 0.1 M β-mercaptoethanol, final pH 5.0. Stored in aliquots at –20°C.
Hybridisation Solution	50% Formamide, 6 x SSC solution, 5 x Denhardt's solution, 0.5% SDS. Salmon sperm DNA added to 200 µg/ml immediately prior to use.
Neutralisation Solution	1.5 M NaCl, 0.6 M Tris-HCl pH 7.5. Stored at room temperature.

RNAse A	RNAse A was dissolved, using 0.01 M sodium acetate (pH 5.2), to a final concentration of 10 mg/ml, incubated at 95°C for 15 minutes, then cooled to room temperature prior to adding 0.1 volumes of 1 M Tris-HCl pH 7.4. Stored at -20°C.
3 M sodium acetate	Prepared as a 3 M solution, pH adjusted accordingly using glacial acetic acid. Autoclaved and stored at room temperature.
Solution I	50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0. Filter sterilised and stored at 4°C.
Solution II	0.2 M NaOH, 1% (w/v) SDS. Made up fresh before use.
Solution III	3 M potassium acetate, adjusted to pH 4.8 with glacial acetic acid. Stored at 4°C.
20 x SSC	175.3 g NaCl, 88.2 g sodium citrate.$2H_20$, volume adjusted to 1 l with water. Stored at room temperature.
TAE (50x)	242 g Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA pH 8.0. Volume adjusted to 1 l with water. Autoclaved and stored at room temperature.
Tris/EDTA (TE)	10 mM Tris-HCl pH8.0, 1 mM EDTA pH 8.0. Stored at room temperature.
TFB 1	100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% (v/v) glycerol. Filter sterilised and stored at 4°C.
TFB 2	10 mM MOPS pH 7.0, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% (v/v) glycerol. Filter sterilised and stored at 4°C.

2.1.1.4. Bacterial Strains

E. coli of the following genotypes were used:

SURE™	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr) 171 end*A1 *sup*E44 *thi*-1 *gyr*A96
(Stratagene)	*rel*A1 *lac rec*B *rec*J *sbc*C *umu*C::Tn*5* (Kan^r) *uvr*C [*F' pro*AB
	*lac*I^{q}ZΔ*M15* Tn*10* (tet^r)]^c

Mutation and deletion of genes involved in DNA rearrangement, deletion, repair and recombination makes this strain suitable for cloning DNA containing inverted repeats or secondary structures.

TOP10	mcrA Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1
One Shot	*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^r) *end*A1 *nup*G
(Invitrogen)	

Ideal for high-efficieny cloning and plasmid propagation.

XL1-Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F'[*pro*AB$^+$
(Stratagene)	.*lac*I^q *lac*ZΔM15 Tn*10* (tet^r)]

Suitable for production of high quality plasmid DNA.

2.1.1.5. Vectors

pECE-EphA2WT: contains the full length cDNA sequence of murine EphA2 (Ganju *et al.*, 1994) in the mammalian expression vector, pECE .

pECE-EphA2Neu: pECE-EphA2WT containing a substitution of the transmembrane region of the EphA2 sequence for the transmembrane domain of the oncogenic form of *neu.*

pJLR-EphA2WT: contains the full length cDNA sequence of murine EphA2 in the retroviral vector pJLR-Gal (a kind gift from Andrew Ziemiecki, University of Berne, Switzerland).

pJLR-Δkin: EphA2 cDNA sequence comprising the intracellular domain of EphA2 (nucleotide 3086 to 3'UTR (*Bam*H I-*Sal* I)).

p2IgFc: contains extracellular domain of murine Nsk2 (5'UTR-nucleotide 673 (*Hind* III-*Bam*H I)) cloned in-frame with the murine IgG1 Fc in the mammalian expression vector, pcDNAI/Amp (Invitrogen) (see Appendix 3).

Signal pIgplus (R&D systems): contains the CD33 signal sequence and the human IgG1 Fc.

pCl.10: contains a ~500 bp *Xba* I-*Xho* I fragment of EphA2 genomic DNA flanking the integration site of the J3A3 provirus (Chen *et al.*, 1996) cloned into pBluescript® II KS-.

pIND (Invitrogen): mammalian expression vector containing the *Drosophila* minimal heat shock promoter under the transcriptional regulation of the VP16 transactivation domain of the retinoid X receptor (RXR)/VgEcR heterodimer that binds to the hybrid ecdysone response element in pIND. See Appendix 3.

pIND-GFP: pIND mammalian expression vector containing the full length sequence of the jellyfish green fluorescent protein (GFP).

pVgRXR (Invitrogen): contains modified ecdysone receptor and retinoid X receptor cDNAs, and *Sh ble* gene to facilitate selection in Zeocin™. See Appendix 3.

pSV2gpt: contains the *E. coli* gpt gene which encodes guanine phosphoribosyltransferase (gpt), facilitating selection in MAX medium (Mulligan and Berg, 1981).

pcDNAI/Amp (Invitrogen)
pcDNA3.1/ Myc-His(+) (Invitrogen)
pcDNA3.1/ Myc-His(+)/ *lacZ* (Invitrogen)
} cloning and expression vectors (See Appendix 3)

2.1.2. Bacterial Culture

2.1.2.1. Establishment of Bacterial Cultures

All bacterial cultures were derived from a single, well-isolated colony on an agar plate established from either a glycerol stock or a plasmid transformation and grown in the appropriate medium at 37°C at 250 rpm for 16 hours.

2.1.2.2. Preparation of Bacterial Glycerol Stocks

1 ml of an overnight bacterial culture was diluted to 10 ml with prewarmed LB broth and grown for a further hour at 37°C with shaking. 200 µl of this was mixed with 200 µl of sterile glycerol in a sterile 1.8 ml cryovial, vortexed to ensure even distribution of the glycerol, and 600µl of water was added prior to a further gentle mixing. Cryovials were then frozen on dry ice before transferral to long term storage at -80°C.

2.1.3. Determination of Nucleic Acid Concentration

Nucleic acid preparations were diluted in water and spectrophotometric readings measured at OD_{260} using a quartz cuvette. An OD_{260} measurement of 1 unit corresponds to ~ 50 µg/ml of double-stranded DNA, ~ 40 µg/ml of RNA and ~ 33 µg/ml of single-stranded DNA.

2.1.4. Horizontal Agarose Gel Electrophoresis

Agarose gels were prepared using electrophoresis grade agarose (UltraPure, Gibco-BRL) at concentrations from 0.6% to 1.5% (w/v) (depending on the size of the fragment(s) being analysed) in 1 x TAE containing 1 µg/ml ethidium bromide. 0.1 volumes of 10 x DNA loading buffer was added to the DNA samples prior to loading. DNA size markers were used to analyse DNA fragment sizes and to approximate DNA concentration. Electrophoresis was carried out at 20-100 V using Bio-Rad horizontal electrophoresis gel equipment. DNA was visualised using a shortwave transilluminator (302 nm) for analysis or longwave (365 nm) for preparatory work. DNA was photographed using the Bio-Rad Gel Doc 1000 system.

2.1.5. DNA Subcloning

2.1.5.1. Restriction Endonuclease Digestion of DNA

Restriction endonucleases and their accompanying buffers were obtained from either Boehringer or Gibco-BRL and digests carried out following the manufacturer's recommendations. Briefly, for an analytical gel, 1 µg of DNA was digested with 1 µl (10 units) of endonuclease and 2.5 µl 10 x buffer in a volume adjusted to 25 µl with water. Reactions were incubated for 70-90 minutes at 37°C. For preparation of DNA fragments, reaction volumes were scaled up accordingly.

2.1.5.2. Cleavage Close to the End of DNA Fragments

Reactions were set up as detailed in Section 2.1.5.1, but incubated for 16 hours at 37°C to allow the restriction endonucleases to attach to the shorter flanking regions of DNA.

2.1.5.3. Isolation of DNA Fragments for Subcloning

Following agarose gel electrophoresis, the required DNA fragment was excised using a clean scalpel blade and transferred into an Eppendorf tube. DNA was isolated from the gel slice using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Briefly, the gel slice was dissolved at 50°C before loading onto a silica-gel membrane spin column in the presence of a high salt, acidic buffer in order to facilitate DNA adsorption to the membrane. The spin columns were centrifuged at 13000 x *g*, room temperature, to remove contaminants and washed with an ethanol based buffer. DNA was then eluted from the membrane using water (pH 7.0-8.5) and collected by briefly centrifuging at 13000 x *g*.

2.1.5.4. Dephosphorylation of Linearised Plasmid DNA

Subsequent to restriction endonuclease digestion of DNA, 1 µl (1 unit) of calf intestinal alkaline phosphatase (CIAP) (Gibco-BRL) was added to the reaction which was incubated at 37°C for a further 30 minutes prior to agarose gel electrophoresis. The required endonuclease- and phosphatase- treated DNA fragment(s) was then isolated according to Section 2.1.5.3.

2.1.5.5. Ligation of DNA Molecules

On ice, 2 µl of 10 mM ATP (Amersham Pharmacia) was mixed with 2 µl of 10 x ligase buffer (NEB). 100-200 ng of vector DNA was then added, followed by the addition of a three-fold molar ratio of insert DNA. The volume was adjusted to 19 µl with water before adding 1 µl of T4 DNA ligase (NEB). The ligation components were gently mixed and divided into 2 tubes with 10 µl in each tube. One tube was incubated at 25°C for 60 minutes while the other was allowed to ligate more slowly overnight at 16°C. 1-5 µl of each ligation mixture was used to transform competent *E. coli* cells.

2.1.6. Preparation of *E. coli* Competent for DNA Transformation

10 ml of an overnight culture was diluted to 100 ml with prewarmed LB broth and incubated at 37°C until the OD_{550} had reached 0.48. At this point, the culture was cooled rapidly on iced water for 5 minutes before being centrifuged at 2500 x *g* for 5 minutes at 4°C. The resulting pellet was resuspended gently in 30 ml TFB 1 and incubated on ice for 15 minutes before a further centrifugation at 2500 x *g* for 5 minutes at 4°C. The pellet was resuspended gently in 4 ml TFB 2 and incubated on ice for 15 minutes before the resulting suspension was aliquoted into precooled Eppendorfs, snap frozen on dry ice and stored at -70°C.

2.1.7. Transformation of DNA into Competent Bacteria

1-5 µl of ligation mix was incubated on ice prior to the addition of 100 µl of freshly thawed competent cells. Following gentle mixing, tubes were incubated on ice for 30 minutes. The bacteria were then heat shocked by incubation at 42°C for 90 seconds before returning to ice for a further 2 minutes. 1 ml LB broth was added to the bacteria which were then incubated at 37°C for 30-60 minutes with gentle shaking to allow the cells to recover. The bacteria were then pelleted at 3000 x *g* for 5 minutes at room temperature. The supernatant was discarded and the pellet gently resuspended in 100 µl LB broth. 70 µl of this bacterial suspension was then spread onto prewarmed LB agar plates containing the appropriate antibiotic for selection and incubated overnight at 37°C.

2.1.8. Small-Scale Preparation of Plasmid DNA

2.1.8.1. Alkaline Lysis and DNA Precipitation

A 10 ml overnight culture was centrifuged at 2500 x *g* for 5 minutes at room temperature. The resulting pellet was resuspended in 200 µl of solution I, transferred to a new tube and 400 µl freshly prepared solution II was added, mixed and incubated on ice for 5 minutes. 200 µl ice-cold solution III was then added, and again mixed and incubated on ice for 5 minutes. The suspension was centrifuged at 13000 x *g* for 10 minutes at room temperature. 700 µl of the resulting supernatant was transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly before a further 5 minute centrifugation at 13000 x *g*. 600 µl of

the supernatant was transferred to a new tube and the DNA was precipitated by adding 360 µl (0.6 volumes) of isopropanol, mixing thoroughly and centrifuging for 10 minutes at room temperature. The resulting pellet was then washed with 70% ethanol before being suspended in 100 µl of TE containing 10 µg/ml RNAse A and incubated at 37°C for 60 minutes. DNA was re-precipitated using 0.1 volumes of 3 M sodium acetate pH 5.5 and 2.5 volumes of 100% ethanol with incubation for 60 minutes on dry ice. Following a 15 minute centrifugation at 13000 x *g*, room temperature, the pellet was washed with 75% ethanol, dried using vacuum centrifuge and resuspended in 50 µl water.

2.1.8.2. QIAprep Miniprep

2 ml overnight cultures were pelleted at 13000 x *g* for 5 minutes before isolating plasmid DNA using the QIAprep miniprep kit according to the manufacturer's instructions. Briefly, bacteria were resuspended and subjected to alkaline lysis, followed by single-step neutralisation and adjustment to high-salt conditions for optimisation of adsorption to the silica-gel membrane spin column. The spin columns were briefly centrifuged at 13000 x *g*, room temperature, to remove contaminants and washed with an ethanol based buffer. DNA was then eluted from the membrane using water (pH 7.0-8.5) and collected by centrifuging for 1 minute at 13000 x *g*, room temperature.

2.1.9. Large-Scale Preparation of Plasmid DNA

200-500 ml overnight cultures were harvested by pelleting at 6000 x *g* for 15 minutes at 4°C before isolating plasmid DNA using the QIAGEN Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instructions. Briefly, the bacteria were lysed under alkaline lysis conditions, and genomic DNA, cell proteins and SDS were separated from the plasmid-containing supernatant by centrifugation. Plasmid DNA bound to the anion-exchange resin under low pH and low salt conditions, allowing removal of contaminants before subsequent elution of plasmid DNA under high salt conditions. A further isopropanol precipitation facilitated the removal of salts, and the concentrated DNA plasmid preparation was then washed with 70% ethanol before being air-dried and resuspended in water.

2.1.10. DNA Sequencing

Nucleotide sequence of double stranded DNA templates was determined by use of the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction kit on the ABI-370A machine from Perkin Elmer according to the manufacturer's instructions. Briefly, 1 µg DNA template was mixed with 20 ng primer and adjusted to 12 µl with water before the addition of 8 µl dye terminator reagent mix (containing A-, C-, G- and T-Dye Terminators, dITP, dATP, dCTP, dTTP, Tris-HCl pH 9.0, $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA Polymerase, FS). The reaction mixture was overlayed with 40 µl mineral oil and the following PCR was carried out for 25 cycles:

96°C for 30 seconds
50°C for 30 seconds
60°C for 4 minutes

The reaction products were then ethanol precipitated by the addition of 2 µl 3M sodium acetate, pH 4.6, and 50 µl 95% ethanol and subsequent incubation on ice for 10 minutes. Precipitated DNA was recovered by centrifugation at 13000 x *g* for 30 minutes at room temperature, rinsed with 250 µl 70% ethanol and dried in a vacuum centrifuge. Samples were resuspended in loading reagent (Perkin Elmer) and run on an automated sequencer (kindly performed by Chris Odell, Ludwig Institute for Cancer Research, UCL). Sequence data was collected using proprietary software associated with the ABI-370A sequencer before further analysis using either the SEQED or DNAStar applications.

2.1.11. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All reagents used in RNA extraction and processing were RNAse free, solutions and glassware were treated overnight with diethyl pyrocarbonate (DEPC) at a final concentration of 0.1% and gloves were worn at all times.

2.1.11.1. Isolation of Total RNA using Caesium Chloride

Tissues were dissected in phosphate-buffered saline (PBS) and chopped finely in 10 ml guanidinium thiocyanate solution per gram of tissue. A homogenate was prepared using a glass Dounce homogeniser and layered over 2.2 ml caesum chloride solution in a

SW41 polyallomer tube (Beckmann). This was then centrifuged in an SW41 swingout rotor at 134000 x *g* for 24 hours at 15°C. The resulting white pellet was washed twice with DEPC-treated water, transferred using a pipette tip to an Eppendorf and dissolved in a minimum volume of 200 µl DEPC-treated water. Any insoluble matter was pelleted by centrifuging the sample at 13000 x *g* for 1 minute at room temperature. The supernatant was transferred to a clean tube, 7.5 µl 4 M NaCl per 100 µl RNA solution was added and the contents mixed. 2.5 volumes of 95% ethanol was added before incubation at -20°C overnight. The resulting pellet was centrifuged at 13000 x *g* for 5 minutes at room temperature, washed twice in 70% ethanol and vacuum dried before being resuspended in a minimal volume of DEPC-treated water. The OD_{260} was measured and samples were stored at -70°C.

2.1.11.2. Assessment of RNA Quality

The quality and integrity of the RNA was assessed by electrophoresis of 2-5 µl of each sample on a 1% (w/v) agarose gel. High quality RNA was characterised by little smearing and the presence of two discrete bands corresponding to the 28S and 18S ribosomal subunits. RNA concentration was determined as described in Section 2.1.3.

2.1.11.3. First Strand cDNA Synthesis

To 1-3 µg total RNA on ice, the following reagents were added; 0.4 µl of a 0.5 mg/ml solution of gelatin, 1.25 µl RNase inhibitor (40 units/µl, Promega), 4 µl of 25 mM $MgCl_2$, 0.5 µl each of 10 mM dNTPs (Amersham Pharmacia), 2 µl of 10 x AMV (avian myeloblastosis virus) buffer (Boehringer) and 1 µl Reverse Transcriptase AMV (20 units/µl (Boehringer). The volume was adjusted to 19 µl with DEPC-treated water before the addition of 1 µl of 100 ng/ul reverse primer. Samples were gently mixed and collected at the bottom of the tube by briefly pulsing them in a centrifuge. The RNA was reverse transcribed by incubation at 25°C for 10 minutes then at 42°C for 60 minutes.

2.1.11.4. Polymerase Chain Reaction of First Strand cDNA

First strand reactions were denatured by incubating at 99°C for 5 minutes and then cooling on ice. 5 µl was used as the reaction template to which the following reagents were added; 10 µl of 10 x Polymerase Buffer (Boehringer), 2.5 µl each of 10 mM

dATP, dCTP, dGTP and dTTP, 0.5 µl (5 unit/µl) Taq DNA polymerase (Boehringer) 1 µl of 1 µg/µl 5' primer, 1 µl of 1 µg/µl 3' primer and water to 100 µl. $MgCl_2$ was added at varying concentrations (2 mM to 6 mM) to optimise conditions. Each tube was mixed and overlayed with 100 µl mineral oil and the following PCR performed for 30 cycles:

93°C 1 minute

55°C 2 minutes

72°C 3 minutes

The final cycle was followed by an additional extension time of 7 minutes at 72°C. 20 µl of each reaction was analysed by gel electrophoresis and, where appropriate, further manipulation was carried out.

2.1.12. Southern Blotting

2.1.12.1. Isolation of Genomic DNA

Tissues were finely chopped in ~ 700 µl of genomic lysis buffer using a scalpel blade prior to incubation for 6-16 hours in a 55°C waterbath with occasional shaking. 700 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the viscous solution and rotated end-over-end for 5 minutes at room temperature prior to a 5 minute centrifugation at 13000 x *g*. Using a wide bore pipette tip, 500 µl of the top aqueous layer was transferred to a clean Eppendorf. Two volumes of 100% ethanol was then added, the contents of the tube were mixed by inversion and the DNA was pelleted by a 5 minute centrifugation at 13000 x *g*. Subsequent to a 70% ethanol wash, the pellet was air-dried before being resuspended in 200 µl TE.

2.1.12.2. Restriction Digest and Agarose Gel Electrophoresis

20 µg of genomic DNA was subjected to restriction endonuclease digestion using a three-fold excess of the appropriate enzyme for a minimum of 2 hours at 37°C. The reaction was subsequently subjected to agarose gel electrophoresis using a low percentage gel. The agarose gel was then photographed prior to undergoing denaturation as described below.

2.1.12.3. Gel Denaturation and Transfer

The gel was placed into a tray containing 0.25 M HCl and was gently shaken for 15 minutes at room temperature. The HCl was then replaced with Denaturation Solution and the gel was again gently shaken for 30 minutes at room temperature. The same procedure was repeated with Neutralisation Solution for 30 minutes. Meanwhile, an appropriately sized piece of Gene Screen™ nylon membrane (NEN) was presoaked in water and then in 10 x SSC for at least 15 minutes at room temperature.

Following incubation in Neutralisation Solution, the gel was carefully inverted before being transferred by capillary action onto the Gene Screen™ membrane overnight. The transfer blot consisted of a 3 MM wick wrapped around a glass plate, on top of which was placed the gel, 4 sheets of 3 MM paper, a large amount of tissues, another glass plate and a weight of approximately 0.5 kg. This was placed on top of a tray holding an ample volume of 10 x SSC, into which the wick was placed (see Figure 2.1). Transfer proceeded overnight at room temperature.

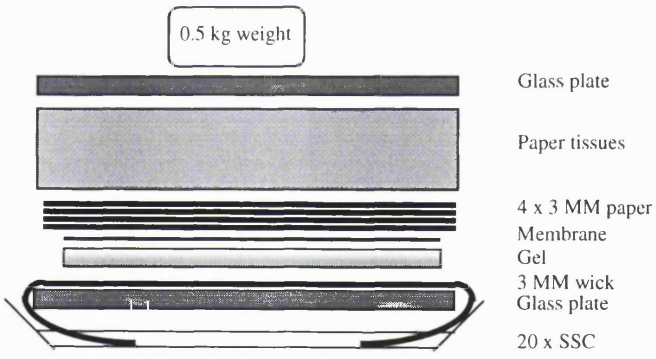


Figure 2.1. Schematic Representation of Southern Blot Transfer

The following morning the blotting apparatus was dismantled and the membrane was appropriately labelled to aid orientation. The membrane was then submerged in 0.4 M NaOH for 1 minute at room temperature before being transferred into a solution of 0.2 M Tris-HCl pH 7.5 and 2 x SSC for 10 minutes.

2.1.12.4. Preparation of Radiolabelled Probe by Random Primed DNA Labelling

25-50 ng of gel purified DNA was labelled by random priming using the Random Primed DNA Labelling Kit (Boehringer) according to the manufacturer's instructions. Briefly, water was added to the DNA probe in an Eppendorf tube to a final volume of

33 µl. The DNA was then denatured by incubation at 95°C for 3 minutes before immediately placing on ice. 10 µl of nucleotide mix was added to the denatured DNA prior to the addition of 50 µCi of [α-^{32}P]dCTP. 1 µl of Klenow enzyme was then added and the contents were incubated at 37°C for 30 minutes. Unincorporated radionucleotides were removed by passing the reaction through a Sephadex G-50 NICK column (Amersham Pharmacia) pre-equilibrated with TE.

2.1.12.5. Probe Hybridisation

The membrane was placed into 20 ml of Hybridisation Solution (prewarmed to 42°C), heat-sealed in a bag and incubated at 42°C for 2.5 hours. Subsequently the probe was denatured by incubation at 95°C for 10 minutes and added to the membrane at 2 x 10^6 counts/minute/ml of Hybridisation Solution. Hybridisation proceeded overnight at 42°C. The following morning the membrane was removed from the bag and washed twice, each for 15 minutes in 2 x SSC, 0.1% SDS at room temperature. Following two further washes at 42°C in 0.1 x SSC, 0.1% SDS, each for 30 minutes, the membrane was then air-dried, wrapped in Saran Wrap and exposed to X-ray film with an intensifier screen at -80°C for 1-3 days.

2.2. Mammalian Cell Culture

2.2.1. Preparation of Stock Solutions and Buffers

2.2.1.1. Media for Cell Culture

Routinely used media (below) were supplied by Gibco-BRL.
Dulbecco's Modified Eagle Medium (DMEM) (high glucose)
OPTIMEM™
RPMI-1640 Medium

Unless otherwise stated, Penicillin/Streptomycin solution (Gibco-BRL) and L-glutamine (Gibco-BRL) were added to all media at a final concentration of 50 units/50 µg per ml and 2 mM, respectively.

Magnesium and calcium free PBS was supplied by Gibco-BRL. Stored at 4°C.

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer was supplied by Gibco-BRL as a 1 M stock solution. Stored at room temperature.

Foetal calf serum (FCS) was supplied by Hyclone and was heat inactivated by incubating in a waterbath at 56°C for 45 minutes. Stored at 4°C.

2.2.1.2. Supplements/Selective Agents

Adenosine	Prepared from 250 mg vial (Sigma) as a 5 mg/ml stock in PBS, filter sterilised and stored at -20°C.
EGF	Supplied by Sigma. Prepared using sterile acidified BSA (4 mM HCl, 1% BSA, pH 4.5) as a 20 µg/ml solution. Stored in aliquots at -20°C.
G418 Sulphate (Geneticin)	25 ml of DMEM was added to a 5 g vial of G418 (Gibco-BRL), filter sterilised and stored in 1 ml aliquots (100 mg activity) at –70°C.
Insulin	Supplied by Sigma, prepared as a 5 mg/ml solution with sterile acidified BSA (4 mM HCl, 1% BSA, pH 4.5). Stored in aliquots at –20°C.
Mycophenolic acid	Prepared from 50 mg vial (Sigma) as a 5 mg/ml stock in 100% ethanol filter sterilised and stored at -20°C.
2 x Trypsin	5 ml PBS was added to a vial of bacto-trypsin (Difco), filter sterilised and stored in 1 ml aliquots at -20°C (100 x stock). To a 100 ml bottle of 1 x Trypsin/EDTA (Gibco-BRL), 1 ml of 100 x stock was added to make a 2 x trypsin solution. Stored at 4°C.
Xanthine	Prepared as a 10 mg/ml stock from 1 g vial (Sigma) in 100 ml PBS, dissolved by the dropwise addition of 10 M NaOH. Filter sterilised and stored at -20°C.
Zeocin™	Supplied by Invitrogen as a 100 mg/ml stock. Stored at -20°C in dark.

2.2.1.3. Transfection Solutions

β- mercapto-ethanol	Prepared as a 10 mM stock in water, filter sterilised and stored at 4°C.
Chloroquine	Prepared as a 5 mM solution in water, filter sterilised and stored in the dark at -20°C.
DEAE-Dextran	Prepared as a 20 mg/ml solution in PBS, sterilised by autoclaving, and stored in the dark at 4°C.
Electroporation buffer	120 mM KCl, 10 mM K_2PO_4/KH_2PO_4, pH 7.6, 25 mM HEPES pH 7.6 2 mM $MgCl_2$, 0.5% (w/v) Ficoll 400. pH adjusted to 7.6 with 1 M KOH, then filter sterilised. Stored at 4°C.
ES trypsin	0.8% (w/v) NaCl, 0.04% (w/v) KCl, 0.01% (w/v)Na_2HPO_4, 0.1% (w/v) glucose, 0.3% (w/v) Trizma base, 0.001% (w/v) Phenol red, 0.006% (w/v) Penicillin G, 0.01% (w/v) Streptomycin, 0.25% (w/v) bacto-tryptone. pH adjusted to 7.6, filter sterilised and stored at -20°C.
Lipofectin™	Supplied by Gibco-BRL.
Methylene Blue stain	0.5% (w/v) methylene blue, 50% (v/v) methanol. Stored in dark at room temperature.
Mitomycin C	Prepared fresh from a 2 mg vial (Boehringer) as a 1 mg/ml solution in PBS, filter sterilised.
Polybrene	Prepared as a 10 mg/ml solution in water. Filter sterilised. Stored at 4°C.

2.2.2. Routine Culture and Passage of Cell Lines

All cells were incubated at 37°C in a humidified atmosphere of 5-10% CO_2.
All cells except MCF-7 cells were routinely grown on 10 cm tissue culture dishes obtained from Becton Dickinson. MCF-7 cells were grown on Collagen I coated tissue culture dishes from Becton Dickinson.

With the exception of EpH4, EpRas and HC11 cells, cells were passaged using 1 x Trypsin-EDTA solution (0.5 g Trypsin and 0.2 g EDTA•4Na/litre) supplied by Gibco-BRL.

EpH4 mouse mammary epithelial cells were a kind gift from Ernst Reichmann (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). The cell line was derived by spontaneous immortalisation from mammary gland tissue of mid-pregnant BALB/c mice (Fialka *et al.*, 1996). Cells were maintained in DMEM containing 8% FCS, 10 mM HEPES buffer, L-glutamine and penicillin/streptomycin.

EpRas cells (also from Ernst Reichmann) were generated by retrovirally infecting EpH4 cells with recombinant v-Ha-*ras* (Oft *et al.*, 1996). EpRas cells were maintained in EpH4 medium containing 200 μg/ml activity G418.

When confluent, but before the appearance of hemicyst domes, cultures were passaged at a ratio of 1 to 3 according to the following regime; medium was aspirated from the cell layer, which was then briefly rinsed in 5 ml 2 x trypsin. 5 ml of 2 x trypsin was then added to the cells and incubated at 37°C until the cell contacts were seen to be broken (but the cells remained adherent), whereupon the trypsin was aspirated off and replaced with 1 ml fresh 2 x trypsin. Cells were then incubated for a further 5 minutes at 37°C before adding 9 ml fresh medium to displace the cells. The 10 ml suspension was then centrifuged at 100 x *g* for 5 minutes at room temperature, the supernatant was removed and the remaining cells were resuspended and split appropriately.

HC11 cells, also derived from mid-pregnant BALB/c mouse mammary gland tissue (Ball *et al.*, 1988), were a kind gift from Clive Dickson (ICRF, London, UK). HC11 cells were maintained in RPMI-1640 supplemented with 10% FCS, 10 ng/ml murine EGF, 5 μg/ml insulin, L-glutamine and penicillin/streptomycin. When confluent,

cultures were passaged at a ratio of 1 to 3 (approximately every other day) using 2 x trypsin as described above for EpH4 cells.

MCF-7 breast adenocarcinoma cells (Soule *et al.*, 1973), obtained from the European Collection of Cell Cultures (ECACC), were maintained in DMEM containing 10% FCS, L-glutamine and penicillin/streptomycin. When 80% confluent, cultures were passaged at a ratio of between 1:5 and 1:10 using 1 ml of 1 x Trypsin/EDTA after washing the cell monolayer with prewarmed sterile PBS. MCF-7 cells stably expressing pVgRXR (MCF-7-pVgRXR) were a kind gift from Jackie Meakin, Molecular Neurobiology Department, SmithKline Beecham Pharmaceuticals, Essex, UK. Stable transfectants were maintained in medium containing 350 µg/ml activity G418 and 400 µg/ml of Zeocin™.

COS-1 and COS-7 cells (Gluzman, 1981), (obtained from Alastair Reith, Ludwig Institute for Cancer Research, UCL, UK and James Blakemore, Molecular Neurobiology Department, SmithKline Beecham Pharmaceuticals, Essex, UK, respectively) were maintained in DMEM containing 10% FCS, L-glutamine and penicillin/streptomycin. When 80% confluent, cell monolayers were washed in prewarmed PBS, trypsinised using 1 ml of 1 x Tryspin/EDTA and split at a ratio of 1 to 10.

NIH3T3 fibroblasts (Jainchill *et al.*, 1969), obtained from Alastair Reith, Ludwig Institute for Cancer Research, London, UK, were maintained in DMEM containing 10% FCS, L-glutamine and penicillin/streptomycin. When 80% confluent, cultures were passaged at a ratio of 1 to 10 using 1 ml Trypsin/EDTA. Stable transfectants were maintained in medium containing 250 µg/ml activity G418.

AmpliGPE retroviral producer lines (Takahara *et al.*, 1992) were maintained in DMEM containing 10% FCS, L-glutamine, penicillin/streptomycin and 250 µg/ml activity G418. When 80% confluent, cultures were passaged as for NIH3T3 cells.

WT38, WT43 and WT62 cells correspond to three clonal cell lines made by retrovirally infecting NIH3T3 cells with pJLR-EphA2WT, and were provided by Alastair Reith (Ludwig Institute for Cancer Research, UCL, UK). These cell lines were maintained in NIH3T3 medium supplemented with 250 µg/ml G418.

MDA-MB-231 cells (Cailleau *et al.*, 1974), (obtained from the ECACC) were maintained in the same medium used for EpH4 cells. When 90% confluent, cells were passaged at a ratio of 1 to 5 using 1 ml 1 x Trypsin/EDTA.

The φ2 retroviral cell line (Mann *et al.*, 1983) transfected with SV2a was a kind gift from Parmjit Jat (Ludwig Institute for Cancer Research, UCL, UK). Cells were maintained in DMEM supplemented with 10% donor calf serum (Gibco-BRL) L-glutamine, and penicillin/streptomycin.

The GP+E 86 retroviral packaging line (Markowitz *et al.*, 1988) transfected with V12Ras and the Ras effector pathway mutants V12S35, V12G37 and V12C40 (Rodriguez-Viciana *et al.*, 1997), were a kind gift from Julian Downward (ICRF, London, UK). GP+E 86 cells were maintained in DMEM containing 10% FCS, L-glutamine and penicillin/streptomycin containing 250 μg/ml G418.

2.2.2.1. Cell Counting

Cells were counted using a Neubauer counting chamber. Cells were trypsinised as detailed previously and the cell pellet was resuspended in a suitable volume of growth medium. A glass Pasteur pipette was used to transfer a small volume of the cell suspension to the counting chamber by capillary action. Cells in the central square (5 x 5 smaller squares) bounded by triple lines were counted on both sides of the chamber, and used to calculate the number of cells/ml of suspension by multiplying the mean result by 1 x 10^4.

2.2.3. Cryogenic Preservation and Recovery of Cell Lines

All exponentially growing cells were removed from their substrates as detailed for each cell line and resuspended in DMEM containing 25% (v/v) FCS and 10% (v/v) dimethylsulphoxide (DMSO), except for MCF-7 cells which were resuspended in 95% (v/v) FCS and 5% (v/v) DMSO. Approximately 1 x 10^6 cells were frozen in 1.5 ml cryovials (Nunc) in cell freezing racks (Nalgene) overnight at -80°C before being transferred to either liquid nitrogen or to -140°C.

Cryogenically preserved cells were brought up from storage by rapid thawing in a 37°C waterbath followed by the addition to 9 ml complete growth medium, centrifugation at

100 x *g* for 5 minutes and aspiration of the supernatant in order to remove the DMSO. Cells were then resuspended in growth medium and transferred to a 10 cm tissue culture dish.

2.2.4. Mammalian Cell Transfection

All DNA used in mammalian cell transfection was prepared using the QIAGEN maxiprep procedure (Section 2.1.9).

2.2.4.1. Transfection by Lipofectin™

Cells were plated at 8 x 10^5 cells per 10 cm dish in complete growth medium and incubated overnight. For each transfection, two 5 ml pop-top tubes (Falcon) containing the following reagents were set up.

Tube A. 10 µg DNA + 1.5 ml OPTIMEM™ + 15 µl 10 mM β-mercaptoethanol
Tube B. 25 µl Lipofectin™ + 1.5 ml OPTIMEM™ + 15 µl 10 mM β-mercaptoethanol

The DNA mixture (tube A) was added dropwise to the Lipofectin™ mixture (tube B) and then left to stand for 15 minutes at room temperature. After the 15 minute incubation, the transfection mixture was added dropwise to the cell layer (which had been washed three times in PBS), ensuring it was evenly distributed over the cells. The cells were then placed back into the 37°C incubator for 6 hours before adding 3 ml of growth medium containing 20% FCS. Following a 16 hour incubation, this was replaced with 10 ml of OPTIMEM™ for proteins expected to be secreted, or regular 10% FCS containing growth medium. Conditioned medium was collected 48 hours and 72 hours after replacement of the medium. Analysis of intracellular proteins was carried out after 48-72 hours.

2.2.4.2. Transfection by DEAE-Dextran

COS-7 cells were seeded at 2.5 x 10^6 cells per 175 cm^2 flask into 10 flasks and incubated at 37°C for 24 hours. 5 ml DEAE-Dextran and 5 ml chloroquine were mixed together. In a separate tube, 400 µg DNA was diluted to 2 ml in TE. 8 ml of the 1:1 DEAE-Dextran/choroquine mix was then added to the DNA solution and incubated at room temperature for 5 minutes before the addition of 190 ml of DMEM containing 2

mM glutamine. Cells were washed twice with DMEM + 2 mM glutamine prior to adding 20 ml of the transfection mixture to the cell monolayer. Cells were then incubated at 37°C for 3 hours, after which the mixture was removed and the cells were osmotically shocked by the addition of 20 ml 10% DMSO in PBS for 2 minutes at room temperature. This mixture was removed and the cells were incubated overnight in 20 ml DMEM/10% FCS + 2 mM L-glutamine. For harvesting secreted proteins, this was replaced 24 hours later with 40 ml of DMEM supplemented with 1% (v/v) Ultra-Low IgG FCS (Gibco-BRL) and 2 mM L-glutamine. Cells were then incubated at 37°C for a further 6 days before the conditioned medium was harvested and secreted proteins purified (Section 2.3.10.2).

2.2.4.3 Transfection by Electroporation

Cells were seeded at 5 x 10^7 cells/15 cm dish. 24 hours later, cells were trypsinised as usual and then washed with 5 ml cold electroporation buffer. Cells were resuspended in 250 µl cold electroporation buffer which was then added to 10 µg DNA in an electroporation cuvette (0.4 cm mammalian, Bio-Rad). The DNA cell suspension was mixed and incubated on ice for 10 minutes before being electroporated at 200 V, 950 µF using the Bio-Rad Gene Pulser II Electroporation System, and returned to ice for 5 minutes. Following a further 5 minute incubation at room temperature, cells were transferred into a 10 cm dish and returned to the incubator. Subsequent to a medium change after 24 hours, protein expression was analysed 24-48 hours later or, where stable cell lines were being created, cells were seeded at 1 x 10^5/15 cm dish before being placed in selection medium.

2.2.4.4. Transfection of Cells by Calcium Phosphate

All solutions were derived from the Stratagene Mammalian Transfection Kit.
Solution I – 2.5 M $CaCl_2$.
Solution II – 2 x BSS, pH 6.95 (50 mM N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid and buffered saline, 280 mM NaCl and 1.5 mM Na_2HPO_4)

Cells were seeded at 5 x 10^5 cells onto 10 cm plates and incubated overnight. For each transfection, 30 µg DNA was diluted to 450 µl water in a 5 ml polypropylene tube. 50 µl solution I was added dropwise with shaking. 500 µl solution II was then added dropwise with shaking. The mixture was incubated at room temperature for 20 minutes

before being added to the medium with gentle mixing. The dish was then returned to the incubator for 8 hours, after which the cells were washed once with PBS before being refed with normal medium. For generation of stable transfectants, 48 hours following transfection the cells were split 1:10 into 10 cm dishes and 24 hours later cells were fed with selective medium.

2.2.4.5. Retroviral Mediated Gene Transfer

2.2.4.5.1.Generation of Retroviral Producer Lines

AmpliGPE viral producer lines were co-transfected with the appropriate retroviral vector and pSV2gpt using calcium phosphate and selected for the presence of *gpt* using MAX medium (DMEM containing 25 μg/ml mycophenolic acid, 25 μg/ml adenosine and 250 μg/ml xanthine, 10% FCS, 2 mM glutamine) every 3-4 days. After 10-14 days, when the mock transfected cells had died, well-isolated clones were picked using a P200 tip and transferred into selective medium in 4 well dishes (Nunc). The clones were gradually grown up to duplicate 60 mm dishes, one of which was used to assess the viral titre while the other was cryogenically preserved.

2.2.4.5.2. Production of Viral Supernatants

Medium was removed from 60 mm dishes and debris was pelleted by centrifugation at 200 x *g* for 15 minutes at 4°C. The supernatant was either frozen at -80°C or used directly for infection of NIH3T3 cells.

2.2.4.5.3. Retroviral Infection of Fibroblasts

NIH3T3 cells were plated out at 4 x 10^5/60 mm dish. The following day, the medium was removed and replaced with 1 ml fresh medium containing 8 μg polybrene plus two 10-fold dilutions of viral stock (25 μl and 250 μl). The cells were incubated for 2 hours at 37°C with occasional gentle shaking, then 3 ml regular medium was added and the cells were left 16-18 hours. The cells were then split 1:10 onto 10 cm dishes and after a further 24 hours cells were fed with medium containing 300 μg/ml G418. Once the mock-infections were cleared of cells, the viral titre was calculated by counting the number of colonies stained with methylene blue.

2.2.4.5.4. Methylene Blue Staining of Cells

Cells were rinsed with 5 ml PBS and then fixed and stained for 10 minutes at room temperature with 5 ml Methylene Blue stain. Dishes were gently washed with water until the stained colonies were visible, then dried at room temperature. The number of colonies was counted and the viral titre (colony forming units (cfu)) calculated using the following equation;

cfu/ml = number of colonies x initial split ratio x (1/initial infection volume)

2.2.4.5.5. Retroviral Infection of Mammary Epithelial Cells by Co-Culture

Retroviral producer lines were seeded at 1 x 10^7/cells per 15 cm dish. 24 hours later, medium was removed and replaced with 10 ml fresh medium lacking selective agents. 100 µl of mitomycin C solution was added and the cells were incubated for 8 hours at 37°C. Following three washes with PBS, cells were trypsinised and split equally into 3 x 10 cm plates containing fresh medium. The following day, cells were washed once with EpH4 medium and overlaid with EpH4 cells in the normal splitting regime. Control dishes of either mitomycin C-treated cells alone or EpH4 cells alone were plated out. After 48 hours, dishes were trypsined and split 1:10 into 10 cm dishes. The following day, cells were fed with medium containing 200 µg/ml activity G418. When the control dish of EpH4 cells plated alone had died, the number of colonies was counted. Clones were picked, grown to confluence and analysed for expression of the infected gene.

2.2.5. Preparation of Primary Embryonic Fibroblasts

Ten 15-17 dpc foetuses were dissected in PBS. The heads, liver and any attached internal organs were removed and the carcusses washed twice in 50 ml PBS to remove excess blood. Using scissors, the carcusses were finely chopped in PBS before adding 50 ml of ES trypsin. This mixture was then stirred using sterile glass beads over a magnetic stirrer for 30 minutes at 37°C. 50 ml of fresh ES trypsin was then added and the mixture was stirred for a further 30 minutes at 37°C. The process was then repeated a third time before the solution was carefully decanted from the beads and filtered through a fine nylon mesh. Cells were pelleted by centrifugation at 100 x *g* for 5 minutes, and washed twice in 50 ml PBS. The cells were then counted (see Section 2.2.4) and large fibroblasts only were plated at a density of 5 x 10^6 cells per 150 mm

dish in DMEM containing 10% FCS, L-glutamine and penicillin/streptomycin. The cells were refed 24 hours later. After 2-3 days, confluent cells were split into five 150 mm dishes, which were then cryopreserved upon reaching confluence.

2.3. Protein Analysis

2.3.1. Preparation of Stock Solutions and Buffers

2.3.1.1. Reagents for SDS-PAGE and Western Blotting

Acrylogel 3 solution	Supplied by BDH as a 40% (w/v) solution of Acrylamide and NN'-Methylenebis-acrylamide (29.1:0.9).
Block buffer 1	TTBS containing 5% (w/v) skimmed milk powder.
Block buffer 2	TTBS containing 2% (w/v) BSA (fraction V).
Coomassie Blue	0.3% (w/v) Coomassie Blue R-250, 10% (v/v) glacial acetic acid, 50% (v/v) methanol. Stored at room temperature.
Destain reagent	10% (v/v) glacial acetic acid, 40% (v/v) methanol. Stored at room temperature.
Molecular Weight Markers	To a vial of prestained molecular weight standards (SDS-7B) (Sigma), 0.24 g urea and 200 µl 5 x sample buffer were added. The volume was adjusted to 1 ml with water. 10-20 µl aliquots were stored at -20°C.
PBS	Five PBS tablets (Sigma) were dissolved in 1 l of water, which was autoclaved and stored at room temperature.
Sample buffer (5 x) (reducing)	100 mM Tris-HCl pH 8.0, 4% (w/v) SDS, 50% (w/v) glycerol, 0.3% (v/v) β-mercaptoethanol and 0.5% (w/v) bromophenol blue. Stored at -20°C.
(non-reducing)	As above with the omission of β-mercaptoethanol.

SDS-PAGE buffer	25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS. Stored at room temperature.
Stripping buffer	Prepared using 0.75% (w/v) glycine, 1% (w/v) SDS and 0.05% (v/v) NP-40 in water. pH adjusted to 2.2 using 1 M HCl. Stored at room temperature.
Transfer buffer	25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol. Stored at room temperature.
TTBS	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20. Stored at room temperature.

2.3.1.2. Reagents for Immunocytochemistry and Staining

Block buffer 3	PBS containing 2% (w/v) BSA (fraction V). Prepared fresh.
Fixative	4% (w/v) paraformaldehyde in PBS. Stored at -20°C in aliquots.
Permeabilising solution	0.5% (v/v) Triton X-100 in PBS. Prepared fresh.
X-gal	Prepared as 40 mg/ml solution in DMSO. Aliquoted and stored at -20°C in dark.
X-gal staining solution	5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal. Prepared in PBS immediately prior to use.
X-gal wash buffer	2 mM $MgCl_2$ in PBS. Stored at room temperature.

2.3.1.3. Protease/Phosphatase Inhibitors

Complete Protease Inhibitor Cocktail Tablets were purchased from Boehringer. One tablet was dissolved in 50 ml lysis buffer.

β-glycerol-phosphate	Prepared as a 1 M stock in water and stored at 4°C.
Leupeptin	Prepared as a 10 mg/ml solution in water and stored at -20°C.
Phenylmethyl-sulphonylfluoride (PMSF)	Prepared as a 100 mM stock in isopropanol and stored at -20°C.
Sodium fluoride	Prepared as a 0.5 M stock in water and stored at 4°C.
Sodium orthovanadate	Prepared as a 200 mM solution. The pH was adjusted to 10 using 1 M HCl and the solution was boiled until it became colourless. Upon cooling to room temperature, the pH was again adjusted to 10 using 1 M HCl prior to boiling. This procedure was repeated until the pH was stable and the solution remained colourless. Aliquots were stored at -20°C.
Sodium pyrophosphate	Prepared as a 200 mM stock in water and stored at room temperature.

2.3.1.4. Cell Lysis Buffers and Protein Kinase Assay Buffers

HBIB	20 mM HEPES pH 7.5, 0.05% (v/v) Triton X-100, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA. Stored at 4°C. 100 μM sodium orthovanadate was added fresh prior to use.
JNK assay buffer	JNK kinase buffer containing 100 μM ATP, 66 μg/ml GST-ATF2 (UBI) and 33 μCi/ml [γ-^{32}P]ATP. Prepared fresh.

JNK kinase buffer	20 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM EDTA, 1 mM ethyleneglycol-bis(β-aminoethylether)-N, N, N, N'-tetraacetic acid (EGTA), 0.1% (v/v) β-mercaptoethanol. Prepared fresh.
JNK lysis buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100. Stored at 4°C. 1 mM PMSF, 10 μg/ml leupeptin and 1 mM sodium orthovanadate added prior to use.
Jun assay buffer	Jun kinase buffer containing 10 μM ATP, 1 mM dithiothreitol (DTT), 200 μM sodium orthovanadate and 66 μCi/ml [γ-^{32}P]ATP. Prepared fresh.
Jun kinase buffer	20 mM HEPES pH 8.0, 20 mM $MgCl_2$, 20 mM β-glycerolphosphate. Prepared fresh.
Jun lysis buffer	20 mM HEPES pH 7.5, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.05% (v/v) Triton X-100, 20 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 x Complete Protease Inhibitor Cocktail tablet/50 ml. Prepared fresh.
Kinase buffer I	10 mM $MnCl_2$, 1% (v/v) Triton X-100. Prepared fresh before use.
MAPK assay buffer	Standard kinase buffer containing 10 μM ATP, 0.25 mg/ml myelin basic protein (MBP) (Sigma) and 66 μCi/ml [γ-^{32}P]ATP. Prepared fresh.
MAPK lysis buffer	10 mM sodium glycerophosphate, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 20 mM Tris-HCl pH 7.5, 0.1% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol and 1 mM sodium orthovanadate. Stored at -20°C.
MEK assay buffer	MEK kinase buffer containing 200 μM ATP and 40 μg/ml GST-Erk 2 (NEB). Prepared fresh.

MEK kinase buffer	25 mM Tris-HCl pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$. Stored at -20°C.
MEK lysis buffer	20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin and 1 mM PMSF. Stored at -20°C.
RIPA buffer (Radioimmuno-precipitation assay)	10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1%(v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS. Stored at 4°C without protease inhibitors. 100 μM sodium orthovanadate, 100 μg/ml leupeptin, 1 mM PMSF were added fresh before use.
Standard kinase buffer	30 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$. Stored at 4°C.

2.3.1.5. Miscellaneous Reagents

Anisomycin	Prepared as a 10 mg/ml solution in 10% (v/v) DMSO and 90% (v/v) ethanol, aliquoted and stored at -80°C.
DMSO soluble inhibitors	U0126 (Promega), SB-203580 and SB-386023 were prepared at 10 mM in DMSO (1000 x working concentration), aliquoted and stored at –20°C. LY294002 (Calbiochem) was prepared at 50 mM in DMSO (2500 x working concentration), aliquoted and stored at -20°C.
Human IgG	A 4.8 mg/ml solution of purified human IgG (Sigma) was diluted in PBS to a 0.1 mg/ml solution, aliquoted and stored at -20°C.
Protein elution buffer	0.1 M glycine-HCl. Prepared fresh by a 1 in 10 dilution in water of a 1 M stock of glycine-HCl, pH 2.7 (stored at 4°C).

2.3.2. Protein Assays

For samples prepared directly in 5 x sample buffer, quantitation of proteins was performed using the modified Lowry procedure (Sigma Protein Assay Kit) and samples were measured at 570 nm. Unless otherwise stated, the protein concentration of all other samples was measured at 595 nm using the modified Bradford procedure (Bio-Rad Protein Standard Assay). Samples were measured in duplicate or triplicate. BSA was used to formulate a standard curve in both assays.

2.3.3. Preparation of Whole Cell Lysates

Cells were washed in prewarmed PBS before being lysed in either 200 µl (60 mm dishes) or 500 µl (10 cm dishes) of 95°C 5 x sample buffer. The cell layer was then scraped using a cell scraper (Costar) and transferred to an Eppendorf before being incubated at 95°C for 5 minutes. DNA was sheared by passing the lysate through a 25 gauge needle five times. The protein concentration of each lysate was determined using the Sigma protein assay.

2.3.4. Immunoprecipitation

Following treatment, cell layers were washed once in ice-cold PBS. During the entire procedure, all lysates and buffers were kept at 4°C.

Subsequent to the addition of the appropriate lysis buffer, cell monolayers were scraped using a cell scraper and lysates were transferred to precooled Eppendorfs. Lysates were precleared by centrifugation at 13000 x *g*, 4°C, for 15 minutes. The protein concentration of the supernatant was then measured according to Section 2.3.2. Essentially, all immunoprecipitations were carried out in equal volumes by incubating equivalent concentrations of lysate with 1-5 µg of an immunoprecipitating antibody and a suitable capturing matrix. For experiments with a small number of samples, lysates were incubated with an appropriate volume of antibody for 60 minutes at 4°C on a rotating wheel, prior to the addition of 30 µl of a 50% (v/v) slurry of protein A- or protein G- Sepharose (Amersham Pharmacia) (prewashed 3 times in lysis buffer) for a further 2 hours at 4°C. For larger scale assays, antibodies were precoupled to beads by preparing 125% of the required amounts of both antibody and beads and incubating for 2–16 hours at 4°C on a rotating wheel. After this period, beads were washed 3 times in the appropriate buffer to remove any uncoupled antibodies, resuspended in 1 bead

volume of buffer and the 50% (v/v) bead/antibody slurry was aliquoted into the appropriate number of tubes. These were then incubated with lysates for 2 hours at 4°C.

Following immunoprecipitation, beads were washed by brief centrifugation, removal of supernatant and resuspension of the beads in a suitable buffer, and either boiled in sample buffer and subjected to SDS-PAGE and western blotting, or to an *in vitro* kinase assay using [$\gamma^{32/33}$P]ATP.

For SDS-PAGE, an appropriate volume (10-50 µl) of sample buffer was added and samples were electrophoresed following a 5 minute denaturation at 95°C and a brief centrifugation. When assessing both the steady-state level of the protein and its phosphotyrosine status, one single immunoprecipitation per treatment was carried out and a volume of denatured sample was divided equally between two SDS-PAGE gel wells. Samples were then electrophoresed and subjected to western blotting as described in Section 2.3.7.

Assay	**Immunoprecipitating antibody (up to 500 µg lysate)**	**Species**	**Bead Matrix**
Standard Immunoprecipitation	1 µg anti-EphA2 (C-20) (Santa Cruz)	Rabbit	Protein A-Sepharose
MAPK	2 µg anti-ERK1/2 (UBI)	Rabbit	Protein A-agarose
MEK kinase	2 µg anti-MEK 1 (Transduction labs)	Mouse	Protein G-Sepharose
JNK kinase	1 µg anti-JNK 1 (C-17) (Santa Cruz)	Rabbit	Protein A-Sepharose

Table 2.1. Summary of Reagents used in Immunoprecipitation Assays

2.3.4.1. Standard Immunoprecipitation

Cells were lysed as described in Section 2.3.4 using 500 µl of RIPA buffer per 60 mm dish or 1 ml per 10 cm dish. Tissues freshly dissected in PBS were snap frozen in liquid nitrogen and stored at -80°C. Tissues were either thawed in ice-cold RIPA buffer and homogenised using a Dounce glass homogeniser, or ground into a fine powder under liquid nitrogen using a pestle and mortar on dry ice. The powder was then resuspended in RIPA buffer. Cell and tissue lysates were then vortexed before being precleared as described in Section 2.3.4. Protein concentrations of tissue samples were routinely measured using the modified Lowry procedure (Sigma Protein Assay Kit).

Equal volumes of samples containing equivalent protein concentrations were further precleared by incubation with 25 µl of a 50% (v/v) protein A- suspension (prewashed in lysis buffer) for 60 minutes at 4°C on a rotating wheel. The bead suspension was pelleted by brief centrifugation before the supernatant was incubated for 60 minutes, 4°C, with the appropriate dilution of antibody on a rotating wheel. Immunocomplexes were captured by the addition of 30 µl of a 50% (v/v) protein A- or protein G-Sepharose suspension, followed by further incubation at 4°C for 2 hours. The Sepharose bead-immunocomplexes were then washed 3 times in RIPA buffer and subjected to SDS-PAGE or to an *in vitro* kinase assay.

2.3.5. SDS-PAGE

16 x 20 cm 3-12% gradient resolving gels were prepared using 20 ml each of 3% and 12% gel acrylamide solutions (see Table 2.2). Resolving gels were overlaid with a 5% stacking gel (see Table 2.2). The 12% solution was added to the first chamber of a gradient pourer (Anachem) and the 3% solution to the second. A small amount of the 12% solution was poured into the gel plate rig (Gibco-BRL) to allow a seal to form before opening the 3% chamber tap. The solutions were mixed (using a magnetic stirrer) as they were poured. The gel mix was overlaid with water-saturated butan-2-ol to form an even surface. Following gel polymerisation, the butan-2-ol was extensively washed off with distilled water and the resolving gel was overlaid with a 5% stacking gel. An appropriately sized comb was inserted and the stacking gel left to polymerise.

Reagent	**3% resolving gel**	**12% resolving gel**	**5% stacking gel**
Water	10.65 ml	6.15 ml	6 ml
Tris-HCl	7.5 ml (pH 8.9)	7.5 ml (pH 8.9)	2.5 ml (pH 6.8)
Acrylogel-3	1.5 ml	6 ml	1.25 ml
20% (w/v) SDS	100 µl	100 µl	100 µl
10% (w/v) ammonium persulphate	66 µl	66 µl	100 µl
TEMED	12.7 µl	12.7 µl	20 µl

Table 2.2. Summary of Reagents Required for the Preparation of Resolving and Stacking Gels for SDS-PAGE

All minigels were precast 4-15% gradient gels purchased from Bio-Rad. All minigels were subjected to SDS-PAGE using the Bio-Rad Mini Protean II Electrophoresis/Transfer system.

Before loading samples, wells were washed out with SDS-PAGE buffer. All samples were denatured at 95°C for 5 minutes and briefly pulsed down before being loaded into the wells of the stacking gel. Minigels were electrophoresed in SDS-PAGE buffer at 70-140 V for 1-2 hours, and large gels overnight at 12-13 mA until the dye front had reached the bottom of the gel. At this point, gels were either fixed and stained with Coomassie Blue, or transferred onto nitrocellulose membrane for western blotting.

2.3.6. Coomassie Blue Staining of SDS-PAGE Gels

Subsequent to SDS-PAGE, gels were fixed and stained in Coomassie Blue stain for a minimum of 60 minutes before being destained in several changes of destain buffer until bands were clearly visible. The gels were then dried onto 2 sheets of 3 MM Whatman paper for 1-2 hours at 80°C using a Bio-Rad gel dryer.

2.3.7. Western Blotting

Electrophoresed proteins were transferred to Protran nitrocellulose membrane (Schleicher and Schuell) by wet blotting, using the Bio-Rad Mini Protean II System for minigels, or the Bio-Rad Trans Blot Cell for larger gels. Gels were pre-incubated in transfer buffer for 10 minutes at room temperature to equilibrate them. A blotting sandwich was then built up (see Figure 2.2), comprising a filter pad, 2 sheets of 3 MM Whatman paper, the gel, 1 sheet of nitrocellulose membrane, 2 further sheets of Whatman paper and another filter pad, all components having been pre-soaked in transfer buffer.

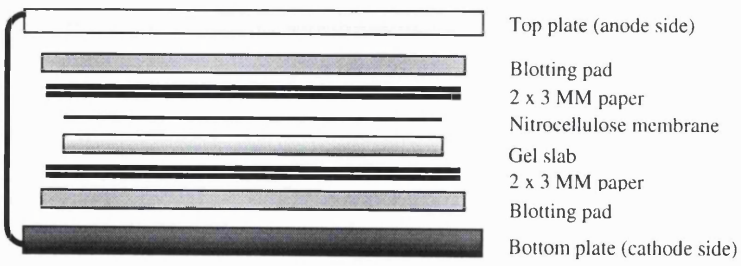


Figure 2.2. Schematic Representation of Western Blot Transfer

Proteins were transferred in transfer buffer for 16 hours at 20 V, 4°C or at 50 V for 90 minutes with an ice-pack in the transfer tank for minigels. Larger gels were blotted at 4°C at 200 mA for 4-16 hours. Following transfer, membranes were briefly stained

with Ponceau S solution (Sigma) to confirm efficient protein transfer. Membranes were then blocked in either block buffer 1 or 2 for a minimum of 60 minutes at room temperature or overnight at 4°C.

Membranes were incubated in primary antibody optimally diluted in block buffer (see Table 2.3) for 60 minutes at room temperature, followed by 5 x 5 minute washes in TTBS to remove excess unbound antibody. Membranes were then incubated in blocking buffer containing an appropriate dilution of secondary horseradish peroxidase (HRP) -conjugated antibodies (Pierce) for 60 minutes at room temperature. The membranes were again washed in TTBS for 5 x 5 minutes before enhanced chemiluminescence (ECL) was carried out according to the manufacturer's instructions (Amersham Pharmacia). Briefly, the membrane was incubated for 1 minute at room temperature with a 1:1 solution of reagent A and reagent B. Excess reagent was then drained off and the membrane was wrapped in Saran Wrap. Using a film cassette, X-ray films (Kodak Biomax MS) were then exposed for varying lengths of time, depending on the intensity of the ECL reaction.

Antibody	**Supplier and catalogue #**	**Dilution**	**2° antibody**	**Block buffer**
EphA2 (C-20)	Santa Cruz #sc-924	1:1000	rabbit 1:5000	1
EphA2 (clone D7)	UBI #05-480	1:1000	mouse 1:5000	1
Phosphotyrosine (clone 4G10)	UBI #05-321	1:5000	mouse 1:5000	2
ephrinA1 (mouse)	R&D systems #AF702	1:5000	goat 1:2500	1
ephrinA1(C-terminus)	Santa Cruz #sc-911	1:500	rabbit 1:5000	1
ephrinA1 (human)	Gift (antisera) (Rick Lindberg)	1:5000	rabbit 1:5000	1
MAPK 1/2	UBI #06-182	1:2500	rabbit 1:5000	1
phosphoMAPK 1/2	Sigma #M8159	1:5000	mouse 1:5000	1
JNK 1 (C-17)	Santa Cruz # sc-474 Santa Cruz # sc-474-G	1:500 1:500	rabbit 1:5000 goat 1:2500	1 1
phosphoJNK	Promega #V7931	1:1000	rabbit 1:5000	1
Ras	Transduction Labs #R02120	1:1000	mouse 1:5000	1
MEK 1	Transduction Labs #M17020	1:1000	mouse 1:5000	1
Meltrinα	Gift (antisera)	1:1000	rabbit 1:5000	1
phosphoMEK	New England Biolabs #9121	1:1000	rabbit 1:5000	1
human IgG (Fc specific)	Sigma #I6760	1:1000	goat 1:2500	1

Table 2.3. Summary of Antibodies used for Western Blot Analysis

2.3.8. Stripping Nitrocellulose Membranes

Membranes were stripped by incubation in stripping buffer with gentle shaking for 25 minutes. Membranes were then washed extensively in TTBS before incubating in blocking buffer. Incubation with primary and secondary antibodies was carried out as previously described.

2.3.9. Protein Kinase Assays

2.3.9.1. *In vitro* Autophosphorylation Kinase Assay

Following immunoprecipitation of the sample and washing of the pellet in RIPA buffer (as described in Section 2.3.4.1), beads were then washed once in kinase buffer I before being resuspended in 10 µl kinase buffer I. 15 µCi [γ-^{32}P]ATP was then added and, after briefly mixing, the samples were incubated at 30°C for 10 minutes. Samples were washed in RIPA buffer, resuspended in 5 x reducing sample buffer, incubated at 95°C for 5 minutes and subjected to SDS-PAGE. The gel was then fixed in 10% acetic acid, 30% methanol with gentle shaking for 20 minutes. To remove non-specific threonine/serine phosphorylation, the gel was incubated in 1 M KOH at room temperature for 10 minutes and then incubated at 55°C for a further 90 minutes in fresh 1 M KOH (Martensen and Levine, 1983). The gel was then re-fixed by incubation in 10% acetic acid, 30% methanol for a further three periods each of 20 minutes. Gels were dried onto 3 MM Whatman paper at 80°C for 2 hours using a Bio-Rad gel dryer, and autoradiography was performed using an intensifier screen at -80°C.

2.3.9.2. MAPK Assay

Cell monolayers were lysed on ice with 250 µl (per 60 mm dish) of MAPK lysis buffer. Equivalent volumes of 50 µg of total cell lysate in HBIB were immunoprecipitated in triplicate according to Table 2.1. Bead samples were then washed twice with HBIB, once with 30 mM Tris-HCl pH 8.0 and once in standard kinase buffer. Beads were resuspended in 30 µl kinase assay buffer and incubated at 30°C for 30 minutes in a shaking incubator. Reactions were terminated by pulsing down the beads and spotting 20 µl of the supernatant onto small squares of P81 paper (Whatman), which were then washed five times, each for 2 minutes, in 75 mM orthophosphoric acid. Following a 2 minute acetone wash, the dried P81 paper squares were transferred into scintillation vials containing 4 ml scintillation fluid (Packard) and incorporated [γ-$^{32/33}$P]ATP was

measured as counts per minute (cpm) in a scintillation counter (Packard). Specific activity (nmol/mg/min) was calculated from the following equation:-

$$\frac{\text{cpm (sample)} - \text{cpm (non-specific binding)}}{\text{total cpm/nmol ATP}} \times \frac{\text{reaction volume}}{\text{vol. spotted out}} \times \frac{1000}{\text{protein (µg)}} \times \frac{1}{\text{time (min)}}$$

For direct autoradiography of MBP phosphorylation, an appropriate volume of 5 x reducing sample buffer was added to the beads following the kinase reaction. Samples were incubated at 95°C for 5 minutes before 10 µl was loaded into the wells of a 15% polyacrylamide gel and subjected to SDS-PAGE. The gel was then stained with Coomassie Blue and dried onto Whatman paper using a Bio-Rad gel dryer. Autoradiography was performed using an intensifier screen at -80°C.

2.3.9.3. MEK Coupled Assay

Cells were lysed in 250 µl (per 60 mm dish) of MEK lysis buffer. 100 µg of precleared lysate was immunoprecipitated in triplicate according to Table 2.1 prior to being washed twice with MEK lysis buffer and twice with MEK kinase buffer. Beads were then resuspended in 50 µl MEK assay buffer and incubated for 30 minutes at 30°C. 10 µl of 5 x reducing sample buffer was added to the sample, which was then incubated at 95°C for 5 minutes and 20 µl subjected to SDS-PAGE and western blotting using an anti-phosphoMAPK antibody.

2.3.9.4. GST-c-Jun Associated Kinase Assay

Cells were lysed in 400 µl (per 10 cm dish) of Jun lysis buffer. 350 µg of precleared lysate was incubated for 3 hours at 4°C with 4 µg of GST-Jun (1-79) (Stratagene) on a rotating wheel, prior to the addition of 30 µl of a 50% slurry of glutathione Sepharose (Amersham Pharmacia) (pre-washed 3 times in lysis buffer) and a further 60 minute incubation at 4°C. The beads were then washed 3 times with HBIB and once with Jun kinase buffer prior to being resuspended in 30 µl of Jun kinase assay buffer. Samples were then incubated for 30 minutes at 30°C in a shaking incubator. The kinase reaction was terminated by the addition of 10 µl of 5 x reducing sample buffer, the samples were boiled for 5 minutes and then subjected to SDS-PAGE. The gel was stained with Coomassie Blue, dried onto Whatman paper and autoradiography was performed using an intensifier screen at -80°C.

2.3.9.5. JNK Assay

200 µg precleared total cell lysate in JNK lysis buffer was immunoprecipitated according to Table 2.1. Beads were washed twice in JNK kinase buffer containing 0.5 M NaCl, then twice in JNK lysis buffer. Samples were then incubated at 30°C for 30 minutes in 30 µl of JNK assay buffer. Reactions were then terminated by the addition of 10 µl of 5 x reducing sample buffer, the samples were incubated at 95°C for 5 minutes and then subjected to SDS-PAGE. The gel was stained with Coomassie Blue, dried onto Whatman paper and autoradiography was performed using an intensifier screen at -80°C.

2.3.10. Production of Fc fusion proteins

2.3.10.1. Small-Scale Production of Fc Fusion Proteins

8 x 10^5 COS cells/10 cm dish were transfected with 10 µg DNA by the Lipofectin™ method. 48 hours after replacing the medium with OPTIMEM™, this conditioned medium was transferred to a Falcon tube on ice and replaced with 10 ml fresh OPTIMEM™. The conditioned medium was centrifuged at 1000 x *g*, 4°C, for 5 minutes to pellet any cell debris. This cleared medium was then added to 100 µl of a 50% slurry of protein A-Sepharose (pre-washed in PBS) and mixed for 16 hours end-over-end at 4°C. The beads were then pelleted at 100 x *g* for 5 minutes, 4°C. The supernatant was removed and the beads washed twice with ice-cold PBS before adding 100 µl sample buffer, boiling for 5 minutes and loading 50 µl on an SDS-PAGE gel. Western blot analysis was then carried out using the appropriate antibody(s).

2.3.10.2. Large-Scale Production of Fc Fusion Proteins

Conditioned medium from DEAE-Dextran mediated COS-7 transfections was pooled and centrifuged at 20000 x *g*, 4°C, for 30 minutes. The supernatant was then filtered through a 0.22 µM low protein binding filter (Nalgene) prior to the addition of 1 ml Protein A-Sepharose (50% slurry, pre-washed in PBS). This was then incubated at 4°C overnight on a tube rotator. After 16 hours, the protein A-Sepharose/conditioned medium mixture was poured through a 25 ml disposable Dispo column (Bio-Rad), which retained the Protein A-Sepharose. The column was then washed extensively with 250 ml PBS before a gel loading tip was attached to the column to slow the flow-through rate down. Proteins were eluted using 6 ml of protein elution buffer, collecting

12 x 0.5 ml fractions into Eppendorfs containing 50 µl 1 M Tris-HCl pH 9.0. The OD_{280} of each fraction was measured, and fractions with an OD_{280} >0.1 were pooled and dialysed against PBS at 4°C overnight using a dialysis cassette (Pierce). The volume of recombinant protein solution was then concentrated in a Centricon 10 spin filter (Amicon) according to the manufacturer's instructions prior to measuring the protein concentration. Purity was then assessed by subjecting 1 µg to SDS-PAGE followed by Coomassie staining and western blotting.

2.3.11. Assay for β-galactosidase Expression by X-gal Staining

Cell monolayers and embryos were washed once in PBS at room temperature before being fixed for 20 minutes at room temperature. Cell monolayers or embryos were then washed three times, each for 5 minutes, in X-gal wash buffer. Staining was then carried out in a darkened chamber in an appropriate volume of X-gal staining solution (5 ml per 10 cm dish or per embryo in a 10 ml glass scintillation vial) for 2-16 hours at 30°C.

2.3.12. Immunocytochemistry

Cells were seeded at 1-5 x 10^5 cells/60 mm dish onto several sterile 13 mm round coverslips, thickness #1 (BDH). The following day the coverslips were transferred into the wells of a 4-well dish (Nunc) and rinsed briefly in PBS. Cells were then fixed using 500 µl per well of fixative. Coverslips were then washed with five changes of PBS before being incubated in permeabilising solution for 5 minutes at room temperature. Following a further five rinses in PBS, the cells were then blocked for 60 minutes at room temperature in block buffer 3 containing 1% (v/v) goat serum (Gibco-BRL) prior to incubation in primary antibody diluted appropriately in block buffer 3 for 60 minutes. After five further 5 minute PBS washes, coverslips were subsequently incubated in Alexafluor (488 nm) -conjugated secondary antibody (Molecular Probes), also diluted in block buffer 3, for 45 minutes. Foil was wrapped around the outside of the incubation chamber to prevent degradation of the signal. Following 5 x 5 minute washes in PBS, coverslips were briefly rinsed in distilled water to remove salts, before mounting in Vectashield containing the nuclear stain 4, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc.) on microscope slides (BDH). The edges of the coverslips were sealed using clear nail varnish and the cells were visualised by fluorescence microscopy.

2.3.13. Phalloidin Staining of Actin Filaments

Cells on 13 mm coverslips were rinsed briefly with room temperature PBS before being fixed and permeabilised as described in Section 2.3.12. Cells were then incubated in a 1:250 dilution of Alexafluor- (488 nm) conjugated phalloidin (Molecular Probes) in block buffer 3 for 45 minutes at room temperature prior to washing in PBS for 5 x 5 minutes. Coverslips were then briefly rinsed in distilled water before mounting in Vectashield and sealing with nail varnish, as described above. Actin filaments were visualised by confocal microscopy, using a Leica TCS SP Laser Scanning microscope. Images were collected using the Leica TCS SP NT software package and processed using Adobe® Photoshop® 5.0.

2.4 Quantitative RT-PCR

2.4.1. Preparation of Total RNA From Tissue

Tissue samples were homogenised in 1 ml of Trizol™ reagent (Gibco-BRL) per 50 mg tissue using an Ultra-Turrax homogeniser (BDH). Total RNA was extracted from the tissue following a 5 minute room temperature incubation of the homogenate. 200 µl of chloroform was added to 1 ml homogenate and shaken vigorously before incubating the sample at room temperature for 2 minutes. Following centrifugation at 12000 x *g*, 4°C for 15 minutes, the upper aqueous phase was transferred to a clean Eppendorf before being re-centrifuged for 5 minutes at 12000 x *g* for 5 minutes, 4°C. 500 µl was then transferred to another fresh Eppendorf and RNA was precipitated by adding 500 µl of isopropanol, mixing thoroughly and incubating at room temperature for 10 minutes. RNA precipitates were then recovered by centrifugation at 12000 x *g*, 4°C for 10 minutes. The supernatant was removed and pellets were subsequently washed twice, each time in 1 ml of 75% ethanol. RNA pellets were then collected at the bottom of the tube by centrifugation at 7500 x *g* for 5 minutes at 4°C before being air-dried briefly. RNA was then resuspended in DEPC-treated water and the concentration calculated by OD_{260} measurement. RNA quality was assessed by electrophoresis on a 1% (w/v) agarose gel as described in Section 2.1.4.

2.4.2. DNAse Treatment of RNA Samples

4 µg of total RNA was incubated with 4 µl of 10 x DNAse buffer and 4 µl DNAse (Gibco-BRL) in a total volume of 40 µl (made up in water). This was incubated at

room temperature for 15 minutes before the addition of 4 µl of 25 mM EDTA. The samples were then heated to 65°C for 10 minutes before undergoing reverse transcription.

2.4.3. Reverse Transcription of RNA (oligo(dT) primed)

All reagents for first strand synthesis were purchased from Gibco-BRL. 9 µl of the above DNAse treated RNA was pipetted into 4 wells of a 96-well plate. 1 µl of oligo(dT) primer was subsequently added before the plate was gently pulsed down, heated to 90°C for 2 minutes and then chilled on ice. A 100 x mastermix of the following was then prepared; 4 µl first strand buffer, 2 µl DTT, 0.5 µl each of 10 mM dATP, dCTP, dGTP and dTTP. This 800 µl mastermix was then subdivided into a 600 µl aliquot, to which 75 µl Superscript II™ and 75 µl RNAseOUT were added, and a 200 µl portion, to which 25 µl of water and 25 µl RNAseOUT were added. 10 µl of the mixture containing Superscript II™ was added to each of 3 wells per sample of the 96-well plate (for triplicate reverse transcription reactions), whereas 10 µl of the mixture containing water was added to the fourth well, forming a 'no reverse transcription' control. The plate was then incubated at 42°C for 60 minutes, and 95°C for 2 minutes using a PTC-200 Peltier Thermal Cycler (MJ Research) before being chilled on ice. Following the reverse transcription reaction, 60 µl of water was added to each of the samples on the plate, forming a total volume of 80 µl per sample. Using a Hydra 96 robot (Robbins Scientific), 4 µl was then aliquoted into 20 identical plates which were sealed with optical caps (Perkin Elmer) and stored at –80°C until further use.

2.4.4. SYBR® Green PCR Primer Design

Primers for SYBR® Green PCR were designed using the Primer Express software package (Perkin Elmer). In addition, the melting temperature of the primers was required to be ≥ 59°C. Primer sets that gave rise to amplicon sizes of 80-120 bp were selected. Primers sets used in this study are detailed in Table 2.4.

Prior to performing SYBR® Green PCR, the primers were tested for their specificity. PCR was performed using genomic/plasmid/cDNA template according to the conditions described in Section 2.4.5. Reaction products were then subjected to horizontal gel electrophoresis to verify the presence and purity of the appropriate sized amplicon.

Sequence	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
Neomycin phosphotransferase (Primer set #1)	V00618	GTGCTCGACGTTG TCACTGAA	GCAAGGTGAGATG ACAGGAGATC
EphA2 (Primer set #2)	X78339	ATGTCTTGCACGC GTCCA	GTTCTACTTTGGC ACCCATGC
exon 5.2/nucleotide 179 (Primer set #3)	U28385 and (Chen *et al.*, 1996)	CAAAGTAAAACCA GTCTCAACTCCTG	CTCCCTTCATTGCT GCGAA
β-Actin	M12481	ACGGCCAGGTCAT CACTATTG	CAAGAAGGAAGG CTGGAAAAG

Table 2.4. Summary of Primers used in SYBR® Green PCR Analysis

2.4.5. SYBR® Green PCR Analysis of Gene Expression

A 100 x mastermix was prepared using (per reaction); 12.5 µl of 2 x SYBR® Green PCR Universal Master Mix (Perkin Elmer) (containing SYBR® Green I dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP and optimised buffer components), 7.5 µl water, and 0.5 µl each of 10 µM forward primer and 10 µM reverse primer. 21 µl of this mastermix was added to each of the 4 µl cDNA samples in the 96-well plate. The plate was briefly spun and the wells sealed with optical lids (Perkin Elmer).

Five 10-fold dilutions of standards were made from an initial starting concentration of 10 µl of mouse genomic DNA (Promega) and 90 µl water in order to generate a standard curve. Where genomic DNA was not suitable for use with the primers, standards were prepared using dilutions of plasmid or cDNA. 4 µl of water was placed into one well to act as a 'no template control'. 21 µl of mastermix was added to the wells which were then sealed with optical caps.

The samples were then subjected to SYBR® Green PCR using an ABI 7700 Prism Sequence Detector (Perkin Elmer) according to the following conditions: (1) 50°C for 2 minutes, (2) 95°C for 10 minutes followed by (3) 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Measurements were taken at step (3) only.

2.5. Transgenic Mouse Analysis

Mice were maintained under the supervision of Jeff Packman, University College, London, UK.

Fertilisation was taken to occur at midnight prior to the detection of vaginal plugs the following morning.

Tissues were dissected into PBS and snap frozen in cryovials in liquid nitrogen.

2.5.1. Mouse Strains

WAP*ras* mice were obtained from The Jackson Laboratory, Bar Harbor, ME, USA, #002410, strain FVB/N-TgN(WapHRAS)69LlnYSJL (Nielsen *et al.*, 1992).

eck^{i} mice (Chen *et al.*, 1996) were obtained from Jin Chen, Vanderbilt University School of Medicine, Nashville, Tennessee, USA. Strain 129/SVC57BL/6.

2.5.2. Genotyping

For verification of genotype, ~ 2 cm tail snips were taken from the animals and Southern blotting was performed as described in Section 2.1.12.

Routine genotyping of genomic DNA prepared from tails was carried out by Steven Hayes, Ludwig Institute for Cancer Research, UCL, UK.

Chapter 3

Upregulation of EphA2 Expression by Oncogenic Ras

3.1. Introduction

Ras is overexpressed in a number of different types of tumour, including breast cancer. Overexpression of the transcription factor, c-Myc, also contributes to cancer, being amplified in up to 40% of breast tumours. The *myc* oncogene was one of the first genes to be expressed in a tissue-specific manner in transgenic mice (Stewart *et al.*, 1984). Together with a number of subsequent studies (Leder *et al.*, 1986; Andres *et al.*, 1988; Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995), the ability of c-*myc* to induce mammary gland transformation in transgenic mice has been demonstrated. Andres and colleagues have made use of the whey acidic protein (WAP) gene promoter to study the expression of *myc* and *ras* in a mammary-specific manner in transgenic mice. The activity of this promoter is regulated by prolactin, and gene expression is therefore induced in the mammary epithelial cells of female mice during the oestrous and reproductive cycles. This results in the development of mammary tumours in both WAP*ras* and WAP*myc* transgenic mice, however, the tumours types differ markedly in their morphologies. WAP*ras* mammary tumours are undifferentiated and metastatic, in contrast to the well-differentiated, poorly invasive mammary tumours induced in WAP*myc* mice. Andres and colleagues (Andres *et al.*, 1994) have also demonstrated that EphA2 mRNA expression is significantly upregulated in mammary tumours of WAP*ras* mice compared with low levels in the normal mammary gland and in mammary tumours of WAP*myc* mice.

The aims of the experiments carried out in this chapter were to i) investigate whether the observed increase in EphA2 mRNA in Ras-induced mammary tumours corresponds to increased levels of active EphA2 protein and ii) to further characterise the molecular mechanism(s) by which Ras upregulates EphA2 expression.

3.2. Results

3.2.1. EphA2 Protein is Overexpressed in WAP*ras* Transgenic Mouse Mammary Tumours

Overexpression of EphA2 at the mRNA level in mammary tumours derived from WAP*ras* transgenic mice has previously been documented (Andres *et al.*, 1994). To analyse the effect of oncogenic *ras* on levels of functional EphA2 protein, transgenic mice in which the WAP*ras* transgene is integrated into the Y chromosome were obtained (Section 2.5.1). The presence of the transgene on the Y chromosome facilitates the identification of transgenic animals. Expression of the transgene is thought to be constitutive, resulting in the development of mammary tumours in male mice (Andres *et al.*, 1987). Although male breast cancer is much less prevalent than female breast cancer, it exhibits similar histology, biology and prognosis (Section 1.3.3).

A polyclonal anti-EphA2 antibody (Santa Cruz) was used to immunoprecipitate EphA2 protein from lysates of normal male mammary tissue and mammary tumours from male WAP*ras* transgenic mice (Section 2.3.4.1). Steady-state protein levels were assessed by western blotting (Section 2.3.7) using a monoclonal anti-EphA2 antibody (UBI), whereas receptor autophosphorylation, as a measure of its catalytic activity, was measured by an *in vitro* kinase assay (Section 2.3.9.1).

The anti-EphA2 antibody immunoprecipitated significant levels of a 120-130 kDa protein, corresponding to the glycosylated form of EphA2 (Ganju *et al.*, 1994) in mammary tumours, whereas EphA2 protein was barely detectable in the normal male mammary gland (Figure 3.1a). In accordance with an increase in protein levels, an increase in the amount of receptor autophosphorylation was observed in protein derived from the tumour compared to normal mammary tissue (Figure 3.1b). In addition to the strong band at 130 kDa, a number of additional bands were observed, which may correspond to degradation products of the EphA2 receptor.

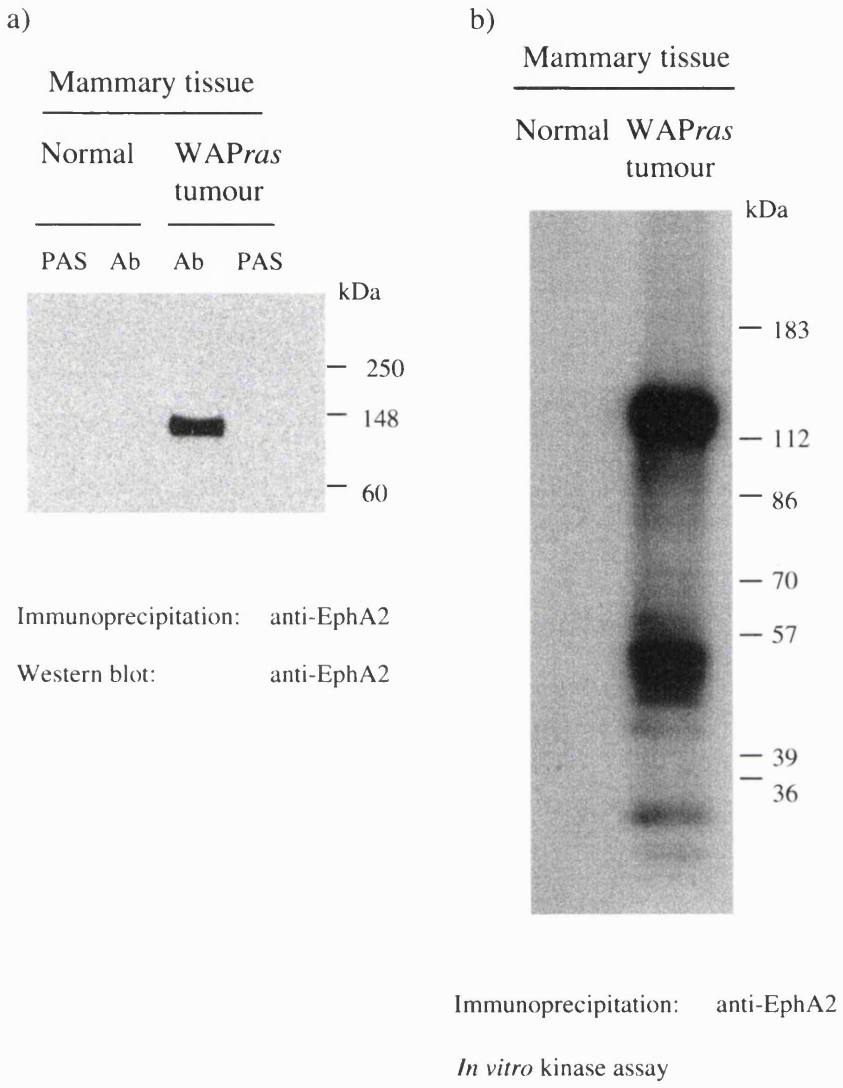


Figure 3.1. Functional EphA2 is Overexpressed in WAP*ras* Mammary Tumours
Normal male mammary gland or WAP*ras* mammary tumours were lysed in RIPA buffer. (a) 1 mg of total protein lysate was immunoprecipitated using a polyclonal anti-EphA2 antibody (Santa Cruz) (Ab), or using protein A-Sepharose alone (PAS). Following SDS-PAGE, samples were western blotted using a monoclonal anti-EphA2 antibody (UBI). (b) 1 mg of total protein lysate was immunoprecipitated using a polyclonal anti-EphA2 antibody (Santa Cruz) and subjected to an *in vitro* kinase assay.

3.2.2. Ras Induces EphA2 mRNA and Protein Expression in a Mammary Epithelial Cell Line

EpH4 is a spontaneously immortalised cell line derived from the mouse mammary gland. EpRas cells were previously generated by retroviral infection of EpH4 cells with recombinant v-Ha-*ras* (V12Ras) (Oft *et al.*, 1996) (see Section 2.2.2). EpRas cells displayed a more fibroblastic, less regular morphology compared to the more ordered cuboidal patterning of EpH4 cells (Figure 3.2a). Furthermore, when cultured at high density, EpH4 cells gave rise to hemicyst domes, which are indicative of the polarised phenotype, whereas EpRas cells failed to form these structures (data not shown). To compare the levels of EphA2 mRNA in EpH4 and EpRas cells, total and poly(A)$^+$ RNA was prepared from both cell lines, and northern blotting was performed using a [α-^{32}P]dCTP-labelled probe corresponding to amino acids 330-593 of the mouse EphA2 cDNA sequence (Ganju *et al.*, 1994). Levels of a single ~ 4.5 kb EphA2 transcript were significantly increased in EpRas cells compared with parental EpH4 cells (Figure 3.2b). Furthermore, the steady-state level of EphA2 protein in EpRas cells was also upregulated compared to EpH4 cells, as detected by western blotting of whole cell lysates and EphA2 immunoprecipitates using two different EphA2 antibodies (Figure 3.2c-f). When *in vitro* kinase assays were performed on EphA2 immunoprecipitates prepared from EpH4 and EpRas cells, an increased level of EphA2 autophosphorylation was observed in EpRas cells (Figure 3.2g), confirming that EphA2 was still active.

3.2.3.1. Stable Expression of Ras Effector Pathway Mutants in EpH4 Cells

As outlined in Section 1.2.3, three major Ras effector pathways have been described. To investigate the mechanism by which the *ras* oncogene upregulates EphA2 expression, EpH4 cells were stably infected with recombinant retroviruses encoding either V12Ras or the V12 mutation in combination with mutations that differentially separate the ability of Ras to bind to the three main characterised effectors, RalGDS, PI3K and Raf. The V12S35Ras mutant is reported to be unable to bind either RalGDS or PI3K but still binds Raf, whereas V12G37Ras and V12C40Ras are only able to interact with RalGDS and PI3K, respectively (Rodriguez-Viciana *et al.*, 1997).

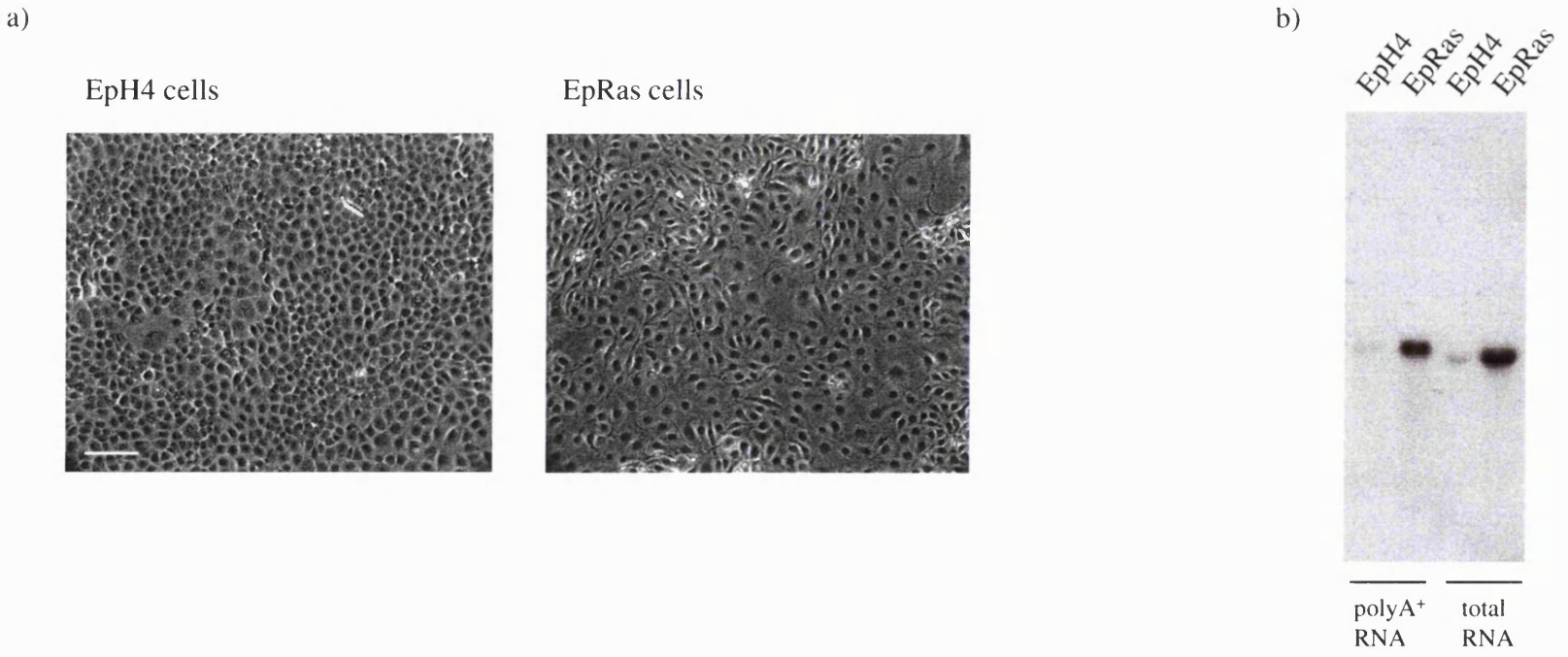


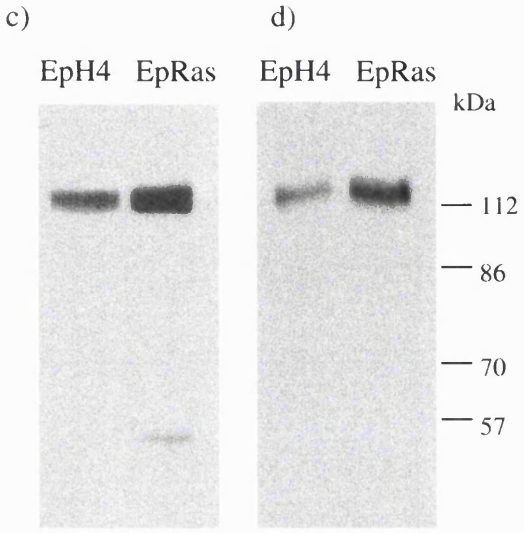
Figure 3.2. EphA2 is Upregulated in EpRas Cells

(a) EpH4 and EpRas cells were fixed in 4% paraformaldehyde and viewed by phase-contrast microscopy. Scalebar represents 50 µm.

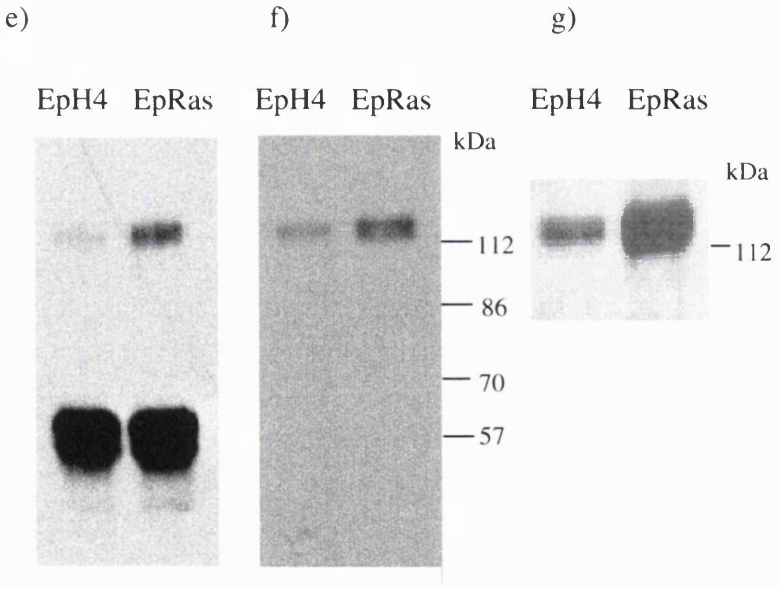
(b) 10 µg of total cellular RNA from EpH4 and EpRas cells was electrophoresed and subjected to northern blotting using a ^{32}P labelled cDNA probe corresponding to residues 330-593 of EphA2. Data courtesy of Dr. Alastair Reith.

EpH4 and EpRas cells were seeded at 5 x 10^6 cells per 10 cm dish and cultured for 16 hours. (c) and (d) 25 µg of whole cell lysate was subjected to SDS-PAGE and western blotting using an anti-EphA2 antibody from either UBI or Santa Cruz, respectively.

(e) and (f) 500 µg of RIPA lysates were immunoprecipitated using an anti-EphA2 antibody from UBI or Santa Cruz, respectively, prior to western blotting with an anti-EphA2 antibody (UBI). (g) 500 µg of RIPA lysate was immunoprecipitated using an anti-EphA2 antibody (Santa Cruz) and subjected to an *in vitro* kinase assay.



Western blot: anti-EphA2 (UBI) anti-EphA2 (SC)



Immunoprecipitation:	anti-EphA2 (UBI)	anti-EphA2 (SC)	anti-EphA2 (SC)
Western blot:	anti-EphA2 (UBI)	anti-EphA2 (UBI)	*In vitro* kinase assay

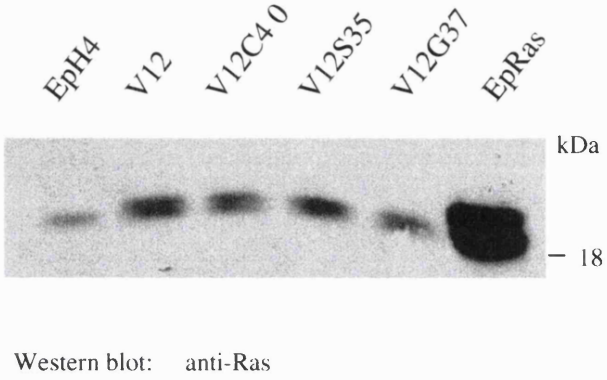
Retroviral infection was chosen as the preferred method for introducing the recombinant DNA into EpH4 cells because, amongst a number of advantages (see Section 5.1) the infection rate using retroviruses is reportedly high, which would eliminate the need to pick individual clones of cells that might otherwise exhibit clonal variation. Furthermore, the previously established EpRas cell line was created using retroviral infection (Oft *et al.*, 1996).

Retroviral infection of EpH4 cells was performed by co-culture with the fibroblast retroviral producer line, GP+E 86 (Section 2.2.2), according to Section 2.2.4.5.5. Subsequent to the 48 hour co-culture period, EpH4 cells were split 1:10 and cells maintained in G418 selective medium for 10-14 days. Bulk populations of G418 resistant cells were used for further experiments. No G418 resistant cells were observed in mock-infected EpH4 cells.

3.2.3.2. Ras Pathway Effector Mutants do not Upregulate EphA2 Protein

Bulk populations of EpH4 cells infected with recombinant V12Ras, V12S35Ras, V12G37Ras or V12C40Ras retroviruses, along with EpH4 cells and EpRas cells, were seeded at equal densities and cultured for 16 hours. Whole cell lysates were prepared and subjected to SDS-PAGE and western blotting using either an anti-Ras antibody or an anti-EphA2 antibody. Infection of EpH4 cells with each of the V12Ras, V12S35Ras, V12G37Ras or V12C40Ras recombinant retroviruses resulted in increased levels of Ras (Figure 3.3a). In all of the recombinant cell lines, Ras was observed as a doublet of approximately 21 kDa, whereas in lysates from EpH4 cells only one band could be detected. The protein of higher molecular weight is thought to correspond to the non-farnesylated form of Ras, suggesting that there is an excess in Ras levels that the endogenous, rate-limiting, farnesyl protein transferase is unable to process. This is a strong indication that recombinant Ras has successfully been introduced into EpH4 cells. However, markedly higher levels of Ras were observed in the previously established EpRas cells (Figure 3.3a). Furthermore, the morphology of the newly-established recombinant cell lines resembled that of EpH4 cells rather than EpRas cells (data not shown). Analysis of the levels of EphA2 protein by western blotting (Figure 3.3b) revealed that EpH4 cells infected with the V12Ras construct had an elevated level of EphA2 protein compared with non- infected EpH4 cells. However, the levels of EphA2 protein in EpH4 cells infected with any of the Ras effector mutants remained

a)



b)

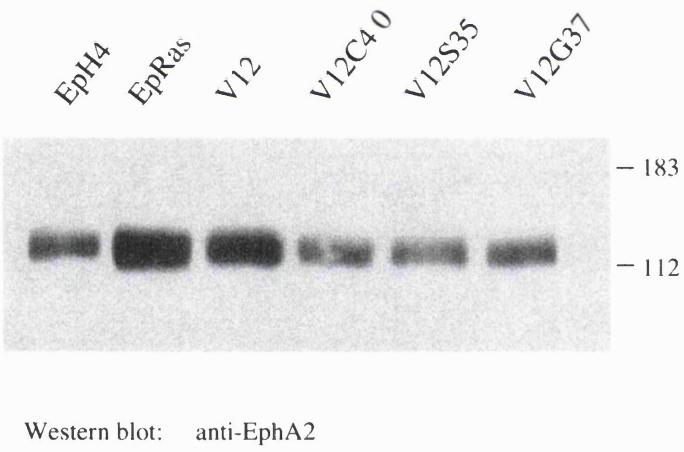


Figure 3.3. Retroviral Infection of EpH4 Cells with Ras Effector Mutants
5 x10^6 EpH4 cells, EpRas cells, and EpH4 cells infected with each of the V12Ras effector domain mutations were plated into 10 cm dishes and cultured for 16 hours. 50 µg of whole cell lysate was subjected to SDS-PAGE and western blotting using (a) an anti-Ras antibody, or (b) an anti-EphA2 antibody (Santa Cruz).

equal to those of non-infected EpH4 cells. This implies that, under these conditions, either Ras-mediated activation of Raf, PI3K or RalGDS alone was incapable of inducing EphA2, or that an alternative Ras effector pathway was utilised. Alternatively, as discussed later (Section 3.3.3), these results may reflect the compromised activity of the V12Ras effector mutants compared to V12Ras.

3.2.4. Inhibition of the Raf/MEK/MAPK Pathway Downregulates EphA2

An alternative strategy to investigate the pathway(s) by which Ras upregulates EphA2 in EpRas cells was to use selective kinase inhibitors. This approach would compromise the activity of a component within an individual effector pathway, but still allow any synergy between the remaining effector pathways to operate. Therefore, cell-permeable compounds were used to investigate whether selective inhibition of individual Ras effector pathways affected EphA2 levels. EpRas cells were cultured for 24 hours in the presence of the selective MEK inhibitor U0126 (Favata *et al.*, 1998), the Raf inhibitor SB-386023 (Yue *et al.*, 2000) or the PI3K inhibitor, LY294002 (Vlahos *et al.*, 1994). Lack of specific inhibitors of RalGDS prevented the study of the involvement of this pathway in Ras-mediated EphA2 induction. DMSO was included as a control. Also included as a control was the p38 inhibitor, SB-203580 (Cuenda *et al.*, 1995), as previous reports have not suggested that p38 acts downstream of Ras.

Confirming previous results, the level of EphA2 protein was higher in EpRas cells than in EpH4 cells (Figure 3.4a). Moreover, the level of MAPK phosphorylation, as measured by an antibody that is specific for dual phosphorylated MAPK (anti-phosphoMAPK), was higher in EpRas cells than in EpH4 cells, indicating that the Raf/MEK/MAPK pathway is upregulated in response to activated Ras (Figure 3.4b). The addition of 0.1% DMSO to EpRas cells had no effect on EphA2 protein levels. However, the presence of the MEK inhibitor, U0126, or the Raf inhibitor, SB-386023, caused a significant decrease in EphA2 protein to levels below those present in EpH4 cells. The p38 inhibitor and the PI3K inhibitor failed to elicit a decrease in EphA2 levels, with protein levels remaining comparable to, or even slightly higher than, those of untreated EpRas cells. Attempts to confirm the efficacy of both of these compounds by western blotting using antibodies designed to recognise the active forms of p38 and PKB/Akt were unsuccessful, therefore changes in cell morphology observed upon

treatment with both inhibitors were used as an indication that the compounds were active. Cells treated with SB-203580 developed a more fibroblastoid phenotype, whereas treatment with LY294002 resulted in the formation of vesicles within the cell bodies (data not shown). To confirm that U0126 and SB-386023 acted as effective inhibitors of the Raf/MEK/MAPK pathway, an anti-phosphoMAPK antibody was used to measure p42 and p44 MAPK phosphorylation. Both compounds effectively inhibited MAPK phosphorylation (Figure 3.4b). Interestingly, levels of MAPK phosphorylation were seen to be markedly increased following treatment with the p38 inhibitor. Steady-state levels of p42 and p44 MAPK protein were unaffected by any of the treatments (Figure 3.4c). This result also serves to confirm that equal amounts of total protein were loaded onto the gel and that the observed decrease in EphA2 protein levels was not a reflection of an overall decrease in protein synthesis (Figure 3.4c).

To assess further the influence of MAPK activity on levels of EphA2 protein, western blotting was performed on whole cell lysates prepared from EpRas cells treated with increasing doses of U0126. A dose-dependent decrease in EphA2 protein levels was observed in response to increasing concentrations (0.5 μM to 10 μM) of U0126 (Figure 3.5a). To confirm that U0126 was effectively inhibiting MAPK phosphorylation, western blotting using the anti-phosphoMAPK antibody was performed. The level of MAPK phosphorylation was inhibited by increasing the dose of U0126 (Figure 3.5b), while the levels of total MAPK remained unaffected by the treatment (Figure 3.5c).

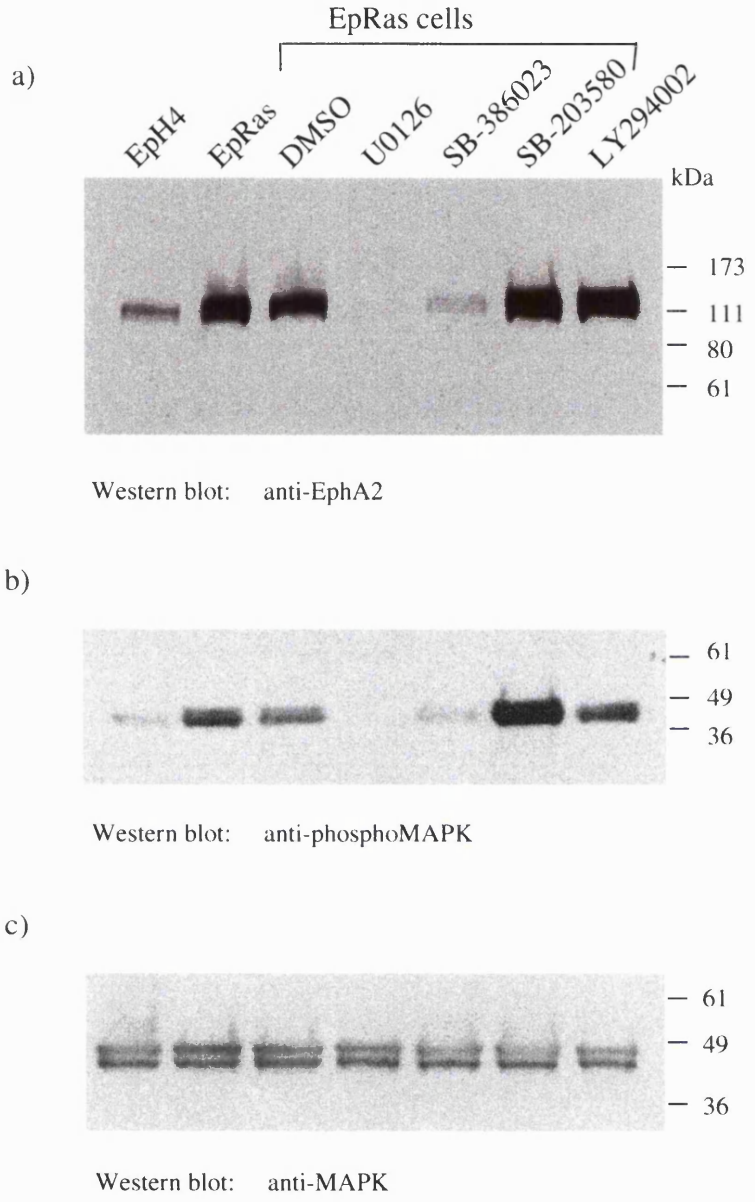


Figure 3.4. MAPK Pathway Inhibitors Downregulate EphA2 Expression
1 x10^5 EpH4 (lane 1) or EpRas cells (lanes 2-7) were plated into 60 mm dishes and cultured for 16 hours prior to incubation for 24 hours with the following treatments; 0.1% DMSO (lane 3), 10 μM U0126 (lane 4), 10 μM SB-386023 (lane 5), 10 μM SB-203580 (lane 6), 20 μM LY294002 (lane 7). Lanes 1 and 2 were left untreated. 25 μg of whole cell lysate was subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI), (b) an anti-phosphoMAPK antibody, or (c) an anti-MAPK antibody.

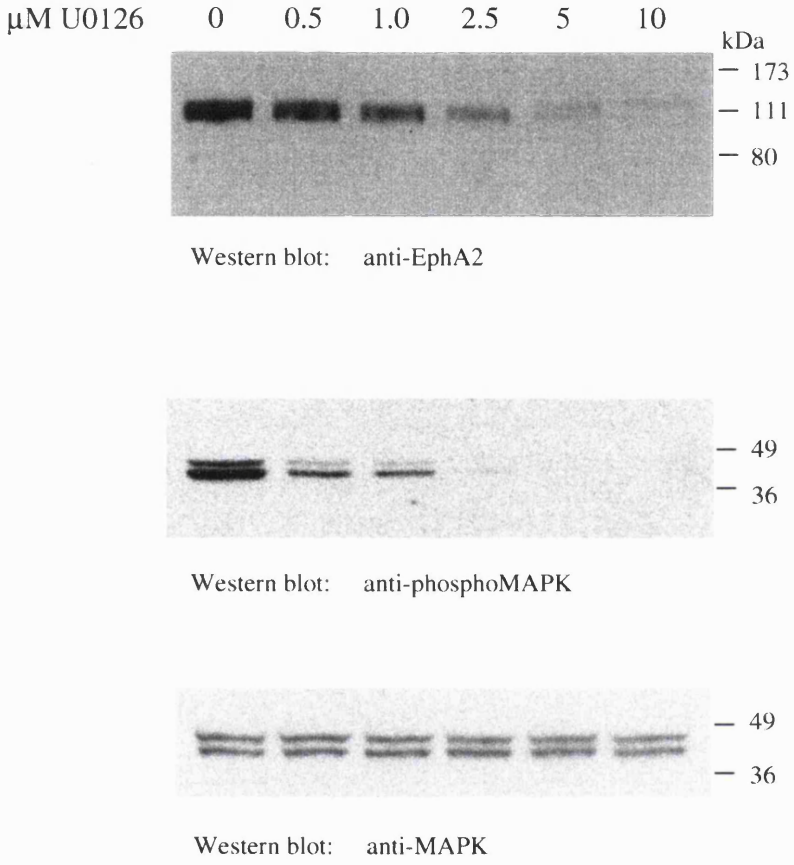


Figure 3.5. Dose-Dependent Inhibition of EphA2 by U0126
1 x10^5 EpRas cells were plated into 60 mm dishes and cultured for 16 hours prior to incubation for 24 hours with 0-10 μM U0126. 25 μg of whole cell lysate was subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI), (b) an anti-phosphoMAPK antibody, or (c) an anti-MAPK antibody.

3.3. Discussion

3.3.1. Upregulation of EphA2 Protein in WAP*ras* Mammary Tumours

The upregulation of EphA2 in mammary tumours of WAP*ras* transgenic mice was originally documented at the mRNA level by Andres and colleagues (Andres *et al.*, 1994). The data presented here are significant in that they confirm that the increase in mRNA described by Andres (Andres *et al.*, 1994) corresponds to an increase in functional protein and is not simply a reflection of changes in EphA2 mRNA regulation as a result of *ras* expression. The observation that tumour-derived EphA2 protein retains catalytic activity is also significant as it implies that EphA2-mediated signalling pathways have the potential to operate. It also suggests that the observed increase in EphA2 protein levels is not due to inhibiting activity-induced receptor degradation, a phenomenon seen in EGF receptors whereby, following activation, receptors are targeted for degradation by Cbl (Levkowitz *et al.*, 1999).

The high steady-state levels of EphA2 receptor protein observed in the WAP*ras* mammary tumours might be expected to result in significant levels of receptor autophosphorylation in the absence of EphA2-ligand stimulation (Lemmon and Schlessinger, 1994), as occurs in human breast tumours expressing high levels of ErbB2 (reviewed in Hynes and Stern, 1994). In a recent report, Ogawa and colleagues have detected ephrinA1 in mammary tumours which may account for their additional finding that EphA2 was tyrosine phosphorylated (Ogawa *et al.*, 2000). As increased transcription of ephrinA1 has recently been found in Ras-transformed fibroblasts (Zuber *et al.*, 2000) it would be interesting to investigate whether ephrinA1 is also upregulated in WAP*ras* mammary tumours. Dysregulated expression of ephrinA1 in tumours could lead to autocrine activation of EphA2, as has been reported in melanoma progression (Easty *et al.*, 1995), TNFα-induced angiogenesis (Pandey *et al.*, 1995b) and adenocarcinoma cell function (Rosenberg *et al.*, 1997). In addition to the co-expression of EphA2 and ephrinA1 in tumour cells of the mammary gland, surrounding endothelial cells were also reported to express both ligand and receptor (Ogawa *et al.*, 2000), suggesting that ligand-receptor interactions could occur both within, and between, the tumour mass and its surrounding stroma. Such interactions have been proposed to play an important role in vasculogenesis (Gale and Yancopoulos, 1999) and, as such, would have a profound effect on tumour metastasis.

3.3.2. Upregulation of EphA2 mRNA and Protein in a Cell Culture Model

The increase in both EphA2 mRNA and active protein in EpRas cells compared to their naïve EpH4 counterparts implies that EphA2 is a target of Ras in mammary epithelial cells, which is significant given that the majority of mammary adenocarcinomas are epithelial. The expression of EphA2 in a mammary epithelial cell line is consistent with the initial naming of EphA2 as epithelial cell kinase (Lindberg and Hunter, 1990) and with previous reports of its expression in both primary mammary cells and established mammary epithelial cell lines (Andres *et al.*, 1994; Andres *et al.*, 1995). Interestingly, in fibroblasts, EphA2 is only expressed at relatively low levels (Lindberg and Hunter, 1990; Andres *et al.*, 1994) and is reportedly not upregulated by Ras (Zuber *et al.*, 2000).

The demonstration that EphA2 expression is upregulated by Ras in a mammary epithelial cell line provides a highly relevant *in vitro* model in which to further study the role of EphA2. The difference in the levels of EphA2 protein between the EpH4 and the EpRas cells is notably not as great as that between the EphA2 levels in normal male mammary tissue and in the WAP*ras* mammary tumours. However, because EpH4 cells are a clonal, spontaneously immortalised epithelial cell line, they are expected to be enriched in epithelial proteins, of which EphA2 is one (Lindberg and Hunter, 1990). This differs from the *in vivo* situation, where the mammary gland comprises both epithelial and stromal components, and, certainly in the male, the epithelial compartment constitutes only a very small proportion of normal mammary tissue, having ceased development prior to birth (see Section 1.3.2). In contrast, mammary tumours derived from WAP*ras* mice consist predominantly of a mass of epithelial cells (as a result of *ras* expression from the epithelial-specific WAP promoter).

A single ~4.5 kb EphA2 mRNA transcript was detected in normal and WAP*ras* mammary tissue (Andres *et al.*, 1994) and in EpH4 and EpRas cells. The increase in EphA2 transcript levels in EpRas cells thus reflects the increase in protein levels, but whether it occurs as a result of increased EphA2 gene transcription or mRNA stabilisation is unknown.

3.3.3. Analysis of EphA2 Protein Levels in Response to Ras Effector Pathway Mutants

In an attempt to investigate which effector pathway is responsible for the upregulation of EphA2 by oncogenic Ras in mammary epithelia, a number of Ras effector domain mutants were used. V12Ras was able to induce levels of EphA2 protein, however, V12Ras mutants that were only able to interact with a single effector pathway were unable to upregulate EphA2. One interpretation of these results would be that a combination of two or more Ras effector pathways are necessary to mediate EphA2 upregulation, or that another less well characterised Ras effector pathway is required. This could be further investigated by infecting combinations of two of the effector mutants at a time. However, although each effector mutant is still able to interact with one effector protein, its ability to activate this protein, and hence the downstream effector pathway is reportedly impaired (Rodriguez-Viciana *et al.*, 1997). Therefore, although the V12Ras and V12Ras effector mutants were expressed at a similar level, this cannot necessarily be used as an indication of their comparable activity. Thus, the possibility that the lack of EphA2 induction by any of the V12Ras effector mutants is due to their impaired ability to activate the appropriate downstream effector cannot be excluded. Indeed, comparison of the level of Ras expression between the bulk populations of V12Ras effector mutant-infected EpH4 cells and the EpRas cells indicates that the effector mutant constructs were expressed at a relatively low level. This is most likely due to the EpRas cells being derived from a single, high-expressing clone (Oft *et al.*, 1996) whereas bulk populations were used during this study.

A further indication that the Ras expression levels were relatively low in the bulk populations was apparent from their morphology. Unlike EpRas cells, V12Ras-infected EpH4 cells continued to display a regular morphology and did not appear mesenchymal. A number of studies have suggested that oncogenes may contribute to the deregulation of proteins involved in maintaining the apical, lateral and basal domains of polarised cells, implying that the levels of Ras may play a critical role in this event. Indeed, studies using both primary mammary epithelial cells and an established mammary epithelial cell line demonstrated that there was a strong correlation between the level of oncogenic Ras and the morphology of the cells, and suggested that levels of oncogenic Ras $\geq$ 20-fold higher than endogenous Ras expression were required to fully transform epithelial cells (Redmond *et al.*, 1988). However, it appears that such high levels of Ras

may not be necessary to induce an increase in the level of EphA2 protein, as a significant induction of EphA2 was attainable in V12Ras-infected cells in which only low levels of Ras were expressed. It would therefore be very interesting to investigate the effect of increasing Ras levels upon EphA2 protein expression.

3.3.4. Regulation of EphA2 by the Raf/MEK/MAPK Pathway

The results presented in this chapter implicate the Raf/MEK/MAPK pathway in the upregulation of EphA2 in EpRas cells. Firstly, EpRas cells displayed a markedly higher level of phosphorylated MAPK than EpH4 cells. This is in accordance with other reports of overexpression, or constitutive phosphorylation, of MAPK in Ras-transformed cell lines and tumours (Amundadottir and Leder, 1998; Martinez-Lacaci *et al.*, 2000). Secondly, inhibition of this pathway by use of the selective MEK inhibitor U0126 or the selective Raf inhibitor SB-386023 resulted in a marked decrease in EphA2 protein levels. Furthermore, EphA2 levels were found to be directly correlated with the activity of MAPK.

Inhibition of either PI3K using LY294002, or the p38 pathway using SB-203580 did not decrease the level of EphA2 protein in EpRas cells. Therefore, PI3K and p38 do not appear to be required for Ras-mediated upregulation of EphA2. Indeed, treatment with these inhibitors elicited a slight increase in EphA2 protein levels which, in the case of the p38 inhibitor SB-203580, correlated with a marked increase in levels of MAPK phosphorylation. This observation again implicates the Raf/MEK/MAPK pathway in the regulation of EphA2 expression. The reason for the increased levels of MAPK phosphorylation following inhibition of p38 activity is unclear. Recent studies by Hall-Jackson and colleagues (Hall-Jackson *et al.*, 1999a; Hall-Jackson *et al.*, 1999b) demonstrated that Raf isolated from cells treated with SB-203580 displayed increased activity when assayed *in vitro*. However, in the same studies, no change in MAPK activity was observed. The increase in EphA2 expression in response to PI3K inhibition appeared to be mediated independently of changes in the activity of MAPK, but the mechanism by which this occurs is again unclear.

The effect of the MEK inhibitor U0126 and the Raf inhibitor SB-386023 on levels of EphA2 mRNA was not analysed, therefore it is not possible to say whether the observed downregulation of EphA2 protein reflected decreased EphA2 gene transcription or mRNA translation. Little is known about EphA2 transcriptional regulation, although

the homeobox proteins Hoxa1 and Hoxb1 have been implicated in rhombomere-specific expression of EphA2 (Chen and Ruley, 1998). Interestingly, the expression of *Hoxa1* is markedly upregulated in mouse mammary tumours compared with normal and preneoplastic tissue (Friedmann *et al.*, 1994). However, a potential link between MAPK signalling and *Hox* gene expression has not been studied to any great extent. A cytoplasmic target for MAPK is the kinase MNK (Section 1.2.4.2), which may mediate the ability of eIF-4E to recruit ribosomes to mRNA and thereby enhance protein synthesis. Consequently, downregulation of EphA2 protein levels may be a reflection of decreased mRNA translation through inhibition of MAPK activity.

The Raf/MEK/MAPK pathway has been demonstrated to be necessary for Ras-induced EphA2 protein expression. However, whether it is sufficient on its own to elicit an increase in EphA2 expression has not been determined. One way to address this would be to introduce constitutively active forms of Raf, MEK, or MAPK into EpH4 cells and compare the levels of EphA2 protein. However, as with many studies undertaken to investigate the role of this pathway in Ras-mediated tumourigenesis, the production of autocrine factors that could result in Ras activation would be difficult to exclude. Identification of the promoter region of EphA2 would also enable the effect of the Raf/MEK/MAPK pathway on EphA2 transcription to be analysed, and analysis of the sequence of the promoter would facilitate the identification of putative AP-1 binding sites. EphA2 overexpression has been reported in mammary tumours and tumour cell lines which have arisen independently of oncogenic *ras* (see Section 1.1.7.6). It would be interesting to determine whether EphA2 expression is also targeted by MAPK in these tumours.

The results presented in this chapter describe how expression of oncogenic *ras* results in the overexpression of EphA2 at the mRNA and protein level in mouse mammary epithelial cells in both *in vivo* and *in vitro* models, and address the possible mechanism(s) by which Ras brings about this upregulation. The Raf/MEK/MAPK pathway was found to be involved in the regulation of EphA2 protein levels. This pathway is also known to play a critical role in Ras-induced transformation by increasing cell proliferation through the upregulation of cyclin D1 expression (Albanese *et al.*, 1995). Furthermore, activation of the AP-1 transcription factor has been implicated in the loss of cell polarity (Reichmann *et al.*, 1992; Fialka *et al.*, 1996), which is important in the process of epithelial-fibroblastoid conversion and Webb and

colleagues have implicated the Raf/MEK/MAPK pathway in experimental metastasis (Webb *et al.*, 1998). Given the proposed roles of Eph receptors in mediating cell positioning, the recent findings that activation of EphA2 inhibits cell-ECM attachment (Dodge-Zantek *et al.*, 1999; Miao *et al.*, 2000), and the lack of evidence for a proliferative effect (Section 1.1.5.4), it is tempting to speculate that one function of the Ras-mediated increase in MAPK activity in tumourigenesis is to induce the levels of EphA2 protein to facilitate anchorage independent growth and tumour cell invasion. This will be further investigated in subsequent chapters using the relevant *in vitro* cell culture model described here.

Chapter 4

Investigation of the Requirement for EphA2 in Ras-Induced Mammary Tumourigenesis *in vivo*.

4.1. Introduction

The results presented in Chapter 3 demonstrated that EphA2 is upregulated by Ras in both *in vivo* mammary gland tumourigenesis and an *in vitro* cell culture model. The aim of the work undertaken in this Chapter is to investigate the requirement for EphA2 in Ras-mediated mammary tumourigenesis using the WAP*ras* model and an additional transgenic line containing the *eck*^{i} mutation. This mutation was generated by the insertion of a gene trap retrovirus into the EphA2 gene. The gene trap retrovirus, U3βgeo, essentially acts as a promoter trap by 'stealing' the promoter of an endogenous gene. The retrovirus carries a sequence comprising an in-frame fusion of β-galactosidase and neomycin phosphotransferase (βgeo), therefore the endogenous gene is both mutated and 'tagged' by the insertional event. This technique thereby facilitates the analysis of both expression and function of the endogenous gene. The *eck*^{i} mutation was initially identified in a search for developmentally regulated genes in ES cells. Subsequent to retroviral infection of ES cells with the U3βgeo retrovirus, selection in G418 was used to identify cells in which the provirus (designated J3A3) was actively transcribed. To identify genes which were developmentally regulated, individual clones of ES cells were induced to differentiate *in vitro* into embryoid bodies. A change in X-gal staining, as a reflection of β-galactosidase activity, during differentiation is indicative of genes that are highly regulated during embryogenesis (Scherer *et al.*, 1996). Having established that EphA2 was one such gene, the mutation was transmitted through the germline and viable homozygous mutant mice (eck$^{i/i}$) were generated. X-gal staining of eck$^{i/i}$ embryos reproduced the pattern of EphA2 mRNA and protein expression as previously detailed (Ganju *et al.*, 1994; Ruiz and Robertson, 1994). Investigation into the consequences of the mutation upon EphA2 protein expression revealed that levels of EphA2 protein were markedly downregulated in adult lung tissue. However, despite showing a severe deficiency in EphA2 protein, eck$^{i/i}$ mice are phenotypically normal (Chen *et al.*, 1996).

Following initial experiments to further characterise the eck$^{i/i}$ mice, work undertaken in this chapter brought together the genotypes of both eck$^{i/i}$ and WAP*ras* transgenic models to address the requirement of EphA2 in Ras-mediated mammary tumourigenesis. In other cases, functional insulin-like growth factor I has been shown to be necessary for transformation of embryonic fibroblasts by the Simian virus 40 (SV40) large tumour antigen (Sell *et al.*, 1993) or by EGF (Coppola *et al.*, 1994), and tumours rarely developed in polyomavirus middle T-antigen-induced mammary tumourigenesis in mice deficient for c-Src (Guy *et al.*, 1994). The main aim of experiments undertaken in this chapter, therefore, was to investigate the ability of Ras to induce mammary tumours in the absence of EphA2.

4.2. Results

4.2.1. eck$^{i/i}$ Mice Express β-galactosidase and are Deficient in EphA2 Protein

Transgenic mice homozygous for the *eck^{i}* allele (eck$^{i/i}$) were obtained (see Section 2.5.1). A schematic diagram depicting the insertion site of the J3A3 provirus within the EphA2 gene is shown in Figure 4.1. Prior to using these mice for further experiments, their molecular genotype was confirmed using two different approaches. Firstly, integration of the gene trap retrovirus into the EphA2 gene was assessed by β-galactosidase staining. 10.5 dpc embryos from eck$^{i/i}$ mice were fixed and stained with X-gal (Section 2.3.11). Positive X-gal staining was clearly visible in a discrete area in the hind limb buds (Figure 4.2a), where expression of endogenous EphA2 mRNA and protein has been previously reported (Ganju *et al.*, 1994; Mori *et al.*, 1995).

Secondly, disruption of the EphA2 gene by the proviral integration event was assessed by western blot analysis of protein samples from adult lung tissue using an anti-EphA2 antibody, or western blot or *in vitro* kinase assay of EphA2 immunoprecipitates from primary embryonic fibroblasts derived from 17 dpc embryos of both eck$^{i/i}$ and wild-type eck$^{+/+}$ mice (Section 2.5.3). The level of EphA2 protein was severely attenuated in lung tissue derived from eck$^{i/i}$ mice compared with wild-type eck$^{+/+}$ mice, but a low level of EphA2 protein was still detectable (Figure 4.3a).

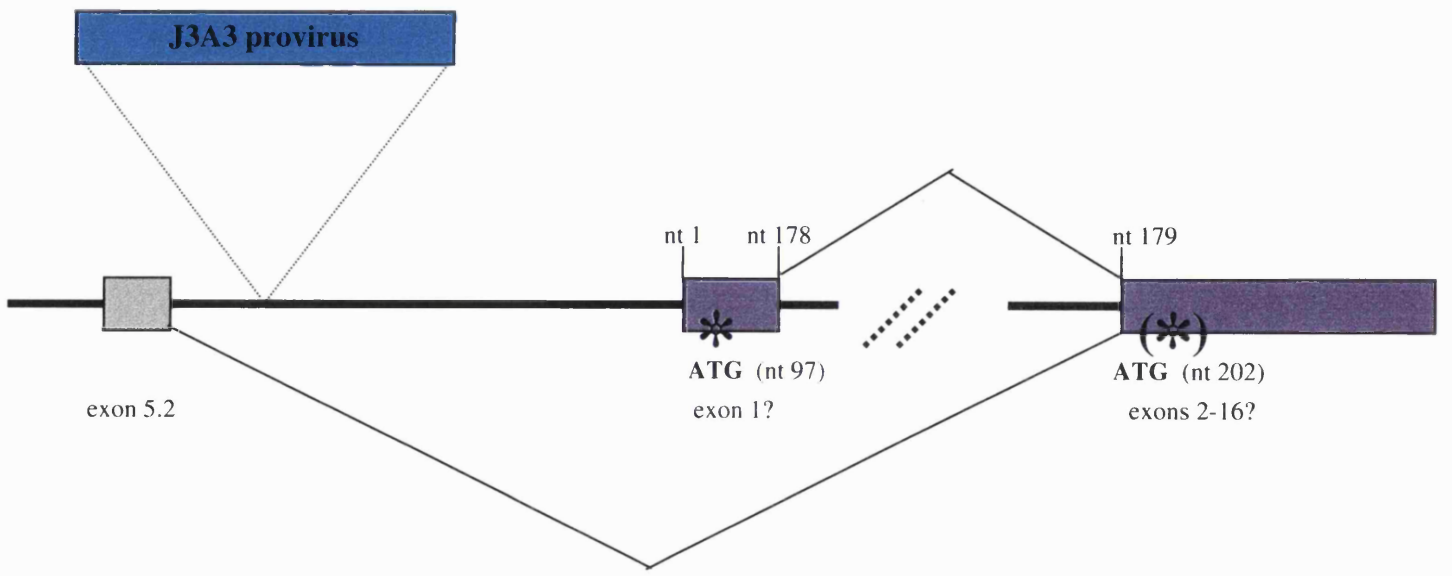


Figure 4.1. The J3A3 Provirus Integrates into the EphA2 Locus
Diagrammatic representation of the insertion of the U3βgeo gene trap retrovirus into the EphA2 locus (not to scale), depicting the previously published EphA2 cDNA sequence (mauve) and the recently identified Exon 5.2 (grey). EphA2 sequences containing nucleotides 178 and 179 are located on separate exons. ✻ indicates initiating methionine. (✻) indicates putative alternative initiation site in exon5.2/nt 179 transcript. The assignment of exon 1 and exons 2-16 is based on the findings of Connor and Pasquale, 1995. Adapted from Chen *et al.*, 1996.

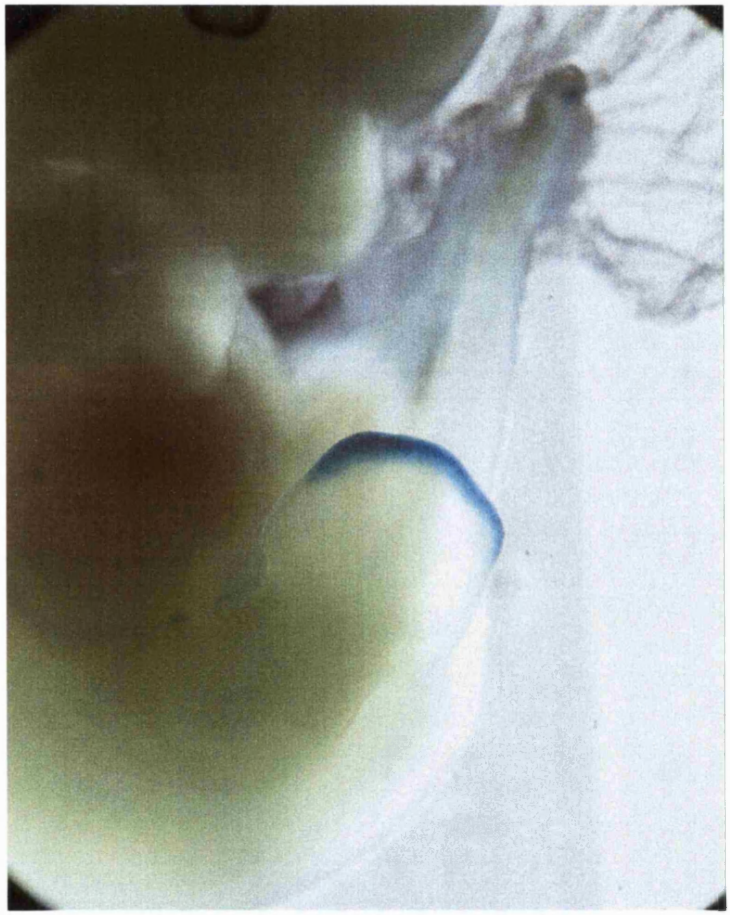


Figure 4.2. eck$^{i/i}$ Mice Express β-galactosidase
A 10.5 dpc eck$^{i/i}$ embryo was stained in X-gal staining buffer for 16 hours. Blue staining indicates ß-galactosidase activity.

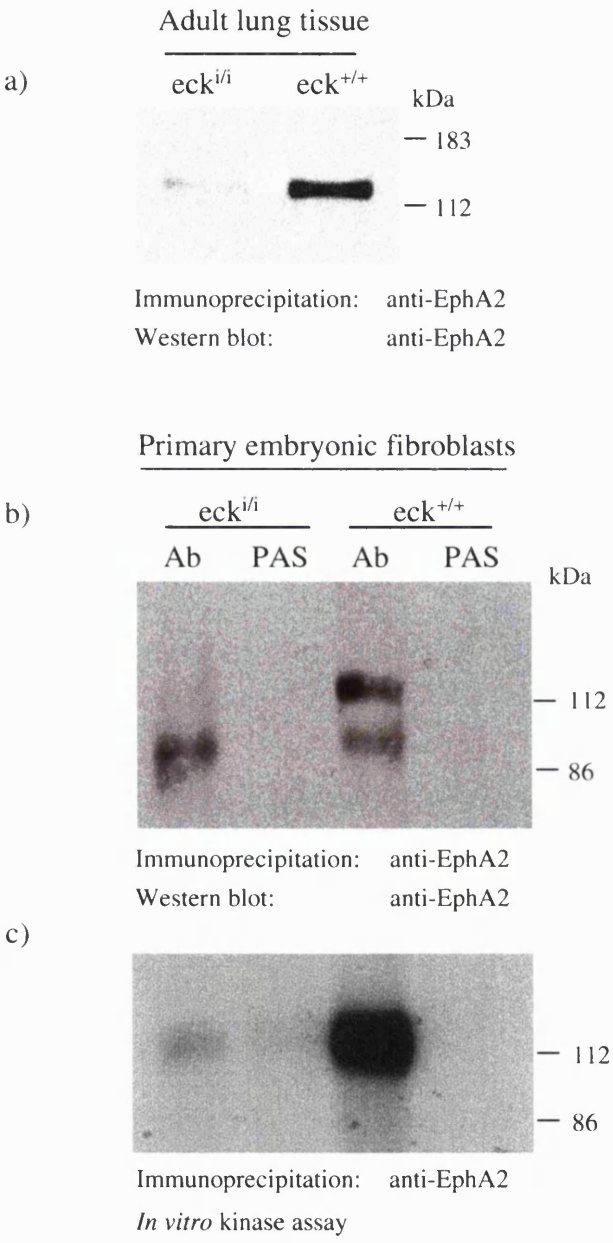


Figure 4.3. eck$^{i/i}$ Mice are Deficient in EphA2 Protein
(a) Adult lung tissue from eck$^{i/i}$ or eck$^{+/+}$ mice was lysed in RIPA buffer. 1 mg total protein lysate was immunoprecipitated using an anti-EphA2 antibody (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE and western blotting with an anti-EphA2 antibody (UBI). (b) and (c) 1 mg of total protein lysate from eck$^{i/i}$ or eck$^{+/+}$ embryonic fibroblasts was immunoprecipitated with either an anti-EphA2 antibody (Santa Cruz) (Ab) or using protein A-Sepharose alone (PAS). Immunoprecipitates were divided equally and subjected to (b) SDS-PAGE and western blotting with an anti-EphA2 antibody (Santa Cruz), or (c) *in vitro* kinase assay followed by SDS-PAGE and autoradiography.

A similar observation was also made in embryonic fibroblasts (EMFs). A larger amount of EphA2 protein was immunoprecipitated from $eck^{+/+}$ EMFs compared with $eck^{i/i}$ EMFs, an observation reflected by the increase in EphA2 catalytic activity from $eck^{+/+}$ EMFs over $eck^{i/i}$ EMFs (Figure 4.3b and c). However, in all experiments, both residual EphA2-immunoreactive protein and catalytic activity were observed in tissues prepared from $eck^{i/i}$ animals.

4.2.2. Breeding of $eck^{i/i}$ and $eck^{+/+}$ Mice with WAP*ras* Mice

It was confirmed in Section 4.2.1 that $eck^{i/i}$ mice express markedly diminished levels of EphA2 protein compared with their wild-type $eck^{+/+}$ counterparts. In Chapter 3, EphA2 expression was seen to be upregulated by oncogenic *ras* in mammary epithelial cells. Therefore, to assess the consequences, in terms of tumour formation, of a deficiency in EphA2 protein on the ability of Ras to induce mammary tumours, $eck^{i/i}$ mice were crossed with WAP*ras* mice.

To this end, WAP*ras* transgenic mice were crossed with either $eck^{i/i}$ or $eck^{+/+}$ transgenic mice. Tumour onset time in the WAP*ras* transgenic mouse line has previously been shown to be sensitive to the genetic background (Nielsen *et al.*, 1992) therefore, as the eck^{i} and WAP*ras* transgenic mice differed in their genetic background (Section 2.5.1), it was first necessary to establish an inbreeding program (as depicted in Figure 4.4). Male mice bearing the WAP*ras* transgene on the Y chromosome (WAP*ras*Y) were crossed with homozygous female $eck^{i/i}$ mice. Sibling mating was then carried out between the offspring of this *F1* generation. The *F2* generation was genotyped using DNA prepared from tail snips (Section 2.5.2). Sibling mating was again carried out between mice homozygous for the wild-type or the mutant eck^{i} allele, enabling WAP*ras*Y mice to be analysed on either an $eck^{i/i}$ or a wild-type ($eck^{+/+}$) background. At this point, several sublines of both $eck^{i/i}$/WAP*ras*Y and $eck^{+/+}$/WAP*ras*Y mice were established.

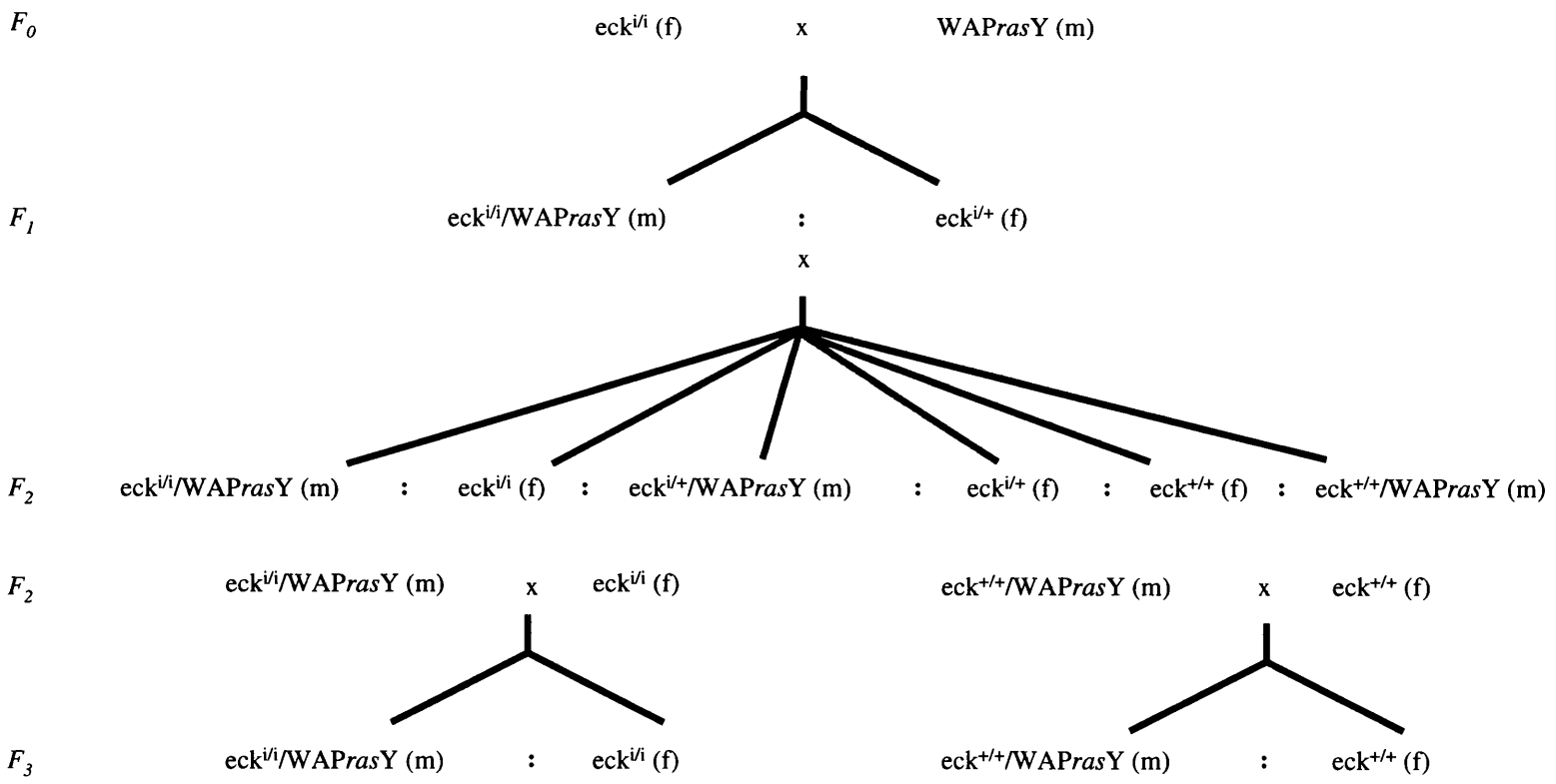


Figure 4.4. Strategy for Breeding WAP*ras* and eck$^{i/i}$ or eck$^{+/+}$ Mice
Male WAP*ras* mice (WAP*ras*Y) were mated with female eck$^{i/i}$ mice. Sibling mating was then carried out between the offspring of this *F1* generation. Following genotyping of the *F2* generation, sibling mating was again carried out between male eck$^{i/i}$/WAP*ras*Y and female eck$^{i/i}$ mice or male eck$^{+/+}$/WAP*ras*Y and female eck$^{+/+}$ mice enabling WAP*ras*Y mice to be analysed on either an eck$^{i/i}$ or an eck$^{+/+}$ background. Male and female mice are denoted by (m) and (f), respectively.

4.2.3. Analysis of Tumour Incidence

Mammary gland tumours were formed in the *F2* generation of eck$^{+/+}$/WAP*ras*Y mice after 60-70 days, in agreement with previous data (Nielsen *et al.*, 1992). However, mammary tumours also developed in eck$^{i/i}$/WAP*ras*Y animals, and there was no noticeable difference in the time that tumours were first observed between these and the eck$^{+/+}$/WAP*ras*Y animals (data not shown). Thus, the presence of the *eck^{i}* allele did not appear to inhibit or delay the onset of mammary tumours in WAP*ras* mice.

4.2.4. Ras Does Not Induce Amplification of the EphA2 Gene

To confirm the integration of the provirus into the EphA2 gene in eck$^{i/i}$/WAP*ras*Y mice, genotyping by Southern blot analysis (Section 2.1.12) was carried out on DNA prepared from mammary tumours of two eck$^{i/i}$/WAP*ras*Y sublines (M66 and M78). Mammary tumours from two eck$^{+/+}$/WAP*ras*Y sublines (M15 and M17) were taken for comparison. Southern blotting was performed using a probe corresponding to a ~ 500 bp *Xba* I-*Xho* I fragment of genomic DNA flanking the proviral integration site (Chen *et al.*, 1996). Genomic DNA from the tumour samples and from tail snips of control homozygous eck$^{i/i}$, heterozygous eck$^{i/+}$ and homozygous eck$^{+/+}$ mice was subjected to restriction digest using *Bgl* II. The presence of the provirus within a *Bgl* II genomic DNA fragment resulted in the altered migration of this fragment by agarose gel electrophoresis, and could therefore be used to distinguish the genotype of homozygous eck$^{i/i}$, heterozygous eck$^{i/+}$ and homozygous eck$^{+/+}$ mice.

The specific probe hybridised to a *Bgl* II fragment of approximately 9 kb in genomic DNA prepared from wild-type animals (Figure 4.5). In mice homozygous for the *eck^{i}* mutation, the probe hybridised to a ~ 15 kb *Bgl* II genomic DNA fragment. In heterozygous animals, genomic fragments of 9 kb and 15 kb were detected by the specific probe. Thus, these results verified the correct genotype of the mammary tumours. In addition, there was no difference in the relative intensity of the hybridisation signal between equal quantities of genomic DNA from tail and tumour tissue, indicating that the EphA2 gene was not amplified in WAP*ras*Y mammary tumours.

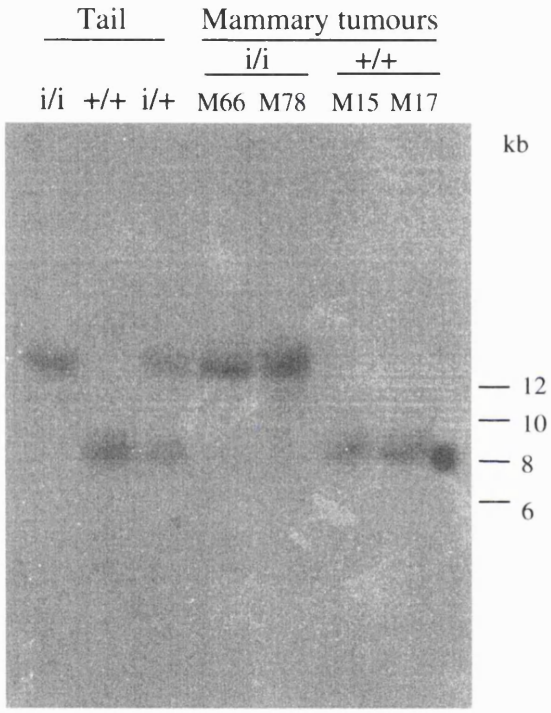


Figure 4.5. Ras does not Induce EphA2 Gene Amplification
Genomic DNA was prepared from tail snips of mice known to be of $eck^{i/i}$, $eck^{i/+}$ or $eck^{+/+}$ genotype, or mammary tumours derived from two sublines of $eck^{i/i}$/WAP*ras*Y mice (M66 and M78) and two sublines of $eck^{+/+}$/WAP*ras*Y mice (M15 and M17). 20 µg was subjected to restriction endonuclease digestion with *Bgl* II prior to agarose gel electrophoresis. Southern blotting was performed using a random primed [α-^{32}P] dCTP-labelled probe corresponding to a ~ 500 bp *Xba* I-*Xho* I fragment of genomic DNA flanking the proviral integration site.

4.2.5. Analysis of EphA2 Protein Levels in eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Mice

Genotypic analysis confirmed that the J3A3 provirus was present in the EphA2 gene in tumours derived from eck$^{i/i}$/WAP*ras*Y mice. Therefore, to investigate whether mammary tumours of eck$^{i/i}$/WAP*ras*Y mice also displayed a marked deficiency of EphA2 protein, as was observed in EMFs and lung tissue of eck$^{i/i}$ mice compared to their wild-type counterparts, the level of EphA2 receptor protein was measured in animals from three sublines of both eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y mice. These sublines were M38, M66 and M78 (eck$^{i/i}$/WAP*ras*Y) and M62, M65 and M69 (eck$^{+/+}$/WAP*ras*Y). Additionally, to confirm the previous observation of a marked downregulation of EphA2 protein in non-tumour tissue, lung and kidney samples were also taken for analysis.

4.2.5.1. Analysis of EphA2 Protein Levels in Lung, Kidney and Tumour Derived from eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Animals

Protein lysates of lung, kidney and tumour tissue from the three sublines of eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y mice were prepared and EphA2 was immunoprecipitated from 1 mg total protein lysate using an anti-EphA2 antibody. The immunoprecipitates were then subjected to SDS-PAGE and western blot analysis using an anti-EphA2 antibody. As previously observed in non-tumour tissues, the amount of EphA2 immunoreactive protein was significantly reduced in the lung and kidney from all eck$^{i/i}$/WAP*ras*Y mice compared with eck$^{+/+}$/WAP*ras*Y animals (Figure 4.6a).

However, in mammary tumours from all three of the eck$^{i/i}$/WAP*ras*Y animals, high levels of an anti-EphA2 immunoreactive protein were observed. High levels of EphA2 protein were also observed in mammary tumours from two of the three eck$^{+/+}$/WAP*ras*Y animals (M62 and M69), whereas only a small amount of EphA2 protein was present in mammary tumour tissue from a third animal (M65).

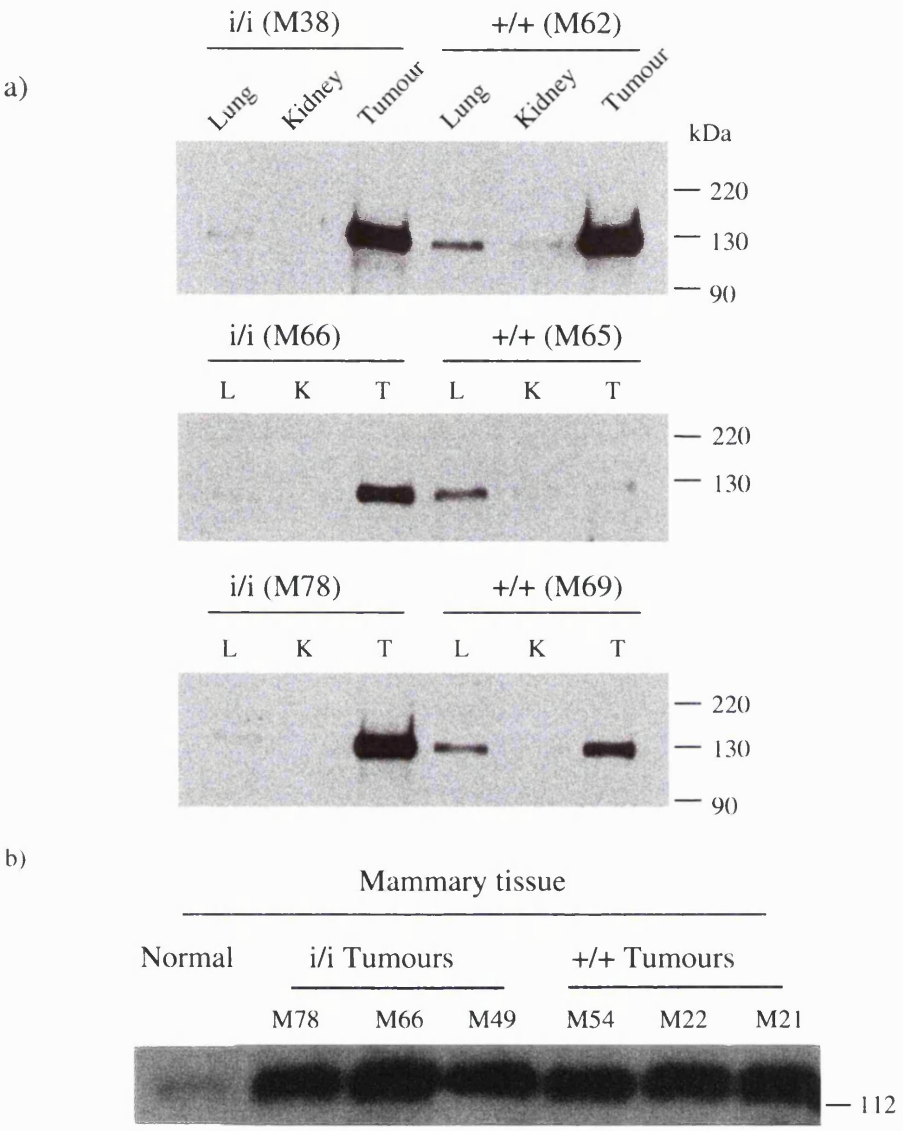


Figure 4.6. eck$^{i/i}$/WAP*ras*Y Mammary Tumours Express Catalytically Active EphA2 Protein

(a) Lung, kidney and mammary tumour from three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and three sublines of eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69) were lysed in RIPA buffer. 1 mg total protein lysate was immunoprecipitated using an anti-EphA2 antibody (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE and western blotting with an anti-EphA2 antibody (UBI). (b) Mammary tumours from three sublines of eck$^{i/i}$/WAP*ras*Y mice (M49, M66 and M78) and three sublines of eck$^{+/+}$/WAP*ras*Y mice (M21, M22 and M54), and normal mammary tissue from an eck$^{+/+}$ animal were lysed in RIPA buffer. 1 mg total protein lysate was immunoprecipitated using an anti-EphA2 antibody (Santa Cruz) and subjected to *in vitro* kinase assay followed by SDS-PAGE and autoradiography.

4.2.5.2. EphA2 Protein from eck$^{i/i}$/WAP*ras*Y Mammary Tumours Retains Catalytic Activity

To determine whether the anti-EphA2 immunoreactive protein detected in the eck$^{i/i}$/WAP*ras*Y tumours was catalytically active, *in vitro* kinase assays were performed on EphA2 immunoprecipitates of lysates prepared from three eck$^{i/i}$/WAP*ras*Y tumours (sublines M49, M66 and M78) and three eck$^{+/+}$/WAP*ras*Y mammary tumours (sublines M21, M22 and M54). A 130 kDa protein immunoprecipitated from lysates of all of the mammary tumours using an anti-EphA2 antibody displayed high levels of autophosphorylation, indicating that it possessed catalytic activity (Figure 4.6b).

4.2.6. Analysis of EphA2 Transcripts in Lung, Kidney and Tumour Derived from eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Animals

Following the observation that considerable levels of a 130 kDa catalytically active anti-EphA2 immunoreactive protein was present in mammary tumours of eck$^{i/i}$/WAP*ras*Y mice, further analysis was undertaken to investigate the possible reasons for this occurence. Expression of this protein in mammary tumours from eck$^{i/i}$/WAP*ras*Y mice could be the result of:-

1. continued expression of the previously published EphA2 cDNA in addition to the provirus.
2. splicing to remove the provirus.

As illustrated in Figure 4.1, transcripts arising in wild-type eck$^{+/+}$ animals may comprise sequences either corresponding to the previously published cDNA or to exon 5.2 spliced to nucleotide 179 of this previously published EphA2 cDNA sequence (Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Chen *et al.*, 1996). However, in eck$^{i/i}$ animals, the strong splice acceptor of the gene trap retrovirus should limit transcripts to those containing the proviral sequence. Thus, transcripts would be expected to comprise exon 5.2 and the J3A3 proviral DNA, as has previously been documented (Chen *et al.*, 1996). Therefore, in order to investigate the occurrence of these transcripts in eck$^{+/+}$/WAP*ras*Y mice and eck$^{i/i}$/WAP*ras*Y mice, RNA was prepared from three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and three sublines of eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69).

To accommodate the large number of samples necessary for the analysis of these transcripts in the different tissues of both sets of animals ($eck^{i/i}$/WAP*ras*Y and $eck^{+/+}$/WAP*ras*Y), RT-PCR was carried out in a 96-well format. The quantitative analysis of gene expression using 'real-time' measurement of PCR products is often used to measure differences in gene expression of large numbers of samples (Harrison *et al.*, 2000; Simpson *et al.*, 2000; Rajeevan *et al.*, 2001). A recently developed method to measure the accumulation of PCR products, SYBR® Green PCR, exploits the ability of the fluorescent SYBR® Green I dye to intercalate into double-stranded DNA. The measurement of the accumulation of PCR products in conjunction with the use of suitable standards enables the initial number of copies of the cDNA template to be determined. This can subsequently be related back to the amount of mRNA transcripts present in the original sample.

4.2.6.1. Preparation of Samples for RT-PCR Analysis

Total RNA was extracted from lung, kidney and mammary tumour tissues from three $eck^{i/i}$/WAP*ras*Y animals (lines M38, M66 and M78) and three $eck^{+/+}$/WAP*ras*Y animals (lines M62, M65 and M69) (Section 2.4.1). Subsequent to DNAse treatment (Section 2.4.2), 1 μg of total RNA from lung, kidney and mammary tumour tissue from each of the six animals was placed in each of four wells of a 96-well plate. Reverse transcription reactions were carried out on three of the RNA samples, while the fourth sample was treated with water to act as a 'no amplification control' (NAC). Twenty identical cDNA 'master plates' (as depicted in Figure 4.7) were then prepared (Section 2.4.3).

4.2.6.2. Analysis of β-actin Gene Expression

To assess the quality of the original RNA and to estimate the quantity of cDNA in the samples, SYBR® Green PCR was performed on a cDNA master plate using primers designed against β-actin (Table 2.4). The expression of β-actin, a 'housekeeping' gene, is necessary for the normal functioning of a cell and should not be greatly influenced by external stimuli. Different tissues, however, may be expected to express different levels of housekeeping genes.

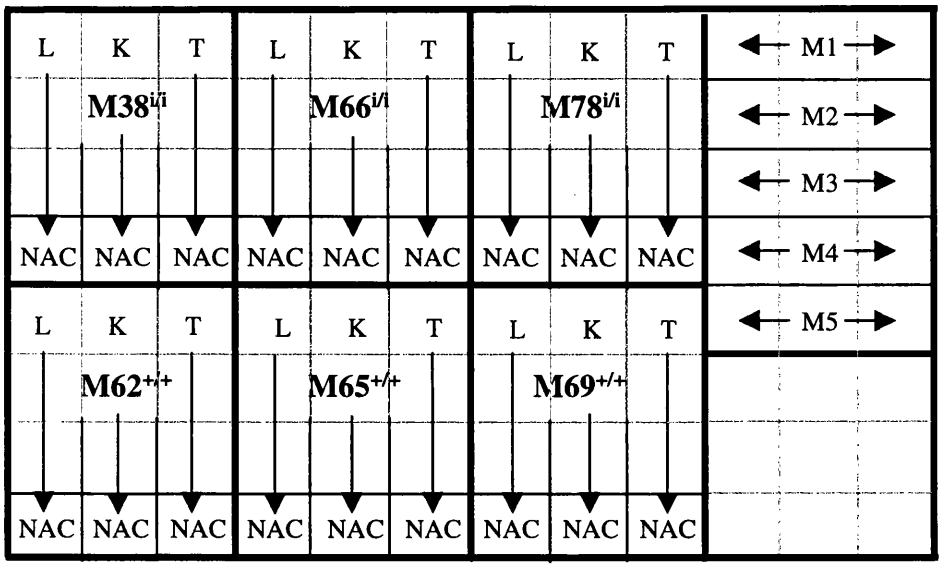


Figure 4.7. Design of Master Plates for SYBR® Green PCR Analysis
The diagram represents a 96-well master plate for SYBR® Green PCR. Total RNA from lung, kidney and mammary tumour was prepared from three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and three sublines of eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69) and 1 μg was added to each of four wells. L represents lung tissue, K corresponds to kidney and T to tumour. Reverse transcription reactions were performed on three of these samples whilst water was added to the fourth in place of the reverse transcriptase enzyme as a 'no amplification control' (NAC). The resulting cDNA was then aliquoted into 20 identical plates. Prior to performing SYBR® Green PCR analysis, five 10-fold dilutions of genomic or cDNA standards were added in triplicate to the plate as indicated (M1-M5).

4.2.6.2.1. Construction of Standard Curves

The raw data obtained from the ABI 7700 Sequence Detector correspond to the mean emission measured during the amplification steps of the PCR. This information can be used to create an amplification plot, where the change in emission values (ΔR) is plotted against the PCR cycle number (Figure 4.8). A threshold is assigned in the exponential phase of PCR. The β-actin standard curve (Figure 4.9) was constructed using data taken from the β-actin SYBR® Green PCR amplification plot. The cycle number (C_t) at which ΔR crossed the threshold value was then plotted against the number of copies of the template in the starting concentration of DNA. There is an inverse linear relationship between the starting quantity of template and the cycle number at which ΔR crosses the assigned threshold. An equation derived from linear regression analysis of the standard curve can therefore be used to calculate the starting quantity of cDNA (which is ultimately dependent on the amount of mRNA transcripts present in the sample) in the unknown samples.

The expression of β-actin was indeed seen to vary between different samples (Figure 4.10). Importantly, however, there was no consistent difference in its expression between $eck^{i/i}$/WAP*ras*Y and $eck^{+/+}$/WAP*ras*Y animals.

4.2.6.3. Design of Primers for SYBR® Green PCR Analysis of EphA2 Transcripts

The primers used to amplify the β-actin were designed within a single exon which enabled genomic DNA to be used both to verify primer function and to construct the standard curve. However, to analyse transcripts comprising both exon 5.2 and EphA2 sequence downstream of nucleotide 179, and for the analysis of transcripts containing the provirus, genomic DNA was unsuitable for testing primer function or constructing the standard curve. Therefore, verification of the specificity of primers and construction of the standard curve was carried out using alternative sources of DNA as described below.

Primer set #1 (Table 2.4) was designed against a short region of the neomycin phosphotransferase gene (accession number V00618), the sequence of which is present in the βgeo fusion of the gene trap retrovirus (Friedrich and Soriano, 1991). The plasmid pcDNA3.1/ Myc-His(+) was used to test the primers and to construct the standard curve.

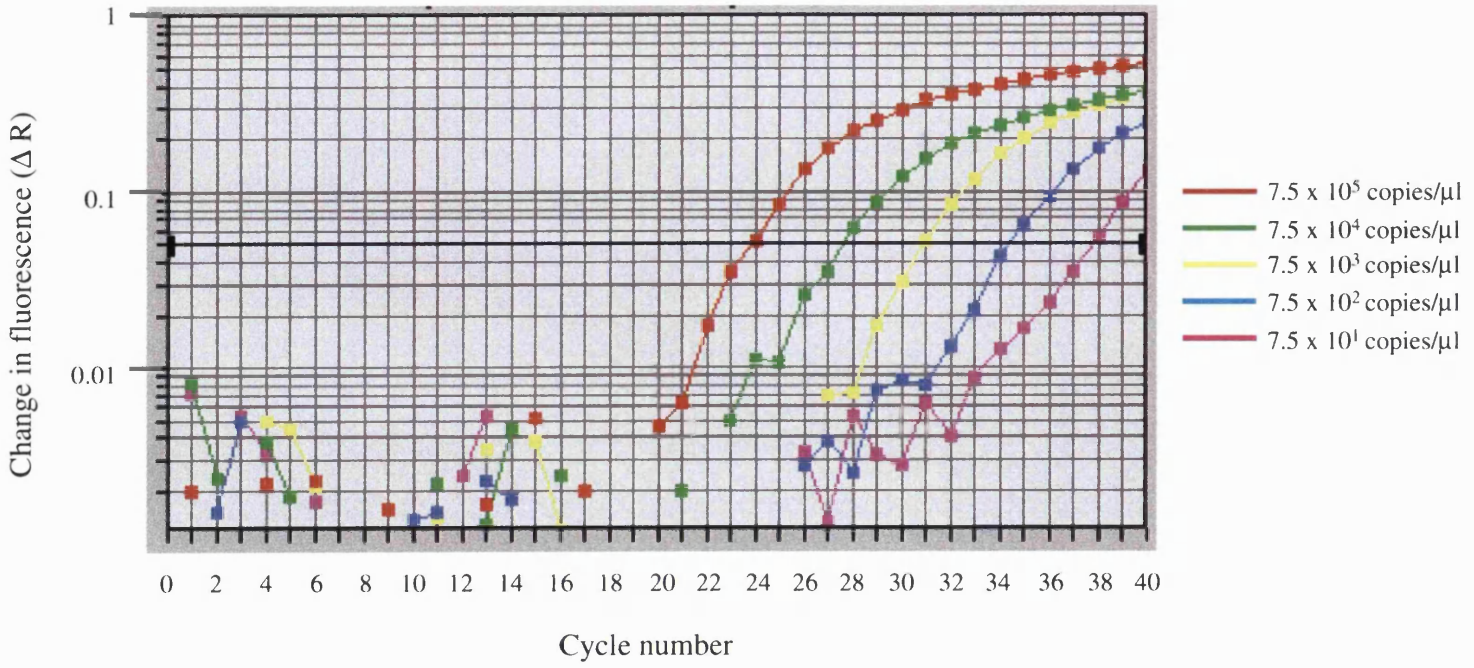


Figure 4.8. Amplification Plot of Genomic DNA using β-actin Primers
Five 10-fold dilutions of mouse genomic DNA were used to perform SYBR® Green PCR analysis with primers designed to amplify β-actin DNA. Analysis was performed using the Sequence Detector software package. The change in fluorescence emission at 521 nm (ΔR) was plotted against the PCR cycle number. The threshold was set at 0.05, the point at which all the reactions were in the exponential phase of amplification. The key indicates the number of copies of genomic DNA present in the starting concentrations of DNA.

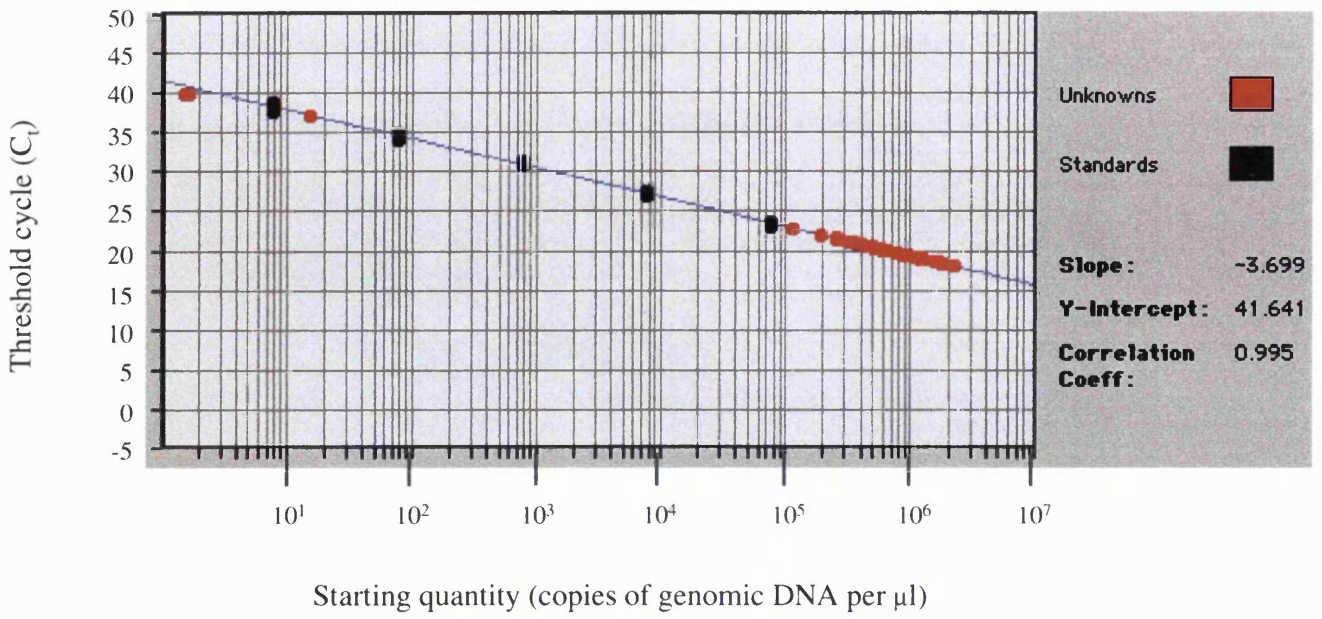


Figure 4.9. β-actin Standard Curve
Data obtained from Figure 4.8 was used to construct a standard curve. The cycle number (C_t) at which ΔR crossed the threshold value in Figure 4.8 was plotted against the number of copies of the template in the starting concentration of genomic DNA (black circles). Linear regression analysis was performed and the starting quantity of cDNA template in the unknown samples (red circles) was determined using the equation derived from this line.

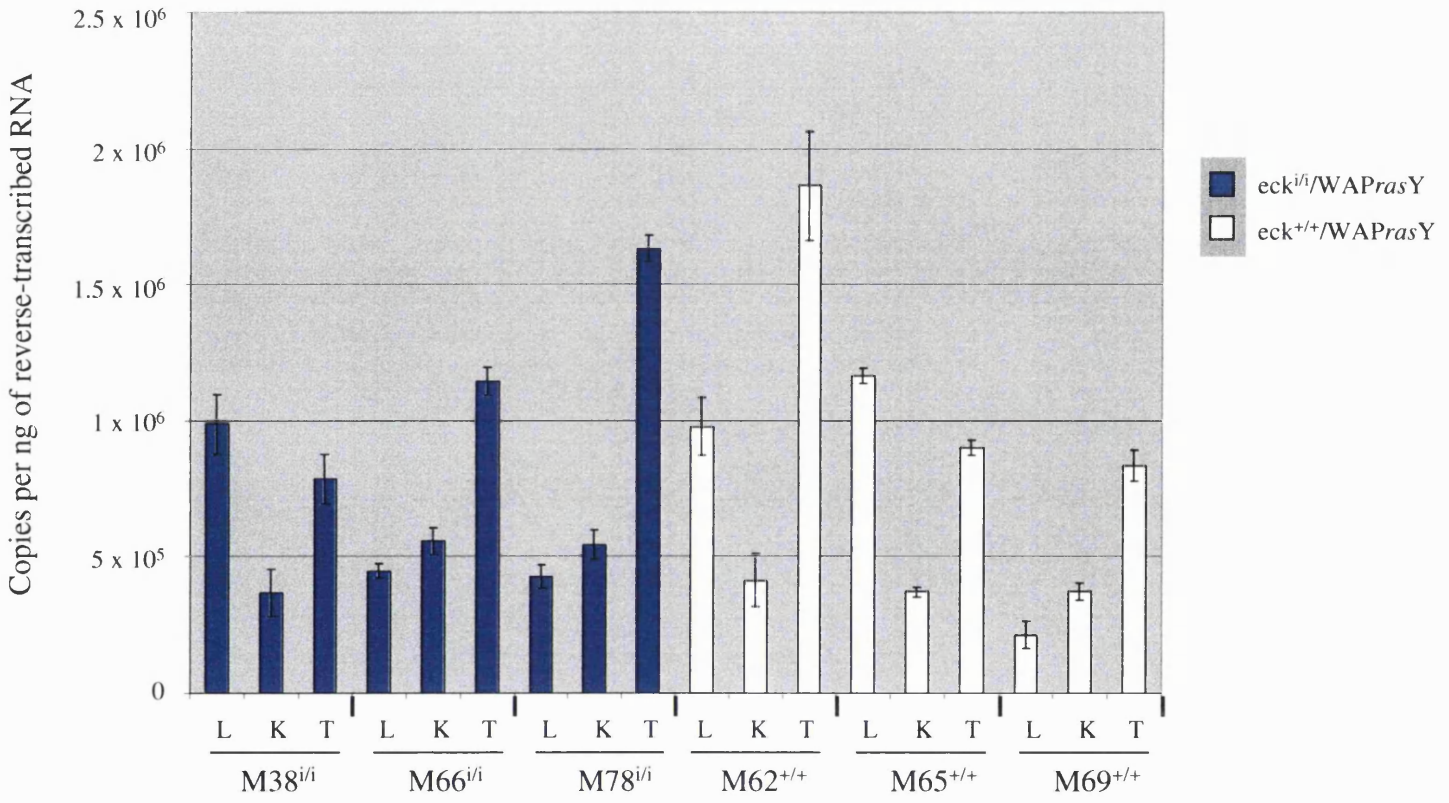


Figure 4.10. Expression of β-actin in Lung, Kidney and Mammary Tumours of eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Mice
Data derived from Figure 4.9 was used to calculate the starting quantity of cDNA template in the lung, kidney and mammary tumours (L, K and T, respectively) of the three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69). Each bar represents a mean of three separate RT-PCR determinations for each RNA sample. Error bars denote standard error of the mean.

Primer set #2 (Table 2.4) was designed to amplify a region of EphA2 cDNA corresponding to nucleotides 1066-1135 of the previously published cDNA sequence (Ganju *et al.*, 1994). The plasmid pECE-EphA2WT was used to test the primers and to construct the standard curve, since nucleotides 1066 and 1135 have been reported to be present on separate exons (Michael *et al.*, 1999), which are likely to correspond to exons 4 and 5 according to Connor and Pasquale (Connor and Pasquale, 1995).

Primer set #3 (Table 2.4) was designed to amplify DNA from the previously unidentified transcript containing sequences derived from both exon 5.2 and EphA2 downstream of nucleotide 179. The transcript sequence was derived from both exon 5.2 and EphA2 sequences spliced together as described in Chen and colleagues (Chen *et al.*, 1996). cDNA obtained from oligo(dT) primed reverse transcription of mouse ES cell RNA was used as a PCR template for testing the primers and constructing the standard curve, since these cells have been reported to contain the exon 5.2/nucleotide 179 transcript (Chen *et al.*, 1996).

4.2.6.4. Analysis of Transcripts in eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Animals

To verify the integration and expression of the J3A3 provirus, SYBR® Green PCR was carried out on the cDNA master plate using primer set #1, designed to amplify a region of the neomycin phosphotransferase gene. The data obtained was normalised against that obtained from β-actin SYBR® Green PCR to correct for differences in RNA expression and quality between different tissues. Neomycin phosphotransferase transcripts could clearly only be detected in tissues from eck$^{i/i}$/WAP*ras*Y animals (Figure 4.11), consistent with the integration of the gene trap retrovirus into eck$^{i/i}$ mice. It is also apparent that high levels of transcript were detected in mammary tumours of two of the eck$^{i/i}$/WAP*ras*Y mice, with markedly lower levels in lung and kidney samples. Since plasmid DNA was used in place of genomic DNA to construct the standard curve, values on the Y-axis (copies per ng of reverse-transcribed RNA) have been assigned arbitrary values.

To analyse the expression of the previously published EphA2 sequence, primer set #2 was used in SYBR® Green PCR analysis of the cDNA master plate. It can be seen clearly (Figure 4.12) that levels of this transcript were affected by the insertion of the J3A3 provirus into the EphA2 gene in eck$^{i/i}$/WAP*ras*Y animals. In these animals, EphA2 transcript levels were decreased by up to 90% in lung and kidney tissues,

although levels in the mammary tumours were only reduced by approximately 50%. However, in both eck$^{i/i}$/WAP*ras*Y and wild-type eck$^{+/+}$/WAP*ras*Y animals, highest levels of this transcript were observed in mammary tumours. Again, values on the Y-axis have been assigned arbitrary values as a result of the use of plasmid DNA, rather than genomic DNA, to construct the standard curve.

Attempts to analyse the levels of the transcript containing sequence derived from both exon 5.2 and downstream of nucleotide 179 of the previously published EphA2 cDNA sequence (Chen *et al.*, 1996) were carried out using primer set #3. However, only a mimimal level of fluorescence was detectable in the wells of the master plate, suggesting that this transcript was not present at significant levels in the lung, kidney or mammary tumours (data not shown). Confirmation of primer function was provided by the amplification of a single 116 nucleotide amplicon from mouse ES cell cDNA by PCR (Figure 4.13). No PCR product was obtained using mouse genomic DNA as a template.

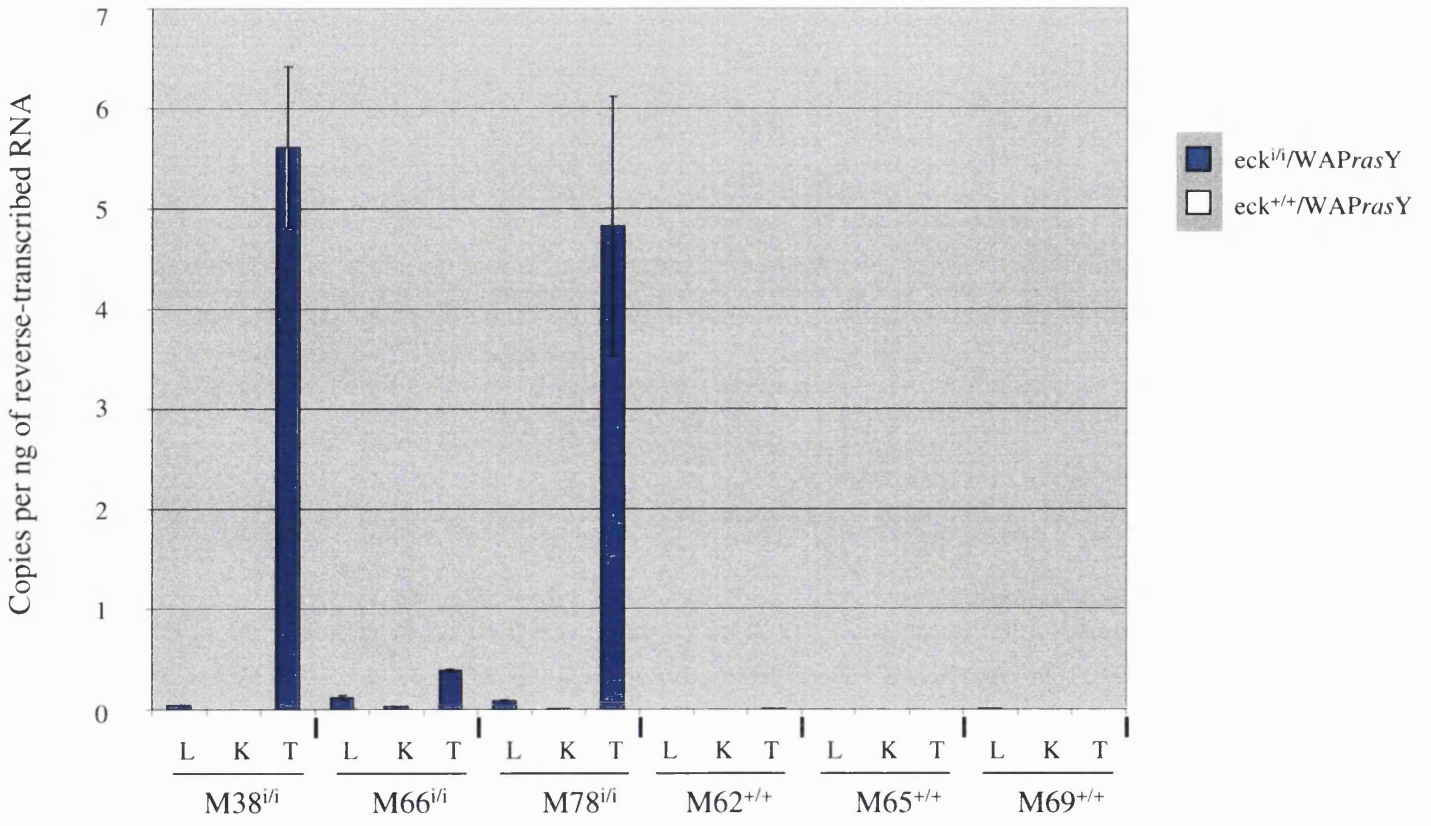


Figure 4.11. Expression of J3A3 Provirus in Lung, Kidney and Mammary Tumours of eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Mice
SYBR® Green PCR was performed on lung, kidney and mammary tumours (L, K and T, respectively) of the three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69) using primer set #1. Each bar represents a mean of three separate RT-PCR determinations for each RNA sample. Error bars denote standard error of the mean. The data obtained has been normalised against the values obtained for β-actin. Values on the Y-axis have been arbitrarily assigned.

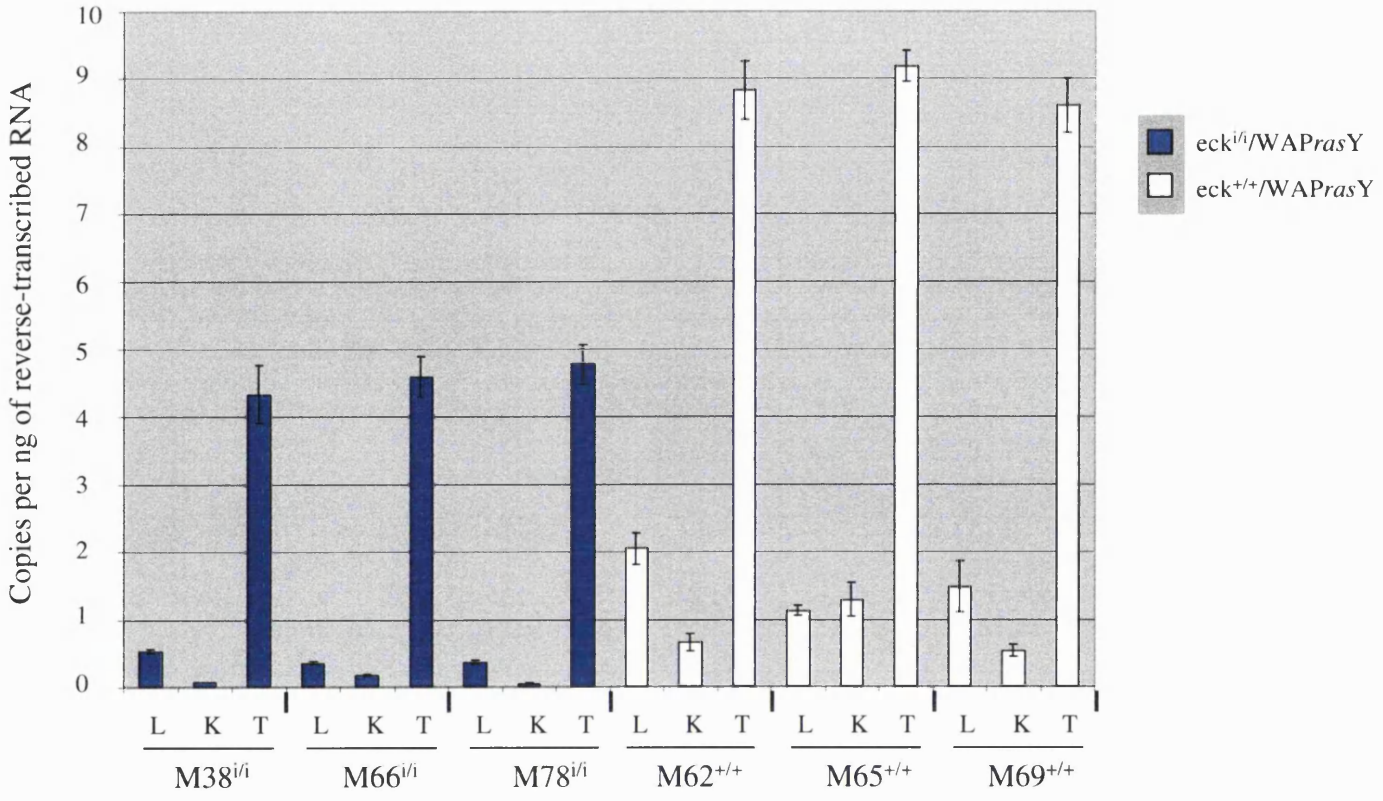


Figure 4.12. Expression of Transcripts Containing Nucleotides 1066-1135 of EphA2 in Lung, Kidney and Mammary Tumours of eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Mice

SYBR® Green PCR was performed on lung, kidney and mammary tumours (L, K and T, respectively) of the three sublines of eck$^{i/i}$/WAP*ras*Y mice (M38, M66 and M78) and eck$^{+/+}$/WAP*ras*Y mice (M62, M65 and M69) using primer set #2. Each bar represents a mean of three separate RT-PCR determinations for each RNA sample. Error bars denote standard error of the mean. The data has been normalised against the values obtained for β-actin. Values on the Y-axis have been arbitrarily assigned.

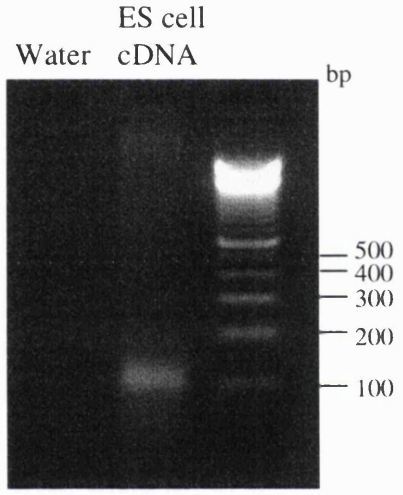


Figure 4.13. Expression of Transcripts Containing Exon 5.2 Spliced to Nucleotide 179 of EphA2 in ES Cells

Total RNA prepared from mouse ES cells was reverse transcribed using oligo(dT) primers and used as a cDNA template from which to amplify a 116 bp product using primer set #3. Water was used as a negative control. PCR products were subject to agarose gel electrophoresis and the gel was stained with ethidium bromide.

4.3. Discussion

4.3.1. Analysis of EphA2 Protein Expression in eck$^{i/i}$ Mice

Insertion of the provirus into the EphA2 gene in eck$^{i/i}$ mice resulted in the expression of β-galactosidase in the hind limb buds of 10.5 dpc eck$^{i/i}$ embryos, an area in which EphA2 expression has previously been reported (Ganju *et al.*, 1994; Mori *et al.*, 1995). Furthermore, EphA2 protein expression was markedly downregulated in EMFs and adult lung tissue of eck$^{i/i}$ mice compared to eck$^{+/+}$ mice. These results are entirely consistent with the use of gene trap retroviruses to disrupt cellular gene expression by effectively 'stealing' the endogenous promoter.

Therefore, having previously observed an upregulation of EphA2 protein in mammary epithelial cells in response to Ras, a cross between WAP*ras* mice and eck$^{i/i}$ mice was undertaken to analyse the consequences of a deficiency in EphA2 protein upon Ras-mediated mammary tumourigenesis. However, mammary tumours were formed at the same frequency in eck$^{i/i}$/WAP*ras*Y as eck$^{+/+}$/WAP*ras*Y mice, implying that the presence of the *eck^{i}* allele had no significant effect on WAP*ras*-induced mammary tumourigenesis. This also initially implied that EphA2 protein was not necessary for Ras-mediated tumourigenesis, given the marked deficiency of EphA2 protein observed in non-tumour tissues of eck$^{i/i}$ mice. However, subsequent analysis revealed that an anti-EphA2 immunoreactive protein continued to be expressed at high levels in mammary tumours from eck$^{i/i}$/WAP*ras*Y mice, in contrast to lung and kidney tissues where only residual EphA2 protein levels were detected. Analysis of one of the three eck$^{+/+}$/WAP*ras*Y mammary tumours revealed a very low level of EphA2 protein expression. This may be due to the sample originating from a necrotic region of the tumour. Since the expression of Ras in this tumour was not assessed, it is also possible that this tumour had arisen independently of the *ras* oncogene.

The reason(s) for the continued expression of anti-EphA2 immunoreactive protein is unclear. The *eck^{i}* mutation was the result of an insertional event, causing disruption to, rather than deletion of, the endogenous EphA2 gene. There is always the potential, therefore, that some form of functional EphA2 protein can be made from the existing coding sequences. An investigation of EphA2 RNA transcripts in lung, kidney and tumour tissue from three eck$^{i/i}$/WAP*ras*Y and three eck$^{+/+}$/WAP*ras*Y animals was undertaken to address this possibility.

4.3.2. Analysis of Transcripts in eck$^{i/i}$/WAP*ras*Y and eck$^{+/+}$/WAP*ras*Y Mice

4.3.2.1. Transcripts Containing βgeo Gene Trap Proviral DNA

Analysis of the expression of the provirus was performed by RT-PCR using primers designed to recognise a short sequence within the neomycin phosphotransferase gene of the βgeo sequence of the U3βgeo gene trap retrovirus. The results indicated that neomycin phosphotransferase transcripts were present in all samples prepared from eck$^{i/i}$ mice, and absent in all eck$^{+/+}$ tissues. This served to confirm the insertion, and subsequent expression, of the J3A3 provirus in the eck$^{i/i}$ mice. Moreover, in two of the three mammary tumours derived from eck$^{i/i}$ mice, significantly more neomycin phosphotransferase transcripts were present than in normal lung and kidney tissue. This indicates that the provirus was expressed at a high level in these tumours, therefore supporting previous observations that the regulatory elements of the EphA2 locus are targeted for upregulation in tumours.

4.3.2.2. Transcripts Containing Exon 5.2 and EphA2 Sequences Downstream of Nucleotide 179

Investigation of the site into which the J3A3 provirus had integrated resulted in the identification of a novel upstream exon, referred to as exon 5.2 (Chen *et al.*, 1996). In a previous study carried out by Connor and Pasquale (Connor and Pasquale, 1995), exon numbering was based on the assumption that exon 1 contains 5' untranslated sequence in addition to the initiating methionine and signal peptide-encoding nucleotide sequence. However, since this was not verified, it is possible that the presence of additional upstream genomic sequence is common to all Eph receptor genes. In some transcripts isolated from ES cells, exon 5.2 was found to be spliced to nucleotide 179 of the previously published EphA2 cDNA (Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Chen *et al.*, 1996). Similarly, such transcripts could be detected in ES cells by RT-PCR and SYBR® Green PCR (data not shown) using primer set #3. However, these primers failed to detect transcripts in RNA prepared from adult lung, kidney or mammary tumour of either eck$^{i/i}$/WAP*ras*Y or eck$^{+/+}$/WAP*ras*Y animals, implying that, in these tissues, the exon 5.2-nucleotide 179 splicing event does not occur to any significant extent. Chen and colleagues (Chen *et al.*, 1996) suggested that transcripts containing exon 5.2 spliced to nucleotide 179 were present in ES cells at a much lower level than

those corresponding to the previously published cDNA sequence. Thus, it is possible that this transcript is only present in ES cells.

Although the exon 5.2-derived sequence of such transcripts would not contribute to a functional protein, a protein lacking the amino terminus of EphA2 may result from translation commencing at an ATG codon at nucleotide 202 of the published EphA2 cDNA (see Figure 4.1) (Ganju *et al.*, 1994; Ruiz and Robertson, 1994; Chen *et al.*, 1996). This truncated protein would lack the signal peptide sequence present in the full length equivalent, which would prevent it passing through the endoplasmic reticulum, and as such, it is expected to be an intracellular rather than a transmembrane protein. The predicted size of this putative protein, based on sequence data alone, is 105 kDa. Therefore it should be distinguishable from its full length, glycosylated counterpart, which is detectable as a 120-130 kDa by SDS-PAGE and western blotting using antibodies directed against the carboxy terminus of EphA2. In accordance with the failure to detect any transcripts containing exon 5.2 and sequence downstream of EphA2 nucleotide 179, western blotting using anti-EphA2 antibodies directed against the carboxy terminus of EphA2 failed to detect a 105 kDa protein in any of the tissue samples from either eck$^{i/i}$/WAP*ras*Y or eck$^{+/+}$/WAP*ras*Y animals.

4.3.2.3. Transcripts Corresponding to Published EphA2 cDNA Sequence

Analysis of EphA2 transcripts corresponding to the published cDNA sequence clearly indicates that insertion of the provirus into the EphA2 gene resulted in a decrease in the levels of this transcript in lung, kidney and tumour tissue from eck$^{i/i}$/WAP*ras*Y mice. However, the reason for the continued presence of EphA2 transcripts in eck$^{i/i}$ animals remains unclear. It has previously been reported that small amounts of transcript corresponding to the βgeo sequence fail to become polyadenylated and are therefore able to extend through the 3' long terminal repeat of the integrated provirus (Scherer *et al.*, 1996). Whether such transcripts could extend as far downstream (approximately 8 kb) as the previously identified EphA2, and indeed, whether this is the mechanism by which EphA2 transcripts continue to be present is unclear. Northern blot analysis could be used to reveal the existence of such transcripts in mammary tumours of eck$^{i/i}$/WAP*ras*Y mice.

The discovery of exon 5.2 located 1.8 kb upstream of the proviral integration site implies that the EphA2 promoter lies even further upstream. Thus, it also remains a

possibility that further exons exist upstream of exon 5.2, and in some cases these may be capable of splicing to the published EphA2 sequence, thereby splicing out the provirus. Interestingly, mRNA transcripts in embryonic and adult tissues detected by northern blot analysis (Andres *et al.*, 1994; Ganju *et al.*, 1994) are larger at 4.7 kb than the actual published cDNA sequence of EphA2 (Ganju *et al.*, 1994; Ruiz and Robertson, 1994) Furthermore, additional transcripts of ~7 kb were detected in embryo and lung (Andres *et al.*, 1994), although it is unclear whether this corresponds to splice variants or cross-reaction of the probe. The size of the major transcript of 4.7 kb suggests the presence of additional, untranslated sequence, which may correspond to poly(A) sequence or potentially to further exons. Analysis of EphA2 transcripts carried out by Chen and colleagues (Chen *et al.*, 1996) implied that sequences may extend further 5' than the previously identified EphA2 cDNA sequence. If alternate exon usage is the mechanism by which EphA2 sequence continues to be expressed in eck$^{i/i}$/WAP*ras*Y mice, the reduced expression may be a consequence of disruption of an enhancer element by the provirus. Similarly, transcription from a putative alternative promoter downstream of the proviral insertion site could be compromised by disruption of an enhancer element. A 0.9 kb fragment located downstream of the proviral insertion site, but upstream of nucleotide 1 of the published EphA2 cDNA sequence was found to confer rhombomere 4-, but not node-, specific expression of a reporter gene in the presence of Pbx1, Hoxa1 and Hoxb1, implying that other enhancer sites must exist. Further investigation is required into the 8 kb region of genomic DNA between the proviral insertion site and the exon containing nucleotide 1 of EphA2, and into the area upstream of exon 5.2, with a view to trying to locate putative promoter elements that may be responsive to Ras.

Whatever the mechanism by which it occurs, the continued upregulation of transcripts containing nucleotides 1066-1135 of the previously published EphA2 sequence in mammary tumours of eck$^{i/i}$/WAP*ras*Y animals strongly implies that the increase in a 130 kDa anti-EphA2 immunoreactive protein observed in these tumours corresponds to EphA2 protein, and not to another Eph family member.

The data presented in this chapter have demonstrated that, despite the presence of a gene trap insertion that results in the attenuation of EphA2 protein expression in EMFs, lung and kidney, EphA2 protein continues to be expressed at significant levels in Ras-induced mammary tumours. Further investigation also revealed the continued presence

of EphA2 transcripts in eck$^{i/i}$/WAP*ras*Y animals. The reason for this is unclear, but may be the result of the existence of transcripts comprising both β-geo and EphA2 sequences, which should be identifiable by northern blot analysis, or the result of alternative splicing giving rise to both proviral and EphA2 transcripts. Sequence analysis of the EphA2 gene should reveal the presence of additional elements of transcriptional regulation. However, since EphA2 continued to be expressed in eck$^{i/i}$/WAP*ras*Y animals, an essential role for EphA2 in WAP*ras*-mediated mammary tumourigenesis cannot be determined from this study. To address this issue, transgenic mice which lack EphA2 coding sequence would be required.

In accordance with previous results, however, (Andres *et al.*, 1994), levels of EphA2 transcripts were upregulated in WAP*ras* mice, providing further evidence that Ras induces the activity of the EphA2 locus *in vivo*. Also in agreement with a number of previous reports that Eph receptor overexpression in tumours is not the result of gene amplification (Maru *et al.*, 1988; Sajjadi *et al.*, 1991; Wicks *et al.*, 1992; Kiyokawa *et al.*, 1994; Easty *et al.*, 1995), Southern blotting indicated that the EphA2 gene was not amplified in WAP*ras* mammary tumour tissue. Therefore, upregulation of EphA2 RNA and protein is likely to occur either as a result of increased EphA2 gene transcription or increased RNA stability.

Chapter 5

Generation of Stable Mammalian Cell Lines Overexpressing Recombinant EphA2

5.1 Introduction

The results presented in Chapter 3 demonstrated that EphA2 expression was upregulated by oncogenic Ras in both mouse mammary tumours and EpH4 mammary epithelial cells. The primary aim of the work presented in this chapter was to establish an experimental system in which to study the functional effects of EphA2 overexpression. Experiments carried out using this system would then provide an increased understanding as to why Ras upregulates EphA2 during mammary tumourigenesis.

One approach to characterise the function of a cloned gene is to introduce it into cells and determine the effects of its expression on various aspects of cellular function. To this end, recombinant wild-type EphA2 (EphA2WT) was used in studies undertaken in this chapter. Overexpression of any receptor tyrosine kinase is likely to create a higher receptor density which favours the formation of stable receptor dimers, thus evoking receptor autophosphorylation and intracellular signalling (Lemmon and Schlessinger, 1994). This is comparable to the stabilisation of transient receptor dimer interactions by the addition of exogenous ligand, however, in the case of Eph receptors, receptor overexpression has the advantage of avoiding the promiscuity of ephrin-Eph interactions (see Section 1.1.3.1). A further approach involves the use of a constitutively active form of EphA2, EphA2Neu, which was made by substituting the EphA2 transmembrane domain with that of the *neu* oncogene (see Section 2.1.1.5). Neu (also known as HER-2, erbB2 and $p185^{neu}$) is a 185 kDa protein whose gene was first isolated from neuroblastomas of ethylnitrosourea-treated rats. The *neu* oncogene product differs from its proto-oncogene counterpart by a single point mutation (T to A) in the transmembrane domain, which confers a valine to glutamate substitution at position 664 (Bargmann *et al.*, 1986). This increases the tyrosine kinase activity of the receptor by enhancing receptor dimerisation (Weiner *et al.*, 1989) although the precise mechanism by which this occurs is unknown. One model is that lipid-mediated protonation of the glutamate residue enables the mutated transmembrane domains to

interact via hydrogen bonds (Sternberg and Gullick, 1989). Alternatively, the mutated protein forms mainly α–helices which are capable of dimerising, in contrast to the sharp-bend conformation of the non-mutated protein which prevents an α-helical transmembrane structure (Brandt-Rauf *et al.*, 1990). A number of experiments have demonstrated that the origin of the transmembrane region does not influence signalling by a receptor chimera provided that the ligand-binding and catalytic domains are of the same origin (reviewed in Ullrich and Schlessinger, 1990), therefore EphA2Neu would be expected to function as a constitutively active EphA2 receptor by favouring receptor aggregation. Indeed, a constitutively active PDGFβ receptor was created by this strategy (Petti *et al.*, 1998). Importantly, the EphA2Neu chimera still possesses the highly conserved Eph juxtamembrane domain which contains two tyrosine sites of significance in catalytic activity (Binns *et al.*, 2000) and in intracellular signalling (Ellis *et al.*, 1996).

Recombinant DNA can be introduced into cells using a number of different techniques and the efficiency with which this occurs will depend on the cell type. Based on a natural biological process, retroviral infection offers a number of advantages, such as directional cloning, broad host range and high transfection efficiencies. However, the process of generating retroviral producer cells by which infectious virus is made is time-consuming, therefore this method is mainly used for creating stable cell lines or infecting a variety of cells with the same construct. In order to characterise constructs and systems, however, long-term expression is not usually required, and since characterisation does not always require use of a specific cell line, cells that are easily transfectable and show high levels of expression, such as COS cells, are often used.

Thus, during the course of this chapter, a number of different cell types and methods of transfection have been used as a means of generating a recombinant EphA2-overexpressing cell line. The advantages of high expression levels attained in transient COS cell transfections have been exploited to demonstrate the validity of the constructs and systems, whilst two different approaches to stable transfection were taken. As EpH4 cells have previously been demonstrated to upregulate EphA2 in response to Ras they were initially chosen as a physiologically relevant cell line in which to perform EphA2 overexpression studies. However, in order to attain a stable, recombinant EphA2-overexpressing cell line, a number of other cell lines were utilised.

5.2. Results

5.2.1. Characterisation of Recombinant EphA2Neu in COS Cells

The mammalian expression vector constructs pECE-EphA2WT and pECE-EphA2Neu encode wild-type (WT) EphA2 and an EphA2 receptor chimera containing the transmembrane domain of the Neu oncogene (Section 2.1.1.5), respectively. To confirm that these constructs encoded functional, recombinant EphA2, they were transfected into COS-1 cells using Lipofectin™ (Section 2.2.5.1). 16 hours before analysing protein levels (72 hours post-transfection) the cells were placed in growth medium containing 0.5% FCS. Subsequently, the cells were lysed in RIPA buffer and EphA2 was immunoprecipitated from the lysates using a polyclonal anti-EphA2 antibody. Following immunoprecipitation, each sample was divided equally between two separate wells and subjected to SDS-PAGE. Western blotting was performed using an anti-EphA2 antibody and an anti-phosphotyrosine antibody to assess both the steady-state level of EphA2 and its tyrosine phosphorylation state. Mock transfected COS-1 cells expressed a low level of endogenous EphA2 protein, which displayed a basal level of tyrosine phosphorylation (Figure 5.1). Transfection of EphA2WT DNA resulted in an increase in the steady-state level of EphA2 protein concomitant with a modest increase in tyrosine phosphorylation, whereas expression of EphA2Neu resulted in only a small increase in the steady-state level of EphA2 protein but a marked increase in tyrosine phosphorylation (Figure 5.1). These results therefore confirmed that i) transfection of the constructs resulted in the expression of recombinant EphA2 protein and ii) that the EphA2Neu protein was tyrosine phosphorylated to a higher level than the EphA2WT protein, indicating an increase in catalytic activity.

5.2.2. Generation of Recombinant EphA2Neu Retroviral Producer Cells

In order to create a retroviral vector construct expressing EphA2Neu, the cDNA encoding the extracellular and N-terminal transmembrane region of EphA2Neu was subcloned into the retroviral vector pJLR-Δkin, which contains the C-terminal region of the transmembrane domain and the cytoplasmic domain of EphA2 (Section 2.1.1.5) (Figure 5.2). A 1.9 kb *Bgl* II-*Bam*H I fragment was excised from pECE-EphA2Neu and ligated into pJLR-Δkin digested with *Bam*H I and treated with calf intestinal alkaline phosphatase (CIAP) (as described in Section 2.1.5.4).

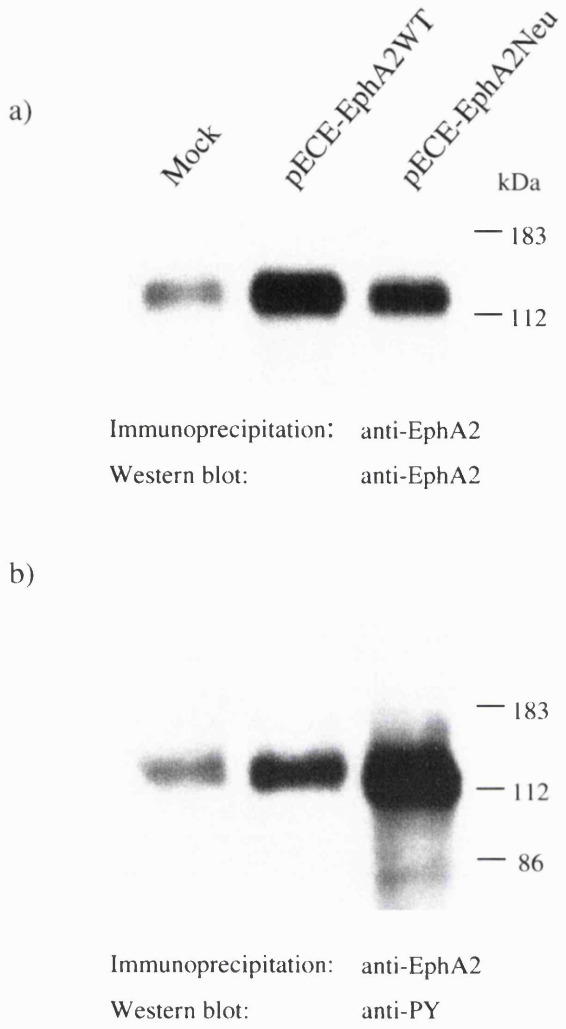


Figure 5.1. EphA2Neu is Constitutively Active
COS-1 cells were transfected with 10 µg of pECE-EphA2WT or pECE-EphA2Neu or mock-transfected using Lipofectin™. Following 16 hours incubation in medium containing 0.5% FCS, EphA2 was immunoprecipitated from RIPA lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine (PY) antibody.

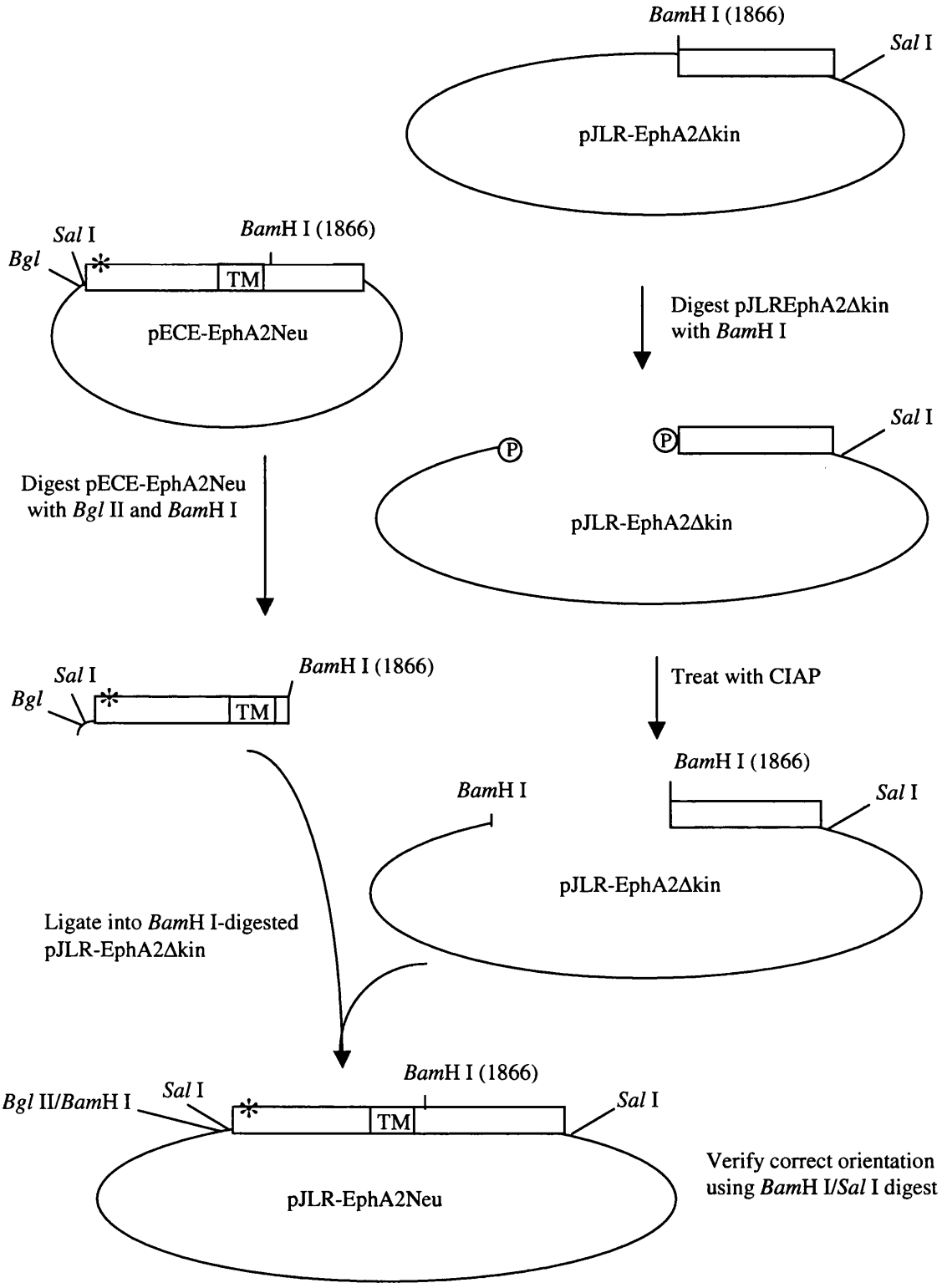


Figure 5.2. Strategy to Subclone the Neu Transmembrane Domain into pJLR-Δkin.
A 1.9 kb fragment was excised from pECE-EphA2Neu using *Bgl* II and *Bam*H I, and ligated into *Bam*H I digested, CIAP-treated pJLR-Δkin. The correct orientation of the 1.9 kb insert was determined by a *Bam*H I-*Sal* I digestion. Asterisks denote the initiating methionine. Numbers in brackets correspond to EphA2 nucleotide sequence according to Appendix 1.

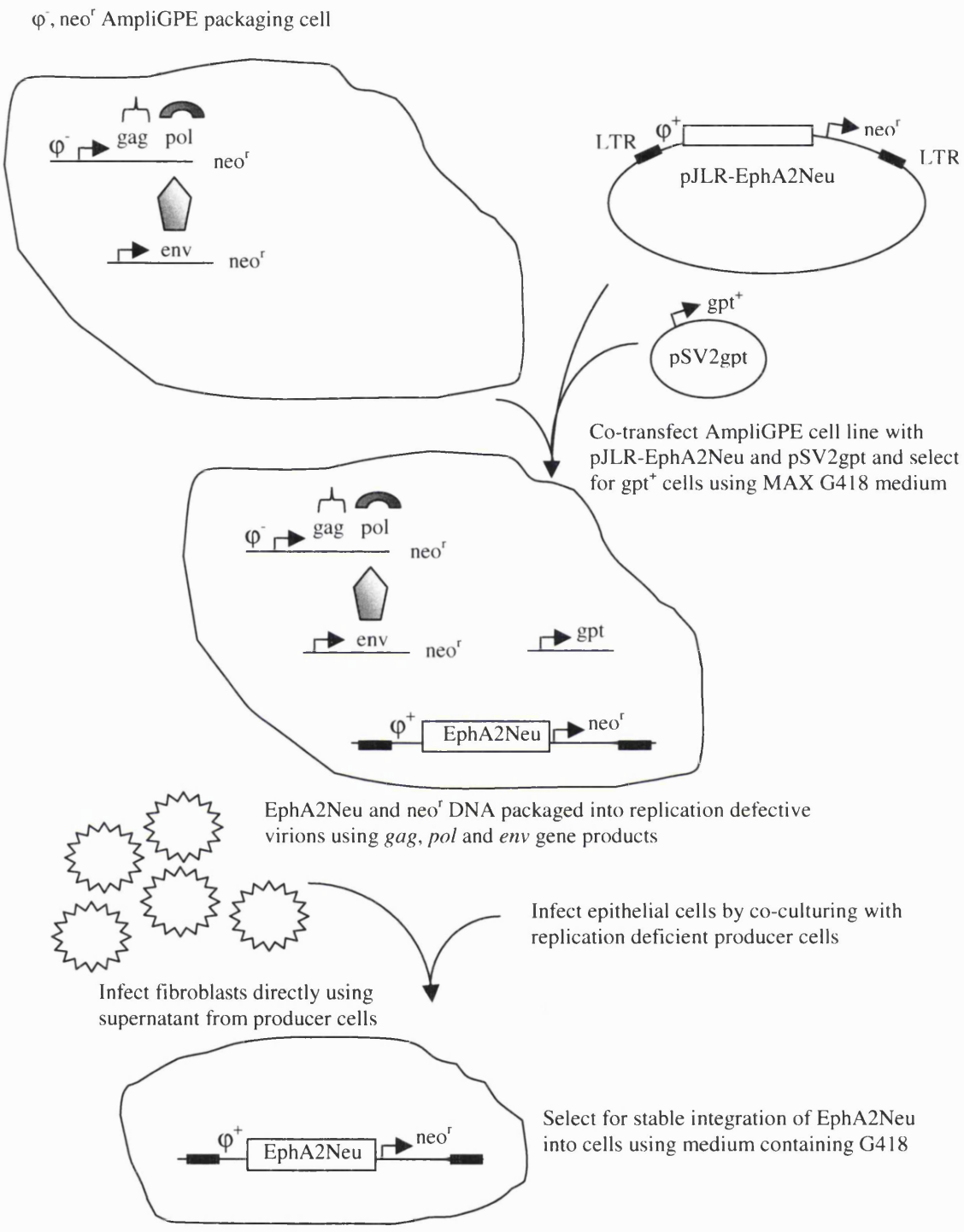


Figure 5.3. Strategy to Retrovirally Infect NIH3T3 Fibroblasts and EpH4 Mammary Epithelial Cells

Packaging deficient (φ⁻) ampliGPE retroviral producer cells were co-transfected with 20 μg of pJLR-EphA2Neu and 2 μg of pSV2gpt using calcium phosphate. Following selection with MAX medium, clones were picked and expanded. Viral titres were assessed by infecting NIH3T3 fibroblasts with the virion-containing supernatant. EpH4 mammary epithelial cells were infected by co-culture.

Verification of the appropriate directional cloning of the *Bgl* II-*Bam*H I fragment into pJLR-Δkin was ascertained by performing a *Bam*H I-*Sal* I restriction digest of pJLR-EphA2Neu (Figure 5.2).

The production of a stable cell line capable of generating retroviral particles enables the subsequent infection of both fibroblasts and epithelial cells, according to Figure 5.3. To create a pJLR-EphA2Neu retroviral producer cell line, ecotropic ampliGPE producer cells were transfected with pJLR-EphA2Neu using calcium phosphate (Section 2.2.5.4). As ampliGPE producer cells were already G418 resistant, pJLR-EphA2Neu was co-transfected with the pSV2gpt plasmid, containing the *gpt* gene, at a ratio of 1:10 (2 μg of pSV2gpt:20 μg of pJLREphA2Neu). This ratio was designed to ensure that cells expressing gpt would also have incorporated the EphA2 retroviral DNA. 48 hours post-transfection, the cells were split 1:10 into MAX medium (Section 2.2.4.5.1) to select for gpt-positive cells (Mulligan and Berg, 1981). Following selection for 10-14 days, individual clones were picked and expanded to duplicate 60 mm dishes. One dish of cells was cryopreserved while the supernatant from the other dish was used to assess the viral titre as described in Section 2.2.4.5.3.

5.2.4. Titration of EphA2Neu Retrovirus on NIH3T3 Cells

Following expansion of individual clones into 60 mm dishes, their virion-containing supernatants were harvested and assayed for their ability to retrovirally infect NIH3T3 cells. NIH3T3 cells were infected with 50 μl of viral supernatant as described in Section 2.2.4.5.3. The following day, cells were split 1:10 and duplicate dishes were selected in medium containing 350 μg/ml G418 for 10-14 days. Dishes were stained with methylene blue (Section 2.2.4.5.4) and viral titres were calculated from the number of G418 resistant colonies (Table 5.1). No G418-resistant cells were observed in the mock-infected cells.

A great deal of variation was observed in the viral titres of the EphA2Neu retroviral producer lines, as seen in Table 5.1. This most likely reflects the genomic position into which the retroviral DNA had integrated. Additionally, it was notable that the supernatants from a number of gpt-positive/G418-resistant clones did not contain detectable levels of infectious viral particles.

Clone number	Average number of colonies/dish	(cfu/ml)
Neu1.42	0	0
Neu1.46	0	0
Neu1.48	0	0
Neu1.49	0	0
Neu1.53	0	0
Neu1.4	0	0
Neu1.7	0	0
Neu1.11	0	0
Neu1.16	0	0
Neu1.20	0.5	1×10^2
Neu1.22	0	0
Neu1.25	5.5	1.1×10^3
Neu1.26	1.5	3×10^2
Neu1.43	0	0
Neu1.44	0.5	1×10^2
Neu1.47	17	3.4×10^3
Neu1.51	0	0
Neu1.53	0	0
Neu1.59	29	5.8×10^3
Neu2.18	0	0
Neu2.24	28	5.6×10^3
Neu2.31	0	0
Neu2.32	0	0
Neu2.33	0	0

Table 5.1. Colony-forming ability of retroviral supernatants from pJLR-EphA2Neu-transfected cells in NIH3T3 cells.

5.2.5. NIH3T3 Fibroblasts Infected with Recombinant EphA2 Retroviruses Overexpress Recombinant EphA2 Protein

Bulk-infected populations of G418 resistant NIH3T3 cells infected with supernatant from the retroviral producer clones Neu1.25, Neu1.47, Neu1.59 and Neu2.24 were subsequently passaged and assayed for both their steady-state and tyrosine phosphorylated levels of recombinant EphA2. These cells were assayed in parallel with naïve NIH3T3 cells and NIH3T3 cells previously stably infected with EphA2WT (Section 2.2.2). All cells were incubated for 16 hours in 0.5% FCS-containing medium to reduce basal phosphorylation levels prior to lysis in RIPA buffer. The EphA2

receptor was then immunoprecipitated from the lysates, and, subsequent to SDS-PAGE, western blotting was performed using an anti-EphA2 antibody and an anti-phosphotyrosine antibody. NIH3T3 cells were not seen to express detectable levels of endogenous EphA2 receptor, and any tyrosine phosphorylation was also undetectable (Figure 5.4). Expression of recombinant EphA2WT resulted in a clear increase in EphA2 protein together with low levels of tyrosine phosphorylation (Figure 5.4). When compared to the EphA2WT-infected NIH3T3 cells, NIH3T3 cells infected using viral supernatants from clones Neu1.47, Neu1.59 and Neu2.24 showed lower steady-state levels of EphA2 protein, with levels below detection in Neu1.25-infected NIH3T3 cells. However, the relative level of tyrosine phosphorylation in NIH3T3 populations infected with Neu1.47, Neu1.59 and Neu2.24 was much higher than that of EphA2WT infected cells. This is consistent with the result previously observed for COS cells transiently transfected with pECE-EphA2WT and pECE-EphA2Neu expression vectors (Figure 5.1).

5.2.6. Retroviral Infection of EpH4 Mammary Epithelial Cells

Cell lines capable of producing recombinant EphA2WT retrovirus had previously been generated (Section 2.2.2). Therefore, having characterised the EphA2Neu-encoding retrovirus in NIH3T3 fibroblasts, EphA2Neu producer lines were used with the previously generated EphA2WT producer cells to infect EpH4 cells. EphA2WT and EphA2Neu retroviral producer lines were titred in parallel in order to identify lines producing equivalent amounts of virus. Fresh viral supernatants were prepared from a number of producer lines and 25 µl was titred on NIH3T3 cells (Table 5.2). In addition, the titre of a control retrovirus, SV2a, produced by the φ2 retroviral producer line (Section 2.2.2) was determined. The producer lines WT43 and Neu1.59 were chosen to infect EpH4 cells, as their titres were similar (cfus of 2.44×10^4 and 2.42×10^4/ml respectively). The control retrovirus, SV2a, gave a titre of approximately twice this amount (5×10^4 cfu/ml).

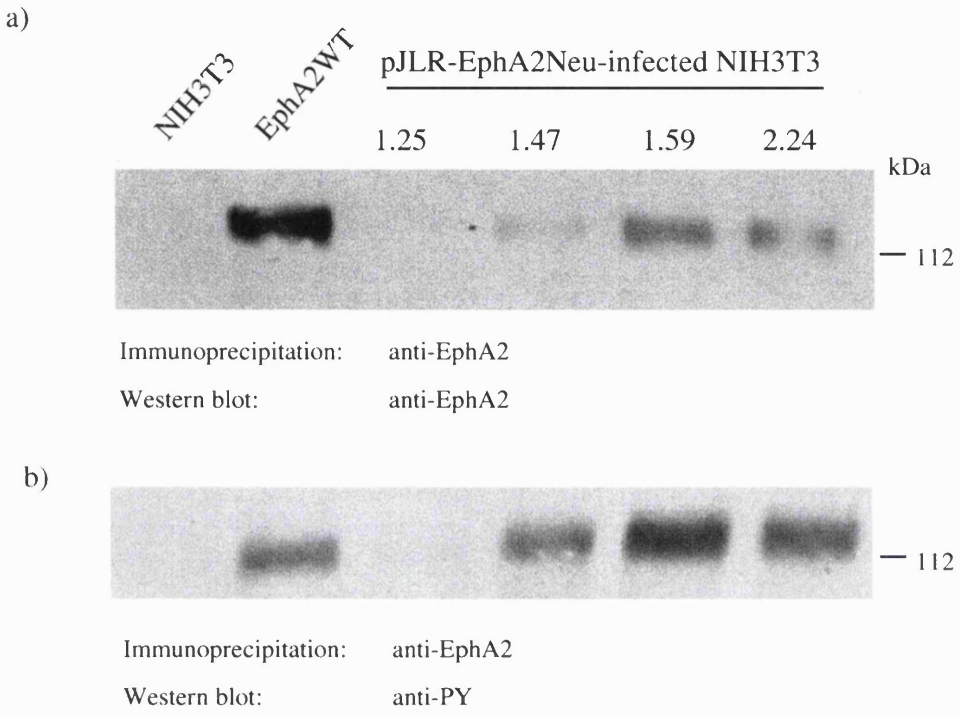


Figure 5.4. NIH3T3 Fibroblasts Infected with Recombinant EphA2 Retroviruses Overexpress Wild-Type and Constitutively Active EphA2
NIH3T3 cells were retrovirally infected with 50 µl of viral supernatant derived from clones Neu1.25, Neu1.47, Neu1.59 and Neu2.24. Following selection, RIPA lysates of bulk populations of G418-resistant cells, uninfected NIH3T3 cells and NIH3T3 cells previously infected with pJLR-EphA2WT were prepared and EphA2 immunoprecipitated using an anti-EphA2 antibody (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine antibody.

Retroviral producer line	Average number of colonies/dish	Viral titre (cfu/ml)
WT43	61	2.44×10^4
WT62	179	7.16×10^4
Neu1.47	42.5	1.7×10^4
Neu1.59	60.5	2.42×10^4
Neu2.24	42.5	1.7×10^4
SV2a	125	5×10^4

Table 5.2. Colony-forming ability of retroviral supernatants from pJLR-EphA2WT-, pJLR-EphA2Neu- and SV2a- transfected cells in NIH3T3 cells

5.2.6.1. Retroviral Infection of EpH4 Cells by Co-Culture

Preliminary experiments suggested that the small volumes of viral supernatants previously used to infect NIH3T3 cells were not sufficient to infect EpH4 cells, therefore retroviral producer lines were co-cultured with EpH4 cells to maximise infection efficiencies. Equal cell numbers of the three producer lines WT43, Neu1.59 and SV2a were seeded onto 15 cm dishes and treated with mitomycin C, as described in Section 2.2.4.5.5. EpH4 cells were then plated onto these retroviral "feeder" layers and the two cell lines co-cultured for 48 hours. The cells were then split 1:10, which served to dilute out the producer cells. Following selection for 10-14 days, it was clear that both the mock-infected EpH4 cells (no producer feeder layer) and the producer feeder cells alone had died. Resistant EpH4 cell colonies were therefore easily distinguishable and present in high numbers. However, during the course of the selection, a number of colonies of a very distinct morphology were observed. Many displayed a "rounded-up" phenotype and appeared to be losing contact with the substratum in a manner distinct from those cells dying through susceptibility to G418 (data not shown). Attempts were made to pick a number of these clones and grow them up to large-scale, but the cells either failed to reattach, or reverted to a more obvious epithelial phenotype.

5.2.7. G418 Resistant EpH4 Cell Populations do not Express Recombinant EphA2

Following the 10-14 day selection, the number of colonies of G418-resistant EpH4 cells remaining on the dishes was counted (Table 5.3). EpH4 cells from the EphA2WT infections gave rise to two and a half times the number of colonies as the EphA2Neu infections, suggesting that infection with EphA2Neu was detrimental to the cells. EpH4 cells infected with the SV2a retrovirus gave rise to the least number of G418 resistant colonies. This may be due to differences in the backbone vectors and/or promoters of the control SV2a retrovirus and the pJLR retrovirus. Alternatively, since the control retrovirus initially gave rise to a two-fold higher viral titre than the recombinant EphA2 retroviruses in NIH3T3 cells, it is possible that a similar higher level of infection in EpH4 cells by the SV2a retrovirus was toxic.

Retroviral vector	Average colony number
SV2a	49.5
pJLREphA2WT	189
pJLREphA2Neu	74.5

Table 5.3. Colony formation in EpH4 cells following co-culture with pJLR-EphA2WT-, pJLR-EphA2Neu- and SV2a- transfected cells

Since a significant number of colonies was attained, whole populations of cells were passaged. An equal number of each retrovirally-infected G418-resistant cell line was seeded and incubated for 16 hours in 0.5% FCS-containing growth medium before being analysed for expression of steady-state and tyrosine phosphorylated EphA2 by immunoprecipitation and western blotting. However, no detectable increase in either steady-state EphA2 protein levels or phosphorylated EphA2 protein could be detected in bulk populations of EpH4 cells retrovirally infected with either recombinant EphA2WT or EphA2Neu (Figure 5.5). Thus, it appeared that although the cells were G418-resistant, the recombinant EphA2 gene was not being expressed at detectable levels.

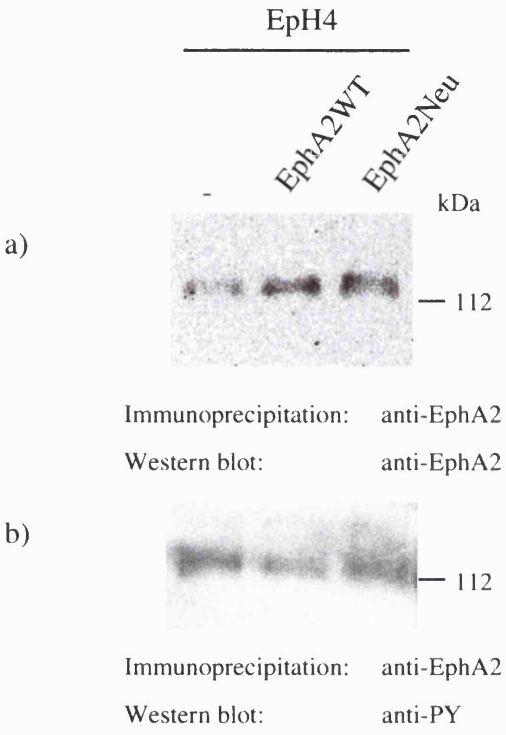


Figure 5.5. EpH4 cells Retrovirally Infected with Recombinant EphA2 Retroviruses do not Express Detectable Recombinant EphA2 Protein

EpH4 cells were retrovirally infected by co-culture with the retroviral producer lines WT43 and Neu1.59. RIPA lysates of bulk populations of G418-resistant cells and uninfected EpH4 cells were prepared and EphA2 immunoprecipitated using an anti-EphA2 antibody (Santa Cruz). Immunoprecipitates were subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine antibody.

5.2.8. Inducible Expression of Recombinant EphA2

To test the hypothesis that the failure to generate EphA2-overexpressing EpH4 cells was a direct consequence of EphA2 expression, an inducible expression system was utilised. This approach would facilitate the generation of stable cell lines as recombinant EphA2 expression should be regulatable, thus enabling expression to be repressed during the generation of the lines. One of the most recent inducible systems comprises two vectors, pIND and pVgRXR (see Figure 5.6), and is based on the ability of the insect steroid moulting hormone, 20-hydroxy-ecdysone, to alter gene expression during *Drosophila* metamorphosis (No *et al.*, 1996). Addition of an ecdysone analogue, ponasterone, induces the formation of a heterodimer comprising the mammalian retinoid X receptor and the *Drosophila* ecdysone receptor. Only as a heterodimer are the DNA binding domains of both receptors able to bind to hormone response elements to drive expression of the gene of interest (see Figure 5.6). Thus, gene expression only occurs upon addition of ponasterone.

5.2.8.1. Subcloning of EphA2WT and EphA2Neu into the Inducible Vector, pIND

A 3.4 kb *Eco*RI fragment from pJLR-EphA2WT or pJLR-EphA2Neu encoding the whole open reading frame of EphA2 was ligated into the *Eco*RI digested and CIAP-treated pIND vector. Verification of the appropriate directional cloning of the *Eco*RI fragment into pIND was ascertained by performing a *Kpn* I digest.

5.2.9. Characterisation of Ecdysone-Inducible System in COS Cell Transient Transfections

COS-7 cells were co-transfected by electroporation with 10 μg of pVgRXR and either 10 μg of pIND-EphA2WT or pIND-EphA2Neu. 24 hours later the cells were given fresh medium containing ponasterone at a final concentration of 5 μM, or an equivalent volume of 100% ethanol for mock inductions, and the cells were incubated for a further 20 hours. EphA2 was then immunoprecipitated from RIPA lysates using an anti-EphA2 antibody, and samples were subjected to SDS-PAGE and western blotting using an anti-EphA2 antibody and an anti-phosphotyrosine antibody.

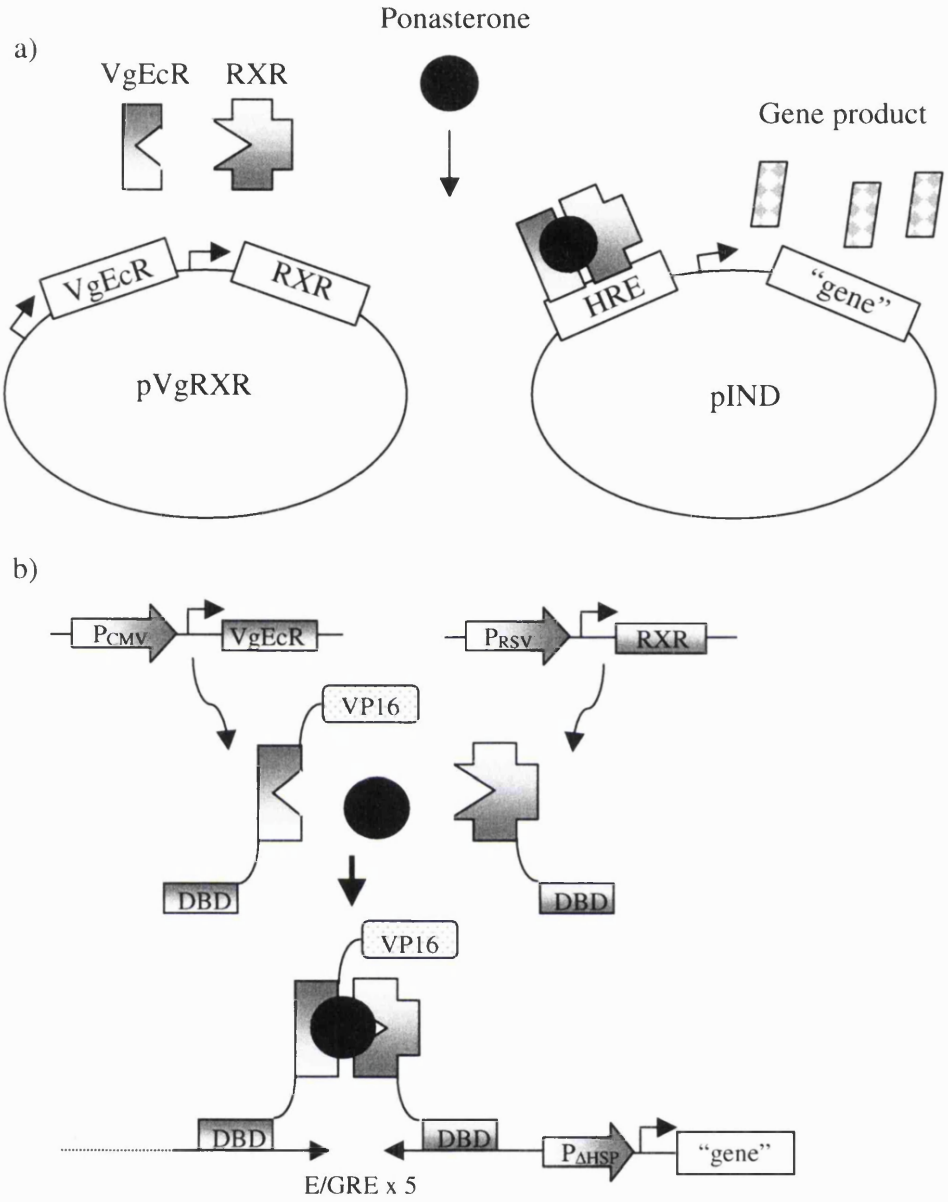


Figure 5.6. Diagrammatic Representation of the Ecdysone-Inducible Mammalian Expression System

(a) The ecdysone system comprises two vectors. The first vector encodes the mammalian retinoid X receptor, RXR and the *Drosophila* ecdysone receptor, VgEcR. The second vector consists of modified hormone response elements (HRE) to which a heterodimer of RXR and VgEcR can bind in the presence of the synthetic ecdysone analogue, ponasterone, to drive expression of the gene of interest.

(b) Among other modifications, the DNA binding specificity of VgEcR has been modified, in conjunction with the response elements of the second vector, to minimise the possibility of any known mammalian transcription factors binding to the response elements of the second vector. The second vector contains 5 hybrid response elements (E/GRE) downstream of the *Drosophila* minimal heat shock promoter. Ponasterone-mediated stabilisation of the RXR-VgEcR heterodimer brings their DNA-binding domains into contact with the E/GREs in the correct orientation. Transactivation of the heat shock promoter via VP16 results in transcription of the gene of interest.

Addition of 5 μM ponasterone for 20 hours resulted in a clear induction of recombinant EphA2 protein in cells transfected with either pIND-EphA2WT and EphA2Neu, whereas expression of recombinant EphA2 was not induced by the addition of 100% ethanol (Figure 5.7). As previously observed (Figure 5.1), mock-transfected COS cells expressed a basal level of endogenous EphA2, which did not display any detectable tyrosine phosphorylation. In contrast, recombinant EphA2 from COS cells transfected with either pIND-EphA2WT or pIND-EphA2Neu showed high levels of tyrosine phosphorylation in the presence of ponasterone.

Furthermore, in accordance with previous observations (Figure 5.1), steady-state levels of EphA2 protein in EphA2Neu transfectants were lower than those of EphA2WT transfectants. The significant levels of tyrosine phosphorylated EphA2 observed in pIND-EphA2WT transfectants was most likely due to the high steady-state levels of EphA2 receptor protein induced by ponasterone, giving rise to high levels of receptor autophosphorylation by spontaneous dimerisation (Lemmon and Schlessinger, 1994).

5.2.10. Optimisation of Transfection of Mammary Epithelial Cells

Although retroviral infection has been demonstrated to be an efficient means of introducing recombinant DNA into cells, the time-scale for creating new retroviral producer lines comprising the two-component system required for regulation of inducible systems prompted the decision to investigate alternative methods of transfection. To this end, transfections mediated by Lipofectin™, calcium phosphate and electroporation were carried out in COS cells, EpH4 cells and an additional mouse mammary epithelial cell line, HC11, to compare the relative efficiencies of each method. All cell types were transfected with 10 μg of pcDNA3.1/Myc-His(+)/*lacZ* by all three methods according to Section 2.2.5. 24 and 48 hours post-transfection, cell monolayers were stained with X-gal to assess the levels of β-galactosidase activity. In all cases, expression levels were higher in the 48 hour samples, therefore only this data is presented. The efficiency of transfection was seen to vary for all three cell lines depending on the method used (Figure 5.8). COS cells were most efficiently transfected, whereas both mammary epithelial cell lines displayed a lower transfection efficiency. However, electroporation appeared to be the most efficient of the three methods to transfect both EpH4 and HC11 cells.

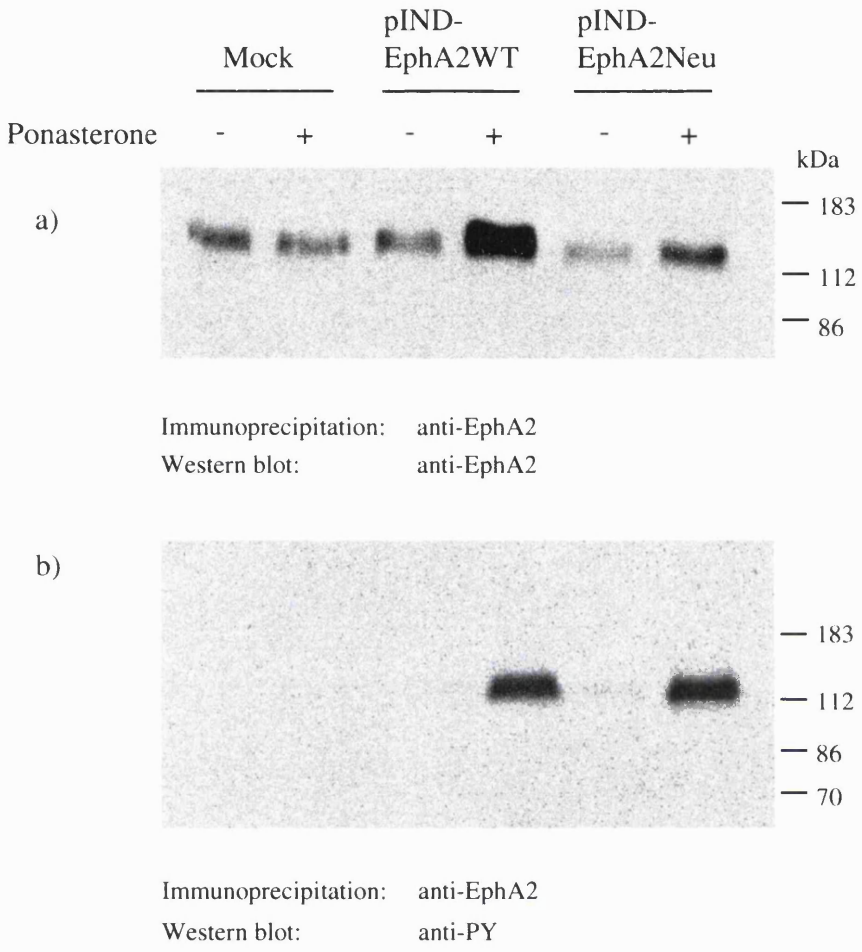


Figure 5.7. Induction of Recombinant EphA2 by Ponasterone in COS Cell Transient Transfections

COS-7 cells were co-transfected with 10 µg of pVgRXR and either 10 µg of pIND-EphA2WT or pIND-EphA2Neu or mock transfected. After 24 hours, ponasterone was added at 5 µM. Following a further 20 hour incubation, EphA2 was immunoprecipitated from RIPA lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI), or (b) an anti-phosphotyrosine antibody.

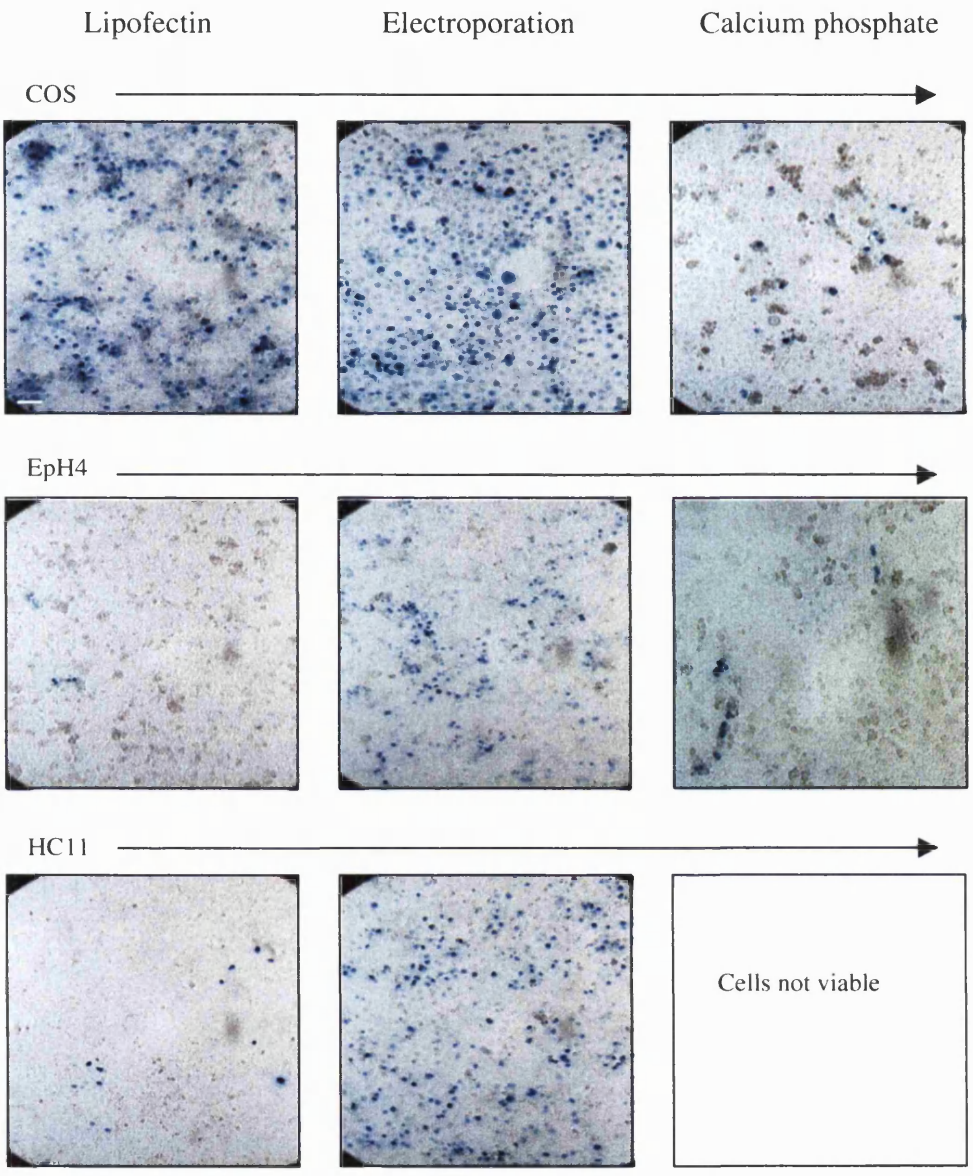


Figure 5.8. Comparison of Three Methods of Transient Transfection in COS, EpH4 and HC11 Cells

COS-1, EpH4 and HC11 cells were transiently transfected with 10 µg of pcDNA3.1/Myc-His(+)/*lacZ* by Lipofectin™, electroporation or calcium phosphate. After 48 hours, cells were stained for β-galactosidase activity using X-gal staining buffer. Images were taken using the Leica DC Viewer software package. Scale bar represents 100 µm.

5.2.11. Characterisation of Ecdysone-Inducible System in Mammary Epithelial Cells

Having characterised the ecdysone-inducible system in transient COS cell transfections, it was then necessary to investigate whether the system would also operate in other cell lines, including EpH4 cells. In addition, it was important to assess the integrity of the ecdysone-inducible system, in terms of "leakiness" of protein expression in the absence of ponasterone, before embarking on the generation of a stable inducible system.

To this end, transfection studies were carried out using EpH4 cells, HC11 mammary epithelial cells and MCF-7 mammary adenocarcinoma cells, using the pIND vector containing green fluorescent protein (GFP), pIND-GFP. Both EpH4 and HC11 cells were transfected by electroporation with either pIND-GFP or pVgRXR, or both pVgRXR and pIND-GFP. MCF-7 cells already stably transfected with pVgRXR (MCF-7-pVgRXR) (Section 2.2.2) were either mock transfected, or transfected with pIND-GFP only. 24 hours post-transfection, cells were incubated with either 5 μM ponasterone, or an identical volume of 100% ethanol, for 20 hours.

Upon the addition of ponasterone, but not 100% ethanol, to HC11 cells and MCF-7 cells transfected with both pVgRXR and pIND-GFP, a clear induction of GFP expression was observed (Figure 5.9). No GFP expression was observed in cells transfected with either the pVgRXR vector or the pIND-GFP vector alone (data not shown). However, in EpH4 cells a significant level of GFP expression was observed both in the absence of ponasterone and in the absence of the pVgRXR vector, suggesting that expression of pIND-GFP could be induced by factors present within the cell or in the medium. A constant leakiness of EphA2 protein expression (equivalent to higher levels of endogenous expression) could influence cell behaviour prior to the addition of ponasterone, therefore providing no advantage over the constitutive expression system previously used. The HC11 and MCF-7 cell lines, which demonstrated a tight regulation of pIND-GFP expression in response to ponasterone, were therefore chosen for further studies. Unfortunately, however, attempts to select for the presence of the pVgRXR vector in HC11 cells using Zeocin™ were unsuccessful due to the inefficient mode of action of this antibiotic, therefore further studies using this cell line were not undertaken.

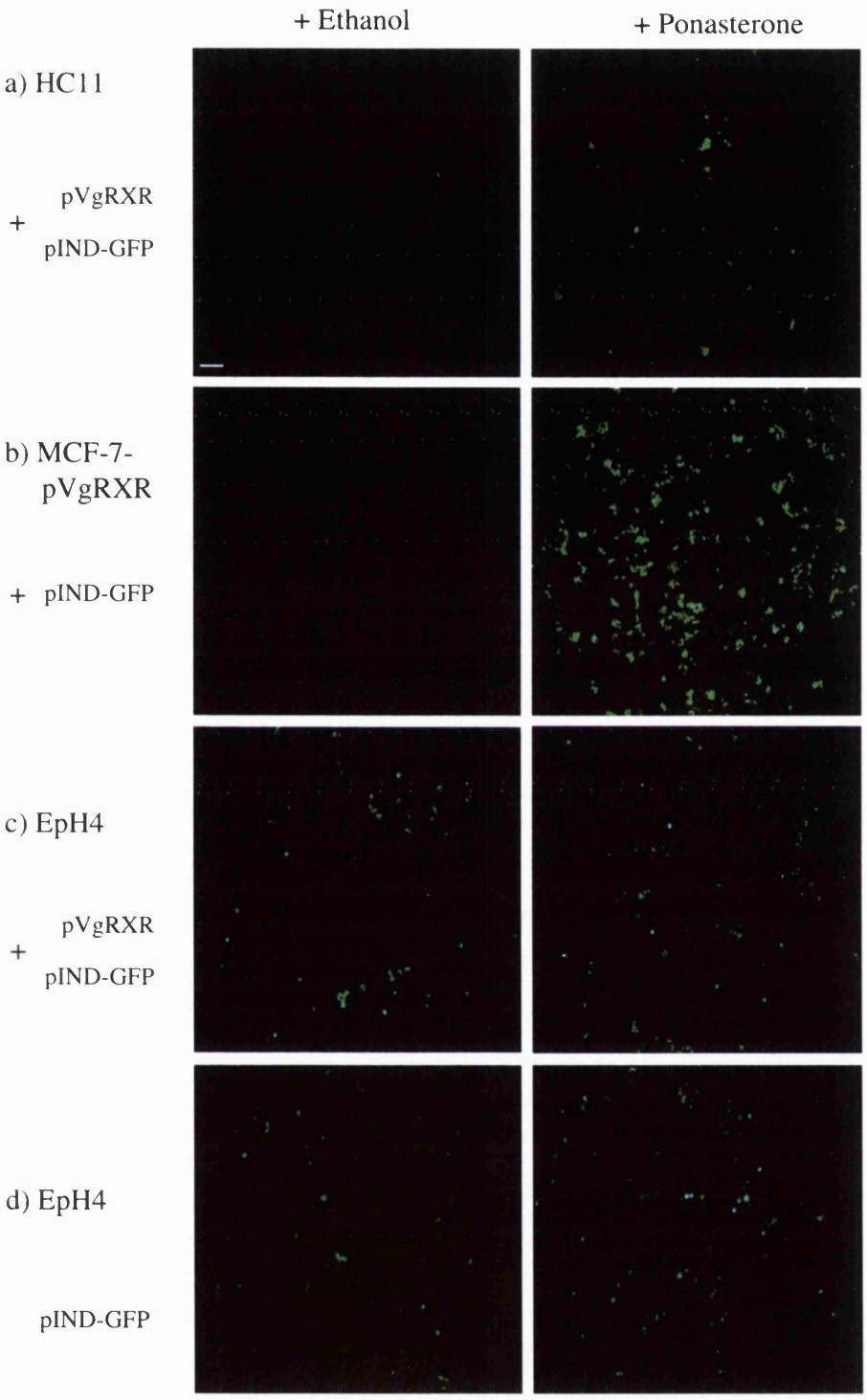


Figure 5.9. Characterisation of the Ecdysone-Inducible Expression System in EpH4, HC11 and MCF-7 Cells

EpH4, HC11 and MCF-7 cells were transfected by electroporation with 10 µg of pVgRXR or 10 µg pIND-GFP, or both vectors together. After 48 hours, cells were incubated for a further 20 hours in medium containing either 5 µM ponasterone or an equivalent volume of 100% ethanol. GFP expression was assessed using fluorescence microscopy and images were taken using the Leica DC Viewer software package. (a), (b) and (c) correspond to GFP expression in cells transfected with both vectors, (d) represents GFP expression in EpH4 cells transfected with pIND-GFP alone. Scale bar represents 100 µm.

5.2.12. Generation of Stable MCF-7 cells Inducibly Expressing Recombinant EphA2

Since an MCF-7 cell line into which the pVgRXR vector had already been stably transfected (MCF-7-pVgRXR) was available, this was used to create a stable cell line expressing inducible, recombinant EphA2. MCF-7-pVgRXR was transfected with pIND-EphA2WT, or the pIND vector as a control, by electroporation. 24 hours later, cells were seeded sparsely (1 x 10^5 cells/dish) in 15 cm dishes and selected for neomycin resistance using 350 μg/ml G418. After 10-14 days, well-separated G418-resistant clones were picked and expanded. No G418 resistant colonies were observed in the mock-transfectants. Duplicate dishes of each cell clone were treated with either 5 μM ponasterone or an equal volume of 100% ethanol for 20 hours, whole cell lysates were prepared, and EphA2WT positive clones were identified by SDS-PAGE and western blotting with an anti-EphA2 antibody. Several clones were identified that expressed recombinant EphA2 protein to varying levels upon induction with ponasterone but not with 100% ethanol (data not shown). Clones pIND-EphA2WT #3 and #17, which were transfected with pIND-EphA2WT,and pIND #1, transfected with the empty pIND vector, were selected for further analysis.

5.2.13. Time- and Dose- Dependent Induction of Recombinant EphA2 in MCF-7 Cells

In order to characterise further the induction of recombinant EphA2, the effect of varying the time of induction and the concentration of ponasterone was assessed. Equal numbers of cells from pIND-EphA2WT clones #3 and #17, and pIND clone #1 were seeded and incubated for 48 hours. Subsequent to the addition of 5 μM ponasterone, cells were incubated for a further 0, 2, 4, 6, 8, 16 or 24 hours and whole cell lyates were then prepared. Samples were subjected to SDS-PAGE and then western blotted with an anti-EphA2 antibody. Increased EphA2 protein levels were detectable in pIND-EphA2WT clone #3 after 4 hours, and continued to increase over the 24 hour period (Figure 5.10). An increase in EphA2 protein levels could already be detected in pIND-EphA2WT clone #17 after addition of ponasterone for 2 hours, and the high steady-state levels of EphA2 protein could not be further induced after 8 hours. Interestingly, an anti-EphA2 immunoreactive protein of approximately 50 kDa was also observed in cell lysates of both pIND-EphA2WT clones #3 and #17 treated for over 16 hours with ponasterone. This most likely represents a degradation product corresponding to the

cytoplasmic domain of EphA2. In contrast, no induction of endogenous EphA2 was observed upon treatment of pIND clone #1 with ponasterone over the 24 hour period.

In addition, levels of recombinant EphA2 protein in MCF-7 cells stably expressing pIND-EphA2WT could be increased by an increasing concentration of ponasterone. Equal numbers of cells were seeded and incubated for 48 hours before increasing concentrations of ponasterone from 0.1 μM to 5 μM were added for 20 hours. EphA2 was then immunoprecipitated from cell lysates using an anti-EphA2 antibody, and immunoprecipitates were then subjected to SDS-PAGE and western blotting with an anti-EphA2 antibody. The levels of recombinant EphA2 protein increased in pIND-EphA2WT clone #3 treated with concentrations of ponasterone above 0.5 μM, whereas concentrations below 0.5 μM had no detectable effect on EphA2 protein levels (Figure 5.11a). No increase in EphA2 protein above basal endogenous levels was seen in clone pIND #1. The high steady-state levels of EphA2 protein attained by induction of pIND-EphA2WT clone #3 for 20 hours with 5 μM ponasterone were thought likely to induce a degree of ligand-independent receptor autophosphorylation by spontaneous dimerisation, therefore the level of tyrosine phosphorylated EphA2 was measured by western blot analysis of EphA2 immunoprecipitates with an anti-phosphotyrosine antibody. Treatment of pIND-EphA2WT clone #17 with ponasterone at 5 or 10 μM for 20 hours did indeed give rise to significant amounts of tyrosine phosphorylated EphA2 (Figure 5.11b). Interestingly, the increase in tyrosine phosphorylation coincided with the appearance of a 50 kDa EphA2 immunoreactive protein.

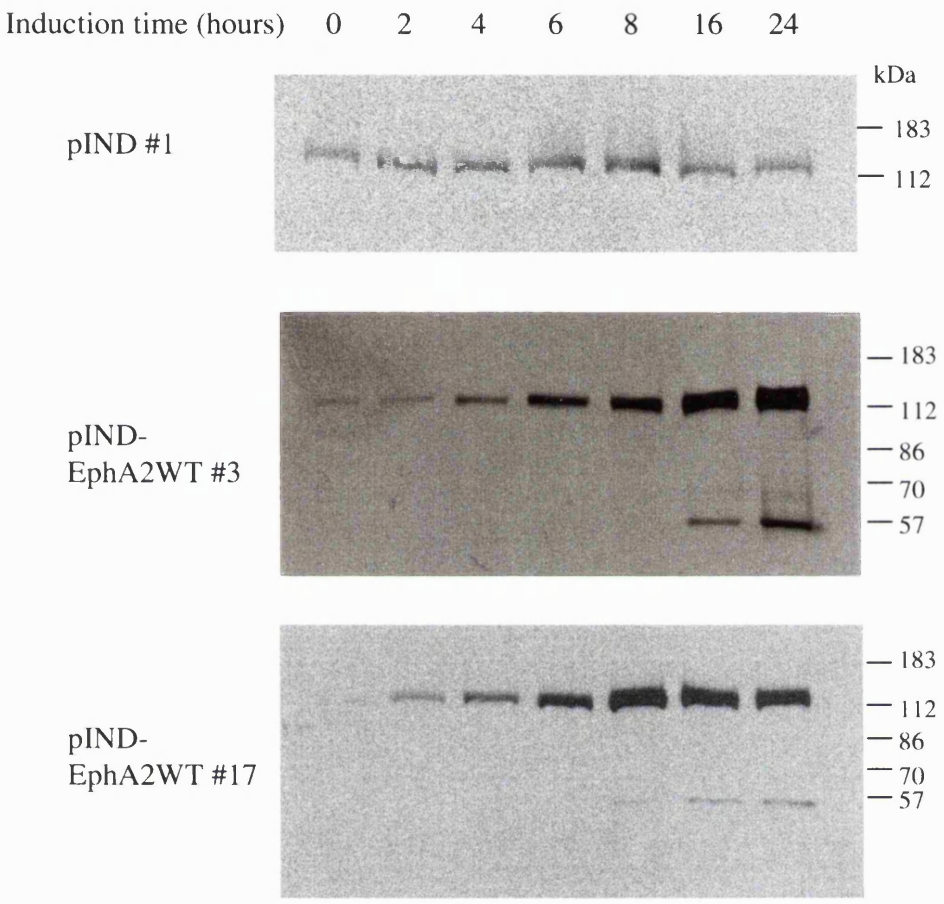


Figure 5.10. Time-Dependent Induction of Recombinant EphA2WT in MCF-7 Cells
MCF-7 cells stably co-transfected with VgRXR and either pIND (clone #1) or pIND-EphA2WT (clone #3 and clone #17) were incubated in 5 μM ponasterone for 0, 2, 4, 8, 16 and 24 hours. 25 μg of whole cell lysate was subjected to SDS-PAGE and western blotting using an anti-EphA2 antibody (UBI).

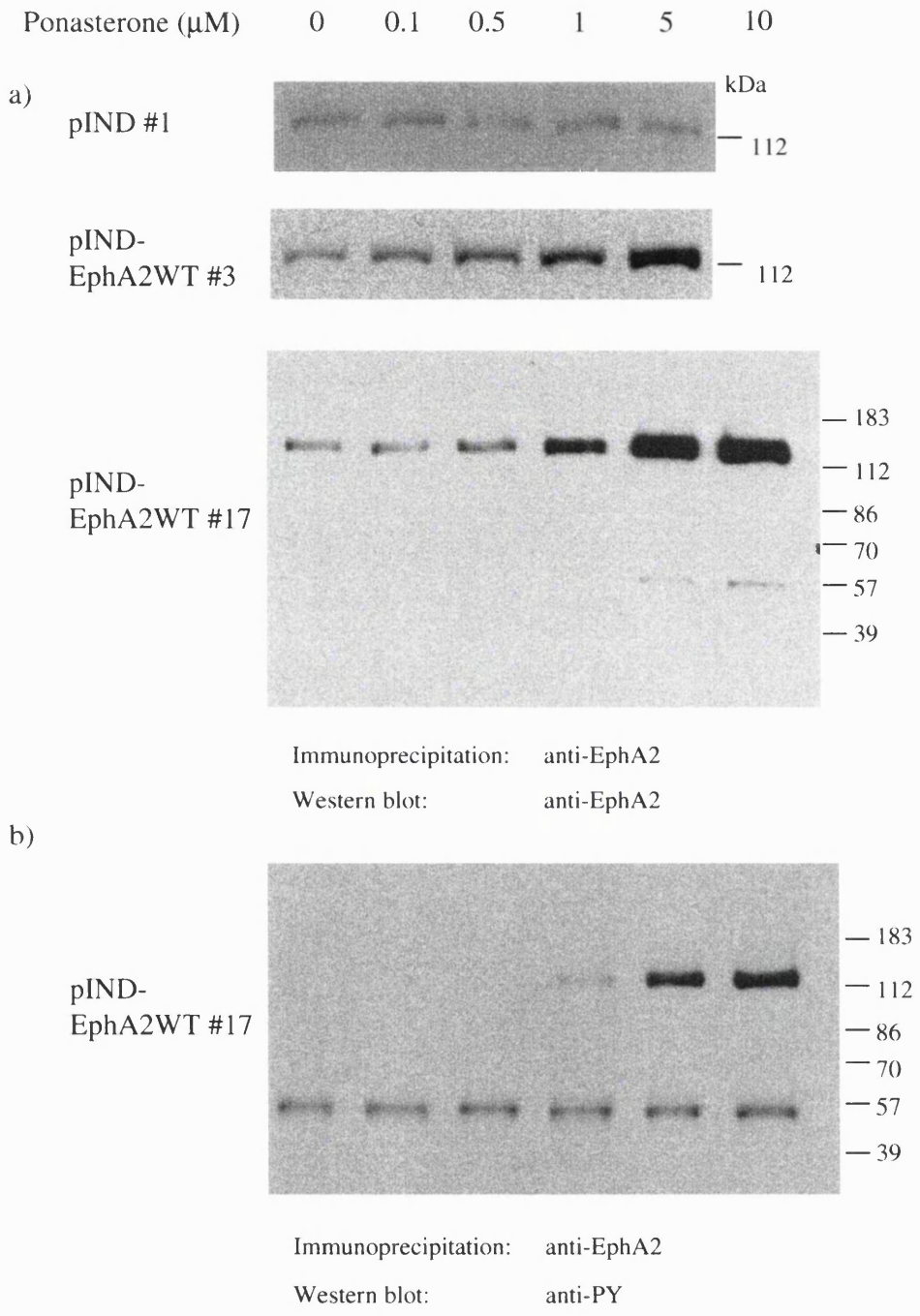


Figure 5.11. Concentration-Dependent Induction of Recombinant EphA2WT in MCF-7 Cells

MCF-7 cells stably co-transfected with VgRXR and either pIND (clone #1) or pIND-EphA2WT (clone #3 and clone #17) were incubated for 20 hours in medium containing 0, 0.1, 0.5, 1 or 5 μM ponasterone (pIND #1 and pIND-EphA2WT clone #3) or 0, 0.1, 0.5, 1, 5 or 10 μM ponasterone (pIND-EphA2WT #17). EphA2 was immunoprecipitated from RIPA lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI), or (b) an anti-phosphotyrosine antibody.

5.2.14. Overexpression of Recombinant EphA2 Retards MCF-7 Cell Growth

While characterising the time- and dose-dependent induction of recombinant EphA2 expression in MCF-7 cells, it was noticed that ponasterone-induced cells from pIND-EphA2WT clone #3 and pIND-EphA2WT clone #17 grew at a slower rate than cells from pIND clone #1. Therefore, cells from pIND clone #1, pIND-EphA2WT clone #3 and pIND-EphA2WT clone #17 were plated at equal densities and incubated for 16 hours prior to the addition of either 5 μM ponasterone or an equal volume of 100% ethanol for 24 hours. Cells were fixed, permeabilised and stained with DAPI to visualise the cell nuclei and thus facilitate cell counting. Preliminary results from 2-3 independent experiments in which DAPI-stained cells were counted suggest that cell growth was impeded upon induction of recombinant EphA2WT protein expression (Figure 5.12). Addition of ponasterone to pIND clone#1 had no effect on the growth rate of the cells.

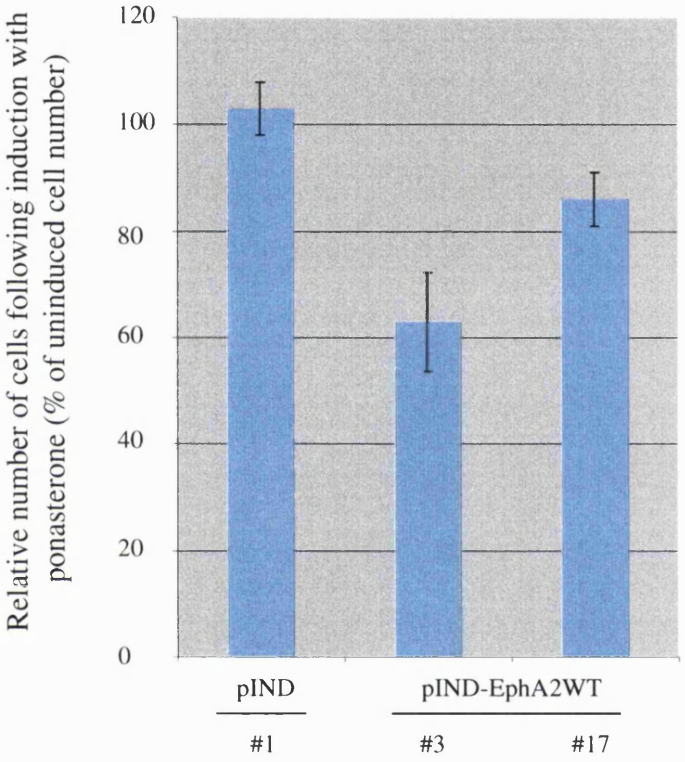


Figure 5.12. Overexpression of Recombinant EphA2WT Inhibits Cell Growth
MCF-7 cells stably co-transfected with VgRXR and either pIND (clone #1) or pIND-EphA2WT (clone #3 and clone #17) were incubated for 24 hours in medium containing 5 μM ponasterone or an equal volume of 100% ethanol. Cells were fixed, permeabilised and stained with DAPI. Cells were viewed by fluorescence microscopy and the number of cells in four random fields counted. The average number of cells for each clone in the presence and absence of ponasterone induction was determined. The average number of ponasterone induced cells was expressed as a percentage of the average number of uninduced cells. Error bars represent standard error of the mean percentage of cells in 2-3 separate experiments.

5.3. Discussion

The work described in this chapter has made use of a number of techniques in order to successfully obtain a mammalian mammary cell line stably overexpressing recombinant EphA2. During the course of this work, a number of significant observations have been made that may have implications in the regulation and function of EphA2.

5.3.1. Steady-State Levels of Active EphA2 Protein

Transient transfection of COS cells with the mammalian expression vectors pECE-EphA2WT and pECE-EphA2Neu demonstrated that these plasmids were able to encode functional, recombinant EphA2 protein. A significant increase in the level of recombinant EphA2WT protein resulted in a modest increase in phosphotyrosine levels, consistent with the formation of an increased number of stable receptor dimers (Lemmon and Schlessinger, 1994). Transfection of constitutively active recombinant EphA2 (EphA2Neu) into COS cells resulted in only a modest increase in the steady-state level of EphA2 protein. This level was above that of endogenous EphA2 expression, but below that occurring as a result of the expression of recombinant EphA2WT. However, the level of tyrosine phosphorylated EphA2 was significantly higher in cells transfected with EphA2Neu than in cells expressing either endogenous EphA2 or recombinant EphA2WT. This phenomenon was repeatedly observed in different cell types under different conditions. In NIH3T3 fibroblasts, retroviral infection with EphA2WT resulted in higher steady-state levels of recombinant EphA2 protein and a modest increase in tyrosine phosphorylated EphA2. NIH3T3 cells infected with EphA2Neu displayed lower steady-state EphA2 protein levels than the EphA2WT-infected cells, but comparatively higher levels of tyrosine phosphorylation (although recombinant EphA2 failed to be expressed in the case of cells infected with supernatant from clone Neu1.25). Similarly, in COS cells transfected with pIND-EphA2Neu and induced with ponasterone, the steady-state levels of recombinant EphA2 were lower than those observed in ponasterone-treated cells transfected with pIND-EphA2WT. However, pIND-EphA2WT-transfected cells had a similar level of EphA2 tyrosine phosphorylation to cells transfected with pIND-EphA2Neu. This was most likely a consequence of the very high level of recombinant EphA2 protein favouring the formation of stable receptor dimers. Induction of MCF-7 cells stably expressing pIND-EphA2WT with high concentrations of ponasterone was also seen to induce high

expression levels of recombinant EphA2 protein and this resulted in the protein being significantly tyrosine phosphorylated.

Thus, introduction of recombinant EphA2WT into cells has been demonstrated to increase the steady-state levels of EphA2 protein, while the amount of tyrosine phosphorylation is dependent on the level of overexpression. A higher level of receptor at the membrane is likely to result in increased formation of stable dimers and consequently autophosphorylation (Lemmon and Schlessinger, 1994). Meanwhile, expression of EphA2Neu markedly increased the phosphotyrosine content of the recombinant EphA2 receptor, while steady-state levels were seen to be more modest. This is consistent with the model of increased dimerisation of the oncogenic Neu protein (Weiner *et al.*, 1989). The decrease in steady-state EphA2 levels in EphA2Neu transfectants is most likely due to an increase in the turnover rate of phosphorylated dimers, as has previously been documented for the oncogenic Neu protein, which has a significantly shorter half-life than its proto-oncogenic counterpart (Stern *et al.*, 1988). However, it cannot be ruled out that the presence of the Neu transmembrane domain had a direct effect on EphA2 protein levels. In a number of cases EphA2 receptor autophosphorylation was coincident with the appearance of a 50 kDa EphA2-immunoreactive protein. This most likely represents a breakdown product corresponding to the cytoplasmic domain of the EphA2 receptor, and will be discussed in more detail in Section 7.3.1.

5.3.2. Phenotypic Consequences of Expressing Recombinant EphA2

Retroviral infection of NIH3T3 cells with EphA2WT and EphA2Neu resulted in the generation of a number of G418-resistant bulk populations of cells that expressed recombinant EphA2 protein (with the exception of Neu1.25) and displayed a spindly morphology. However, retroviral infection of EpH4 cells with EphA2WT and EphA2Neu failed to produce bulk populations of cells that overexpressed either form of recombinant EphA2, despite the presence of a significant number of G418-resistant colonies. Although it remains feasible that recombinant EphA2 was expressed at low levels, it would have been useful to verify the presence of the retroviral DNA by Southern blotting. However, while this would have confirmed the presence and integrity of the retroviral DNA, it would not have excluded the possibility that expression of the retroviral DNA was transcriptionally regulated so that only the G418 resistance gene was expressed. Additionally, to distinguish complete failure to express

the recombinant retroviral constructs from low-level expression, it would have been useful to use a cell line in which EphA2 is not expressed. However, since EphA2 expression has been reported in a wide variety of cell types (Lindberg and Hunter, 1990; Andres *et al.*, 1994; Ganju *et al.*, 1994; Dodge-Zantek *et al.*, 1999; Walker-Daniels *et al.*, 1999; Miao *et al.*, 2000), and a source of mammary epithelial cells from EphA2 knockout mice was not available, this was not feasible. In addition, the control retrovirus should ideally have comprised the pJLR retroviral vector containing an easily identifiable but "neutral" gene, such as GFP or lacZ, rather than the SV2a retrovirus, but due to the length of time required to make the recombinant retrovirus and generate retroviral producer lines, this was also not feasible.

Although it cannot be ruled out that technical problems were responsible for the lack of success in overexpressing recombinant EphA2 in EpH4 cells, the observation that a number of G418 resistant colonies "rounded up" and detached from the substrate during antibiotic selection suggested that cells remaining on the dish after G418 selection may have evolved the ability to suppress the expression of recombinant EphA2, while those that had not succumbed to the consequences of expressing EphA2 above a certain threshold. This implies that, under normal conditions, EpH4 cells possess a means of regulating the expression of EphA2 at a certain level, depending on a number of internal and external factors, otherwise the consequences are detrimental to the cell. Interestingly, one of the known substrates for EphA2, SLAP (Pandey *et al.*, 1995a), when overexpressed in fibroblasts, was found to negatively regulate mitogenesis (Roche *et al.*, 1998). However, the observation that expression of recombinant EphA2 induced changes in cell morphology in NIH3T3 cells and did not appear to inhibit mitogenesis, and that a number of EpH4 cells lost contact with their substratum during the selection procedure implied a distinct mode of action for EphA2 activation.

Consistent with the observations of EpH4 cell rounding and detachment from the substratum, members of the Eph receptor family are implicated in regulating signalling pathways that control changes in cell shape and cell adhesion (see Section 1.1.5). Many groups have observed a decrease in cell-substrate attachment in response to Eph receptor activation (Section 1.1.5.2). Significantly, activation of EphA2 using cross-linking antibodies resulted in the loss of cell-ECM attachment in neoplastic mammary epithelial cells (Dodge-Zantek *et al.*, 1999). Similarly, activation of EphA2 in PC-3 prostate epithelial cells by ephrinA1Fc resulted in cell rounding and a transient

detachment from the substratum (Miao *et al.*, 2000). The morphology of these cells was reminiscent of some of the colonies of retrovirally infected EpH4 epithelial cells seen rounding up during selection. Direct evidence that EphA2 inhibits integrin function was also provided by the studies of Miao and colleagues (Miao *et al.*, 2000) who demonstrated that ligand induced activation of EphA2 inhibited PC-3 cell adhesion to the integrin ligands fibronectin and laminin but had no effect on cell adhesion to poly-L-lysine. The same group implicated FAK in the ephrinA1Fc-induced changes in PC-3 cell morphology (Miao *et al.*, 2000). In unstimulated PC-3 cells, EphA2 and tyrosine phosphorylated FAK were found to co-immunoprecipitate (Miao *et al.*, 2000). However, within 30 seconds of ephrinA1Fc addition, the tyrosine phosphatase Shp2 was recruited to EphA2, dephosphorylation of FAK occurred and, concomitantly, the EphA2-FAK complex was disrupted. Cell adhesion is adversely affected by a number of different agents that cause FAK dephosphorylation (Schwartz *et al.*, 1995). Similarly, embryonic fibroblasts isolated from $FAK^{-/-}$ null animals show a reduced ability to spread (Ilic *et al.*, 1995), and expression of dominant negative FAK impeded cell spreading and migration (Zhao *et al.*, 1998), whereas expression of constitutively active FAK promoted cell spreading (Frisch *et al.*, 1996) and cell migration (Cary *et al.*, 1996).

Thus, the failure to generate EphA2-overexpressing EpH4 cells may be a consequence of these results – chronic EphA2 activation caused inhibition of integrin function resulting in cell rounding and, ultimately, detachment of the cells from the substatum. Only cells that were able to suppress the expression of EphA2 above a certain threshold were able to survive. Discrepancies in the behaviour between the PC-3 and EpH4 cells with respect to complete detachment may be due to several reasons. There may be differences in the extent of the detachment response of the 2 cell types, with the EpH4 cells being more sensitive to a loss of ECM attachment than the PC-3 cells. The transient nature of the cell detachment response in PC-3 cells may have been due to ligand-induced receptor desensitisation, whereas continual overexpression of recombinant EphA2 receptor in the EpH4 cells would prevent such an occurrence. Furthermore, mammary epithelial cells are dependent on ECM-derived signals for survival (see Section 1.3.4.3; Streuli and Gilmore, 1999), and are therefore extremely sensitive to anoikis, a form of apoptosis induced by the disruption of the interaction between cells and their extracellular matrix (Frisch and Francis, 1994). There is a growing body of evidence implicating FAK in the process of anoikis due to its role in

cell spreading and its correlation with anchorage-dependent growth. Activation of FAK by membrane-targeting reportedly confers resistance to anoikis in Madin Darby canine kidney (MDCK) cells (Frisch *et al.*, 1996) whereas injection of embryonic fibroblasts with FAK antagonists induced apoptosis (Hungerford *et al.*, 1996). In cells attached to the ECM, integrin clustering results in an increase in FAK tyrosine phosphorylation (reviewed in Burridge and Chrzanowska-Wodnicka, 1996). Binding of the p85 subunit of PI3K to tyrosine phosphorylated FAK increases the activity of PI3K, thereby increasing the activity of PKB/Akt (see Section 1.2.3.2). Detachment of normal epithelium from the matrix causes a decrease in PI3K-mediated PKB/Akt activity, therefore, even in the presence of growth factors, the cellular apoptotic machinery is activated (Khwaja *et al.*, 1997).

Thus, the disruption of integrin function by EphA2 may account for the inability to generate recombinant EphA2-overexpressing EpH4 cells. An alternative approach to create a stable recombinant EphA2-overexpressing cell line was to use an inducible system. Unfortunately, however, recombinant gene expression using this system was not tightly regulated in EpH4 cells. The reason for this is unclear, but as the EpH4 cells are derived from mammary epithelium and are responsive to lactogenic hormones (Reichmann *et al.*, 1989), it is likely that they contain a number of hormone receptors with the potential to bind to the hormone response elements in pIND and therefore drive recombinant gene expression.

However, subsequent data showed that inducible system was tightly regulated in MCF-7 cells and that recombinant EphA2 expression could be influenced by both the time of induction and the concentration of ponasterone. Thus, in contrast to EpH4 cells, it appears that MCF-7 were capable of overexpressing high levels of recombinant EphA2. However, this could be due to differences in cell type. Like PC-3 cells, MCF-7 cells are transformed. Since anoikis is thought to have evolved as a means of tumour suppression to prevent cells being able to grow in inappropriate environments, it is likely that transformed cells will have developed mechanisms to overcome this. Indeed, cells transformed with Ras do not undergo anoikis (Frisch and Francis, 1994), presumably as a result of an increase in PI3K activity, which might therefore explain why EpRas cells are able to induce high levels of EphA2 expression and remain viable. In addition, fibroblasts are resistant to anoikis (Frisch and Francis, 1994), as are cells that express the SV40 large T antigen (Re *et al.*, 1994), which might also provide an

explanation for the ability to obtain both recombinant EphA2-overexpressing NIH3T3 cells and high levels of recombinant EphA2 overexpression in transient COS cell transfections.

The ability of the induction of recombinant EphA2 expression in MCF-7 cells to be influenced by both time and concentration of ponasterone provides an important extra level of control over the expression of recombinant EphA2. Preliminary data suggested that expression of recombinant EphA2WT to levels which have been demonstrated to induce ligand-independent signalling impairs the growth of cells. This is in accordance with the demonstration that activation of EphA2 in mammary epithelial cells using a cross-linking antibody resulted in a decrease in bromodeoxyuridine (BrdU) staining (Dodge-Zantek *et al.*, 1999), and may be the result of decreased ECM-signalling. Unfortunately, the length of time taken to generate and characterise the EphA2-overexpressing MCF-7 cell line meant that there was limited time in which to use it. In the absence of time restrictions, the effect of EphA2 overexpression on the processes of adhesion and migration, which are influenced by changes in cellular morphology and cell-ECM interactions, and signalling pathways responsible for the observed decrease in the cell growth rate, would have been studied.

Chapter 6

Cloning and Expression of Full Length and Soluble EphrinA1

6.1. Introduction

Recombinant ligands are widely used in the study of many aspects of receptor activation. In contrast to the wide availability of many of these ligands, such as EGF and TGFβ, ephrinA1Fc was not commercially available at the beginning of these studies, possibly due to conflicting information regarding its bioactivity as a soluble ligand. Therefore, in order to analyse the signalling pathways downstream of EphA2 and their potential morphological effects in mammary epithelial cells, it was first necessary to clone ephrinA1.

EphrinA1 was initially identified as B61, a 25 kDa protein secreted into the conditioned medium of endothelial cells treated with TNFα (Holzman *et al.*, 1990). It was subsequently identified as a high affinity ligand for EphA2 both in soluble form (Bartley *et al.*, 1994) and as a full length, GPI-linked membrane-bound form (Shao *et al.*, 1995), in both cases evoking tyrosine phosphorylation of the EphA2 receptor. Therefore, to study the consequences of both forms of ligand on EphA2 activation in mammary epithelial cells, mouse ephrinA1 was cloned as both a soluble protein (without its GPI linkage) and in its full length membrane-bound form.

Soluble recombinant proteins are often tagged with an epitope to facilitate capture on a suitable matrix such as protein A/G- or antibody- coupled beads. Such tags include the Fc portion of immunoglobulins. For ephrinA1, an Fc-tag was cloned in frame at the C-terminus of the extracellular domain. The Fc tag offers a number of unique features; it enables direct isolation of the fusion protein on protein A/G-Sepharose beads, facilitates spontaneous dimerisation through disulphide bonds, and has even been reported to increase the yield of secreted proteins over their non-tagged counterparts by apparently chaperoning them along the secretory pathway (Simmons, 1993). Another advantage that the Fc tag confers is the ability of the fusion protein to be clustered with an anti-Fc antibody into higher order oligomers. This may be especially important for ephrins, as conflicting reports exist in the literature concerning their requirement to be clustered to

induce Eph receptor activation (Section 1.1.3.2). Clustering is thought to enable the ligand to be presented to target cells in a state representative of its membrane bound form. Indeed, it has been reported that ephrins are not effective as a soluble monomers (Davis *et al.*, 1994) and may even act in a dominant negative manner as antagonists, despite the initial identification and subsequent demonstration of ephrinA1 as a bioactive soluble ligand (Holzman *et al.*, 1990; Bartley *et al.*, 1994).

Mammalian cells such as COS cells are often used as a means of producing soluble, recombinant proteins. For proteins in which glycosylation and protein conformation are likely to be important, expression in a mammalian cell is preferable to a bacterial expression system. Therefore, the cloning of ephrinA1 into a mammalian expression vector provided a means of producing both soluble Fc-tagged and full length recombinant bioactive proteins from COS cells for the further analysis of EphA2 signalling.

6.2. Results

6.2.1. First Strand Synthesis of Soluble and Full Length EphrinA1 cDNA

EphA2 and ephrinA1 show similar patterns of distribution in the developing embryo and in many adult tissues (Lindberg and Hunter, 1990; Ganju *et al.*, 1994; Shao *et al.*, 1995; Takahashi and Ikeda, 1995; Rosenberg *et al.*, 1997). Since EphA2 expression has previously been documented in the mammary gland (Andres *et al.*, 1994), virgin mouse mammary gland was chosen as the source of RNA from which to clone ephrinA1. Total cellular RNA was isolated from virgin mouse mammary gland as described in Section 2.1.11.1, and first strand cDNA synthesis was performed (Section 2.1.11.3) using two different specific 3' reverse primers based on the reported sequence of mouse ephrinA1 (accession number U26188). These primers were specifically designed in order to clone ephrinA1 as both full length protein and as a soluble extracellular form fused in frame with the Fc portion of mouse IgG1 (see Figure 6.1).

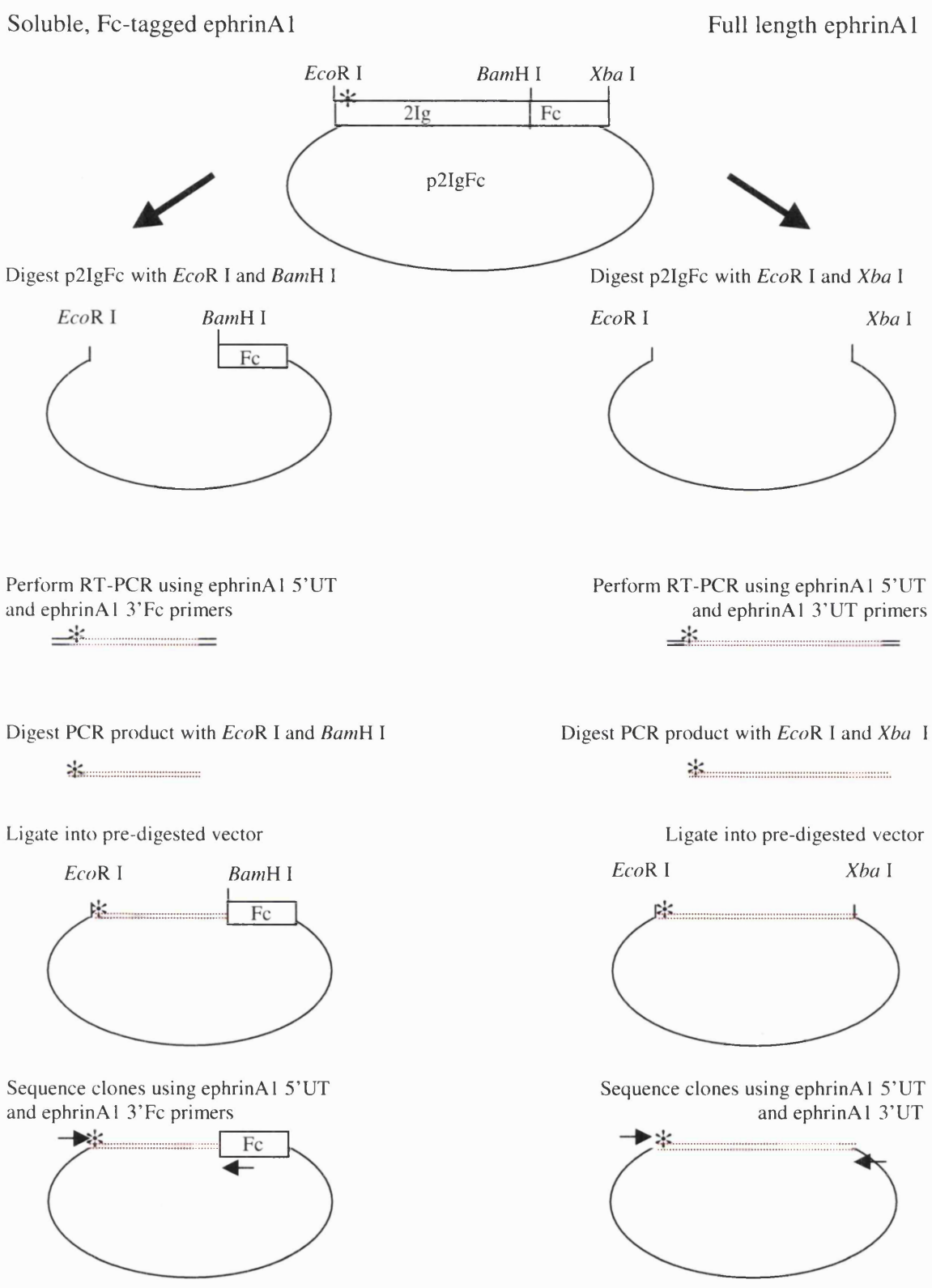


Figure 6.1. Strategy to Clone EphrinA1 in Full Length and Soluble Forms
The full length sequence of ephrinA1 or ephrinA1 lacking its GPI linkage site was amplified by PCR from a cDNA template generated by reverse transcription of mRNA. Subsequent to restriction endonuclease digestion with appropriate enzymes, both DNA fragments were ligated into the p2IgFc vector. The soluble form of ephrinA1 was ligated N-terminal to the Fc region of murine IgG. The Fc region was removed for cloning of the full length form of ephrinA1. Asterisks denote initiating methionine.

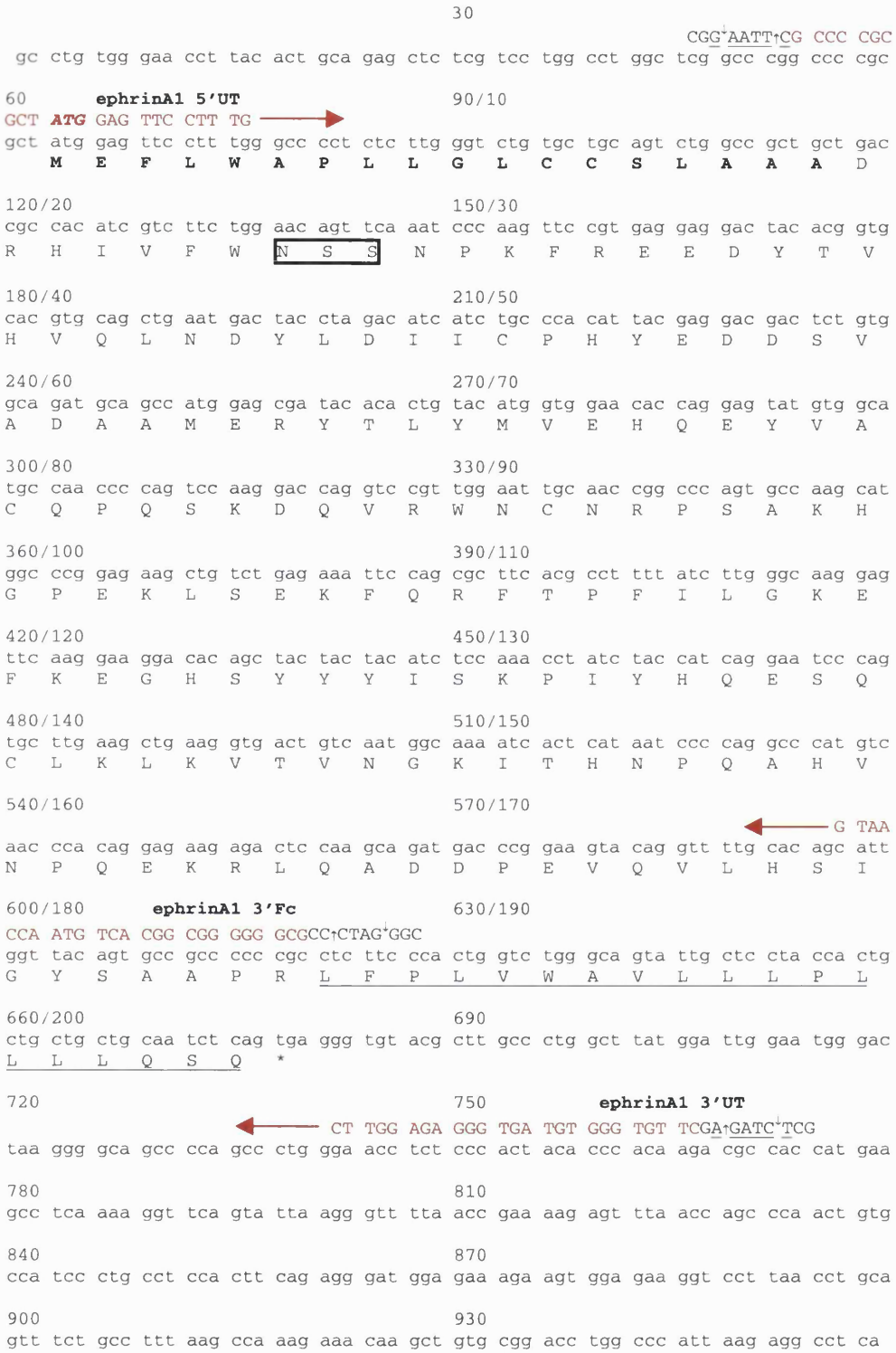


Figure 6.2. Sequence of Mouse EphrinA1
Accession no. U26188. The upper line represents the nucleotide sequence and the lower line represents the amino acid sequence of mouse ephrinA1. Oligonucleotide primers ephrinA1 5'UT, ephrinA1 3'UT and ephrinA1 3'Fc (shown in red) are depicted above the region to which they are designed to anneal, with an arrow indicating the direction of amplification. The signal peptide sequence is shown in bold, the putative N-glycosylation site is boxed, and the GPI recognition sequence is underlined.

The oligonucleotide primer ephrinA1 3'UT was designed to anneal to nucleotides 742-766 in the 5' untranslated end of ephrinA1 and was engineered to include an *Xba* I site (underlined) at the 5' end (see Figure 6.2).

ephrinA1 3'UT $^{5'}$GCTCTAGAGCTTGTGGGTGTAGTGGGAGAGGTTC$^{3'}$

The oligonucleotide primer ephrinA1 3'Fc was designed to anneal to nucleotides 596-620 within the coding region of ephrinA1 before the GPI-recognition site (residues 187-205), and a *Bam*H I restriction site (underlined) included in the 5' end (Figure 6.2).

ephrinA1 3'Fc $^{5'}$CGGGATCCCGCGGGGGGCGGCACTGTAACCAATG$^{3'}$

6.2.2. PCR Cloning of EphrinA1

Subsequent to reverse transcription, first strand cDNA was then amplified by PCR (Section 2.1.11.4) in the presence of 4 mM $MgCl_2$. The appropriate 3' reverse oligonucleotide primer (ephrinA1 3'UT or ephrinA1 3'Fc) was used in combination with ephrinA1 5'UT, a 5' oligonucleotide primer designed to anneal to nucleotides 53-77, and containing an *Eco*R I restriction site (underlined). This primer encompasses the end of the 5' untranslated region and the ATG initiation codon (nucleotides 63-65) (see Figure 6.2.). Hence, the resulting polypeptide would contain the signal peptide sequence required for secretion of the mature protein from the cell.

ephrinA1 5'UT $^{5'}$CGGAATTCGCCCCGCGCTATGGAGTTCCTTTG$^{3'}$

The PCR products were then subjected to horizontal gel electrophoresis and stained with ethidium bromide (see Figure 6.3). For the full length form of ephrinA1, a discrete band corresponding to the predicted size of 730 nucleotides was observed in the presence of the oligonucleotide primer ephrinA1 5'UT alone (Figure 6.3, lane 3). The presence of this band in the absence of further addition of the oligonucleotide primer ephrinA1 3'UT was presumably due to carryover of this primer from the reverse transcription reaction into the PCR. Addition of further ephrinA1 3'UT oligonucleotide primer resulted in the production of an intense band of approximately 200-300 bp as well as a larger product of approximately 650-700 bp (Figure 6.3, lane 2). The identity of the larger band is unclear but may correspond to the full length form of ephrinA1, the electrophoretic mobility of which is altered due to the presence of the lower band. The

presence of the lower 200-300 bp band was also observed in reactions containing the oligonucleotide primer ephrinA1 3'UT together with the cDNA template (Figure 6.3, lane 4), but not in reactions containing both primers but no cDNA template (Figure 6.3, lane 5). This suggests that the lower 200-300 bp band was the result of non-specific priming by the ephrinA1 3'UT primer. For the extracellular domain of the ephrinA1, a well-defined band corresponding to the predicted size of 584 nucleotides was observed both in the presence of ephrinA1 5'UT and ephrinA1 3'Fc (Figure 6.3, lane 7) and ephrinA1 5'UT alone (Figure 6.3, lane 8). No bands were observed in the presence of the oligonucleotide primer ephrinA1 3'Fc alone (Figure 6.3, lane 9). Similarly, no bands were observed in reactions containing ephrinA1 5'UT and ephrinA1 3'Fc in the absence of cDNA template (Figure 6.3, lane 10).

The PCR products from the ephrinA1 full length and ephrinA1 soluble extracellular domain reactions (Figure 6.3, lanes 3 and 7) were digested for 16 hours with the appropriate restriction enzymes (*Eco*R I and *Xba* I for full length ephrinA1 and *Eco*R I and *Bam*H I for soluble ephrinA1). The DNA fragments were gel purified and ligated into the vector p2IgFc which had previously been digested with the appropriate restriction enzymes (*Eco*R I and *Xba* I for full length ephrinA1 and *Eco*R I and *Bam*H I for soluble ephrinA1) (see Figure 6.1).

Both ligation products were then transformed into XL1-Blue competent *E. coli* and selected for ampicillin resistance. Small-scale plasmid preparations were prepared from resultant ampicillin resistant colonies. A number of putative soluble extracellular domain Fc fusion clones (ephrinA1Fc) and several putative full length clones (ephrinA1FL) were identified by restriction digest analysis using *Eco*R I and *Xba* I for full length ephrinA1 and *Eco*R I and *Bam*H I for soluble ephrinA1 (data not shown). Nucleotide sequencing of the cDNA inserts was then performed (Section 2.1.10) using the oligonucleotide primers ephrinA1 5'UT and ephrinA1 3'Fc for ephrinA1Fc, and ephrinA1 5'UT and ephrinA1 3'UT for ephrinA1FL. One clone of ephrinA1Fc and one clone of ephrinA1FL, both containing a cDNA insert sequence identical to the reported sequence of mouse ephrinA1 (accession number U26188), were used for further analysis.

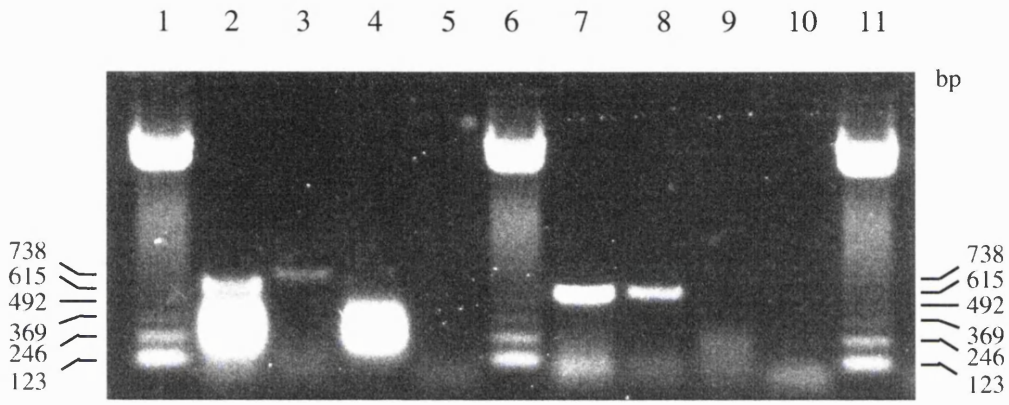


Figure 6.3. RT-PCR of Full Length and Soluble EphrinA1
PCR products obtained from first strand cDNA synthesis using the following oligonucleotide primer combinations were subjected to gel electrophoresis and stained with ethidium bromide.

Lane 1. 123 bp marker
Lane 2. ephrinA1 5'UT and ephrinA1 3'UT
Lane 3. ephrinA1 5'UT only
Lane 4. ephrinA1 3'UT only
} ephrinA1 3'UT reverse transcribed cDNA
Lane 5. No cDNA - ephrinA1 5'UT and ephrinA1 3'UT
Lane 6. 123 bp marker
Lane 7. ephrinA1 5'UT and ephrinA1 3'Fc
Lane 8. ephrinA1 5'UT only
Lane 9. ephrinA1 3'Fc only
} ephrinA1 3'Fc reverse transcribed cDNA
Lane 10. No cDNA - ephrinA15'UT and ephrinA1 3'Fc
Lane 11. 123 bp marker

6.2.3. Optimisation of Small-Scale Production of Soluble EphrinA1Fc

To produce the recombinant ephrinA1Fc fusion protein, COS-1 cells were transfected by lipofection with either ephrinA1Fc DNA or an equivalent volume of water. 16 hours later, the transfection medium was replaced with low serum conditioning medium (OPTIMEM™). Conditioned medium was then harvested from the cells after 24 hours (ephrinA1Fc-CM1), fresh conditioning medium added and cells incubated for a further 24 hours and medium again harvested (ephrinA1Fc-CM2). Fc fusion proteins were captured by incubation for 16 hours with protein A-Sepharose (Section 2.3.10.1). Proteins isolated on protein A-Sepharose were then subjected to western blot analysis using both an anti-mouse secondary antibody, and an anti-human ephrinA1 antibody (Table 2.3) followed by a secondary anti-rabbit antibody. This revealed that a protein of approximately 50-55 kDa, recognised by both the anti-mouse secondary antibody and the anti-human ephrinA1 antibody, was present in conditioned medium 72 hours post-transfection (Figure 6.4, ephrinA1Fc-CM2). The apparent molecular weight of this secreted protein is slightly higher than the molecular weight of 46 kDa predicted from the primary amino acid sequence, but may well be the result of N-linked glycosylation at a single site in the amino terminus (see Figure 6.2). No secreted protein was detected by either antibody 48 hours post-transfection (Figure 6.4 ephrinA1Fc-CM1) or in conditioned medium from the mock-transfected samples (Figure 6.4 mock-CM1 and mock-CM2).

In order to investigate whether ephrinA1Fc protein was still being secreted at 96 hours post-transfection, COS-1 cells were transfected with ephrinA1Fc DNA or mock-transfected as described above. 16 hours post-transfection, the transfection medium was replaced with OPTIMEM™. Conditioned medium was then harvested from one dish of ephrinA1Fc-transfected cells after 48 hours (ephrinA1Fc-CM3), fresh conditioning medium added and cells incubated for a further 24 hours and medium again harvested (ephrinA1Fc-CM4). A duplicate dish of ephrinA1Fc-transfected cells and a dish of mock-transfected cells were incubated in OPTIMEM™ in parallel, without media change, for a total of 72 hours (ephrinA1Fc-CM5 and mock-CM3). Fc fusion proteins were captured by incubation for 16 hours with protein A-Sepharose. Whole cell lysates were prepared from the three dishes of COS-1 cells after harvesting the final conditioned medium (96 hours post-transfection).

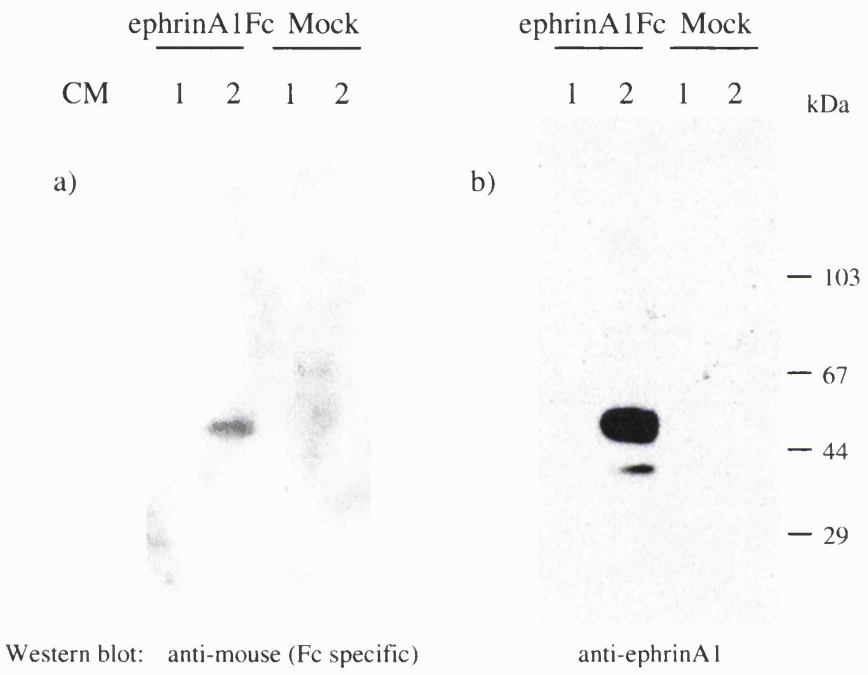
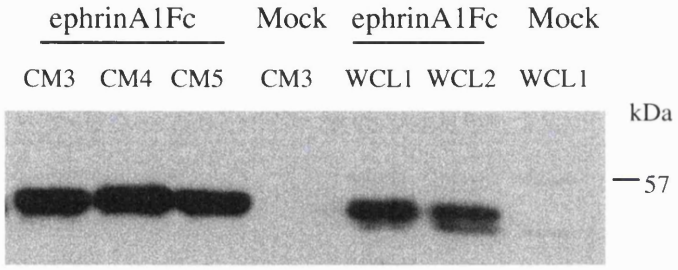


Figure 6.4. EphrinA1Fc is Secreted into COS Cell Medium
COS-1 cells were transfected with either 10 µg of ephrinA1Fc DNA or mock transfected. 16 hours later, the medium was replaced with OPTIMEM™. Conditioned medium was harvested 24 hours later (ephrinA1Fc-CM1 and mock-CM1), fresh OPTIMEM™ added and harvested 24 hours later (ephrinA1Fc-CM2 and mock-CM2). Fc fusion protein was isolated on protein A-Sepharose and subjected to SDS-PAGE and western blotting using (a) an Fc specific anti-mouse antibody, or (b) an anti-human ephrinA1 antibody.



Western blot: anti-human ephrinA1

Figure 6.5. Optimisation of the Yield of EphrinA1Fc from COS Cell Conditioned Medium

COS-1 cells were transfected in duplicate with 10 μg of ephrinA1Fc DNA, or mock transfected. 16 hours later, the medium was replaced with OPTIMEM™. Conditioned medium from one of the ephrinA1Fc-transfected dishes was harvested 48 hours later (ephrinA1Fc-CM3), fresh OPTIMEM™ added and medium again harvested after a further 24 hours (CM4) and whole cell lysate was prepared (ephrinA1Fc-WCL1). For the second dish of ephrinA1Fc-transfected cells and for mock-transfected cells, conditioned medium was harvested after a continuous 72 hour period (ephrinA1Fc-CM5 and mock-CM3) and whole cell lysates prepared (ephrinA1Fc-WCL2 and mock-WCL1). Whole cell lysates and Fc fusion protein isolated on protein A-Sepharose were subjected to SDS-PAGE and western blotting using an anti-human ephrinA1 antibody.

Whole cell lysates and secreted protein eluted from protein A-Sepharose were then subjected to SDS-PAGE and analysed by western blotting using an anti-human ephrinA1 antibody. It can be clearly seen that a significant amount of ephrinA1Fc was secreted over the 72 hour conditioning period (Figure 6.5). However, the quantity of recombinant ephrinA1 Fc secreted into the medium was not cumulative, as conditioning of the medium for a continuous 72 hour period did not result in significantly more protein being secreted compared to the 48 hour conditioning period. Maximum yield of antibody-detectable protein was obtained by removing the medium after 48 hours of conditioning, adding fresh OPTIMEM™ and harvesting again after a further 24 hours. Although ephrinA1Fc protein was still present in the whole cell lysates 96 hours post-transfection, timepoints exceeding 96 hours were not investigated. No protein was detected by the anti-ephrinA1 antibody in the conditioned medium or whole cell lysates of the mock-transfected cells.

6.2.4. Expression of Full Length Recombinant EphrinA1 in COS Cells

In order to evaluate the expression of the full length form of ephrinA1, ephrinA1FL DNA was transfected into COS-7 cells using Lipofectin™. 72 hours post-transfection, whole cell lysates were prepared and subjected to SDS-PAGE and western blotting using three independent antibodies as follows; the anti-human ephrinA1 antibody was raised in rabbit against bacterially expressed recombinant human ephrinA1 and was a kind gift from Richard Lindberg (Magal *et al.*, 1996); the anti-mouse ephrinA1 antibody (R&D systems) is a goat polyclonal antibody raised against recombinant mouse ephrinA1 extracellular domain; the rabbit anti-ephrinA1(C-terminus) antibody (Santa Cruz) was raised against a carboxy terminal peptide of human ephrinA1 (see Table 2.3). A 25 kDa protein corresponding to the predicted molecular weight of full length ephrinA1 was detected in whole cell lysates using the anti-human ephrinA1 antibody and the anti-mouse ephrinA1 antibody but not the anti-ephrinA1(C-terminus) antibody (Figure 6.6a-c). However, four distinct bands of considerably higher molecular weight were additionally detected by both the anti-human ephrinA1 and the anti-mouse ephrinA1 antibodies (Figure 6.6a and b). Two of these bands were also detected by the anti-ephrinA1(C-terminus) antibody (Figure 6.6c).

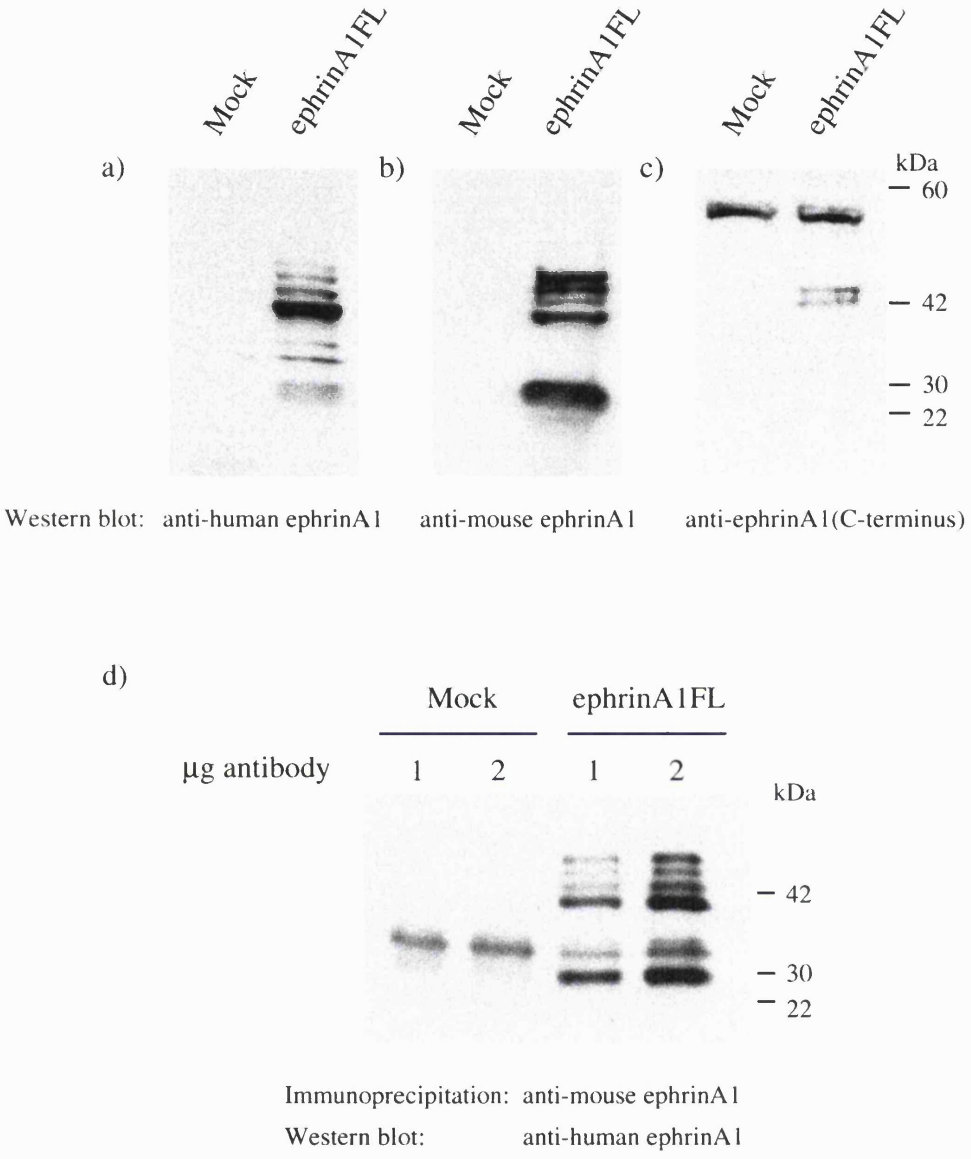


Figure 6.6. Expression of Full Length EphrinA1 in COS Cells
COS-7 cells were transfected with ephrinA1FL or mock-transfected. 72 hours post-transfection, whole cell lysates were prepared and subjected to SDS-PAGE and western blotting using (a) an anti-human ephrinA1 antibody, (b) an anti-mouse ephrinA1 antibody or (c) an anti-ephrinA1(C-terminus) antibody.
d) EphrinA1FL- or mock- transfected COS-7 cells were lysed 72 hours post-transfection and immunoprecipitated using either 1 µg or 2 µg of an anti-mouse ephrinA1 antibody. Western blot analysis was performed using an anti-human ephrinA1 antibody.

Immunoprecipitation using the anti-mouse ephrinA1 antibody followed by western blotting using the anti-human ephrinA1 antibody also yielded four higher molecular weight protein species in addition to the expected 25 kDa protein (Figure 6.6d). It is possible that the higher molecular weight bands represent glycosylated forms of ephrinA1, as the ephrinA1 sequence contains a putative N-glycosylation sequence in the amino terminus (Holzman *et al.*, 1990) (see Figure 6.2).

6.2.5. Bioactivity of EphrinA1Fc in Conditioned Medium

To assess the biological activity of recombinant mouse ephrinA1Fc protein, 48 hour conditioned medium from ephrinA1Fc- or mock- transfected COS-1 cells was added to an 80% confluent dish of recombinant EphA2WT-expressing NIH3T3 cells (WT38) (Section 2.2.2). Before addition to the cells, the conditioned medium was incubated for 3 hours at 4°C on a rotating wheel with either 1 or 10 µg/ml of anti-mouse IgG antibody (Fc specific, Sigma) to artificially cluster the ligand. The clustered conditioned medium was then prewarmed to 37°C before addition to the cells for 10 minutes at 37°C. Cells were lysed and the EphA2 receptor was immunoprecipitated using an anti-EphA2 antibody. Immunoprecipitates were then subjected to SDS-PAGE and western blotting with an anti-EphA2 antibody to analyse steady-state levels of the protein and an anti-phosphotyrosine antibody to assess tyrosine phosphorylation. Incubation of WT38 cells with conditioned medium from ephrinA1Fc-transfected cells resulted in an increase in the phosphotyrosine content of the EphA2 receptor, while steady-state receptor levels remained the same as the mock conditioned medium treated cells (Figure 6.7). No increase in phosphotyrosine content was observed in cells incubated with mock conditioned medium.

It is apparent that a 10-fold increase in clustering antibody made no difference to receptor phosphorylation. This could be due to a number of reasons; firstly, ephrinA1Fc could be maximally clustered at the lower antibody concentration of 1 µg/ml, or alternatively, a component of the conditioned medium might serve as a clustering agent. Another explanation is that ephrinA1Fc monomers are dimerising via Fc intermolecular disulphide bonds which evoke the same response, with respect to receptor phosphorylation, as higher magnitude oligomers.

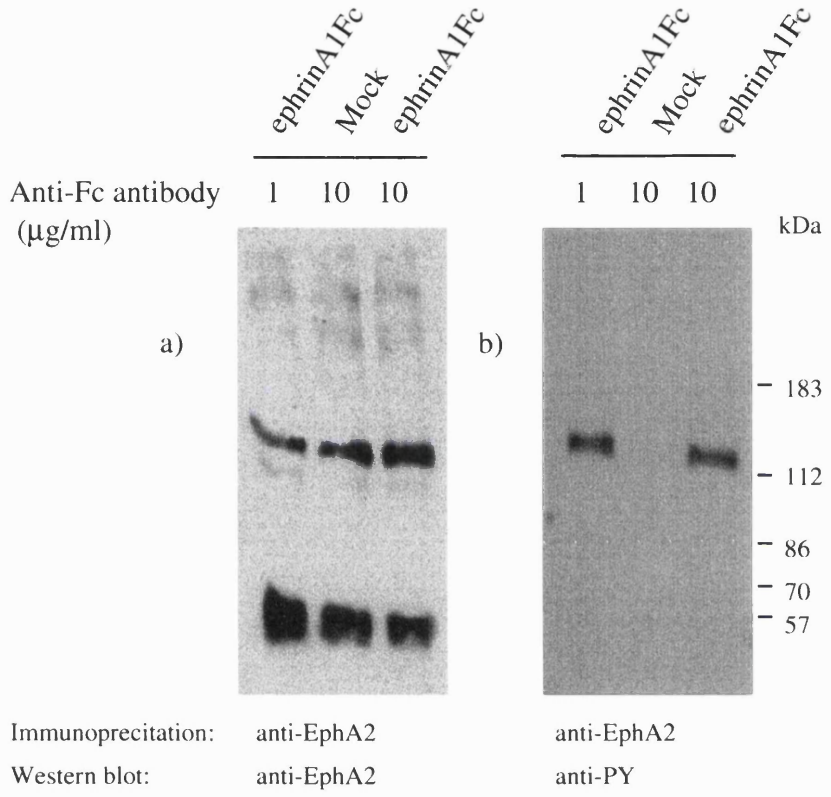


Figure 6.7. Clustered Soluble EphrinA1Fc is Bioactive
Conditioned medium from ephrinA1Fc- or mock- transfected COS-1 cells was clustered with either 1 μg/ml or 10 μg/ml of an Fc specific anti-mouse IgG antibody and added to WT38 cells for 10 minutes. EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to western blot analysis using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine antibody.

In receptor tyrosine kinase-mediated signalling, one effect of ligand binding is to stabilise receptor complexes within the target cell membrane in order to initiate intracellular signalling (Lemmon and Schlessinger, 1994; Lackmann *et al.*, 1998; Stapleton *et al.*, 1999). Since WT38 cells express high levels of EphA2, this may preclude the need for ephrinA1Fc-mediated receptor stabilisation. However, ephrinA1Fc conditioned medium was subsequently demonstrated to induce EphA2 receptor phosphorylation, in the absence of a clustering antibody, in NIH3T3 fibroblasts which have low endogenous levels of EphA2 (Figure 6.8).

EphrinA1Fc conditioned medium also effectively induced endogenous EphA2 receptor tyrosine phosphorylation in EpH4 mouse mammary epithelial cells (Figure 6.9). Conditioned medium from COS cells transfected with Fc-tagged meltrinα, a protein involved in myoblast fusion, did not induce autophosphorylation of the EphA2 receptor (Figure 6.10), as expected.

6.2.6. Bioactivity of Purified, Recombinant EphrinA1Fc

To undertake futher studies using ephrinA1Fc, it was necessary to purify the fusion protein from the conditioned medium. This would serve to eliminate other proteins, thus ensuring that any biochemical or morphological changes observed were due to the addition of ephrinA1Fc alone. Additionally, it would enable accurate quantification and standardisation of the ephrinA1Fc concentration, an important consideration for future experiments, as differences in protein concentration could potentially evoke different cell responses, such as occurs for the transcription factor Krüppel (Sauer *et al.*, 1995).

Large-scale transfection of ephrinA1Fc and subsequent protein purification was performed as described in Section 2.3.10.2. However, significant amounts of ephrinA1Fc could not be purified using either lipofection or DEAE-Dextran methods of transfection, presumably due to poor expression levels. More substantial protein yields of human Fc were attained using the pIgsig$^+$ vector (Section 2.1.1.5), a commercial vector designed specifically for the production of Fc-fusion proteins. A typical elution profile from the purification of human Fc using protein A-Sepharose beads is shown (Figure 6.11a). Purified human Fc was subjected to SDS-PAGE and stained with Coomassie Blue to assess the purity of the protein preparation (Figure 6.11b) or western blotted with an anti-human (Fc specific) antibody (Figure 6.11c).

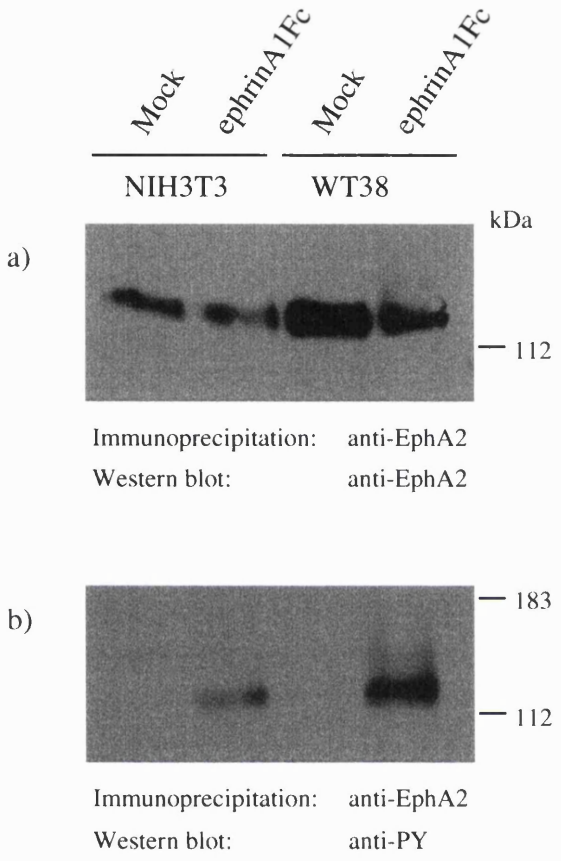


Figure 6.8. EphrinA1Fc Activates EphA2 in the Absence of a Clustering Agent
Conditioned medium from ephrinA1Fc- or mock- transfected COS-1 cells was added to NIH3T3 cells or WT38 cells for 10 minutes. EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to western blot analysis using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine antibody.

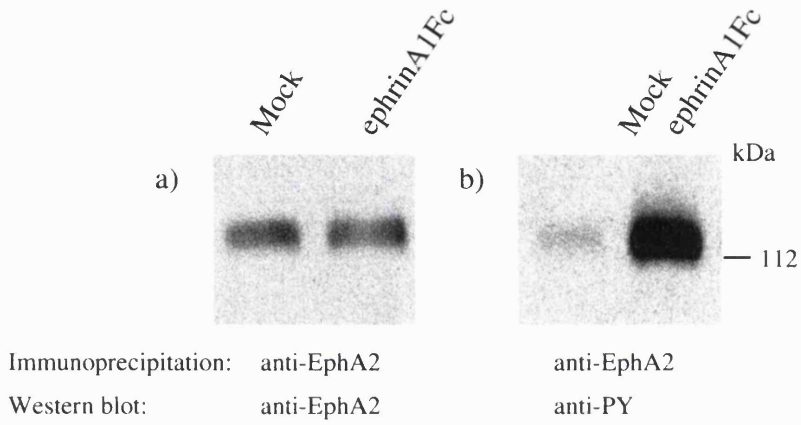


Figure 6.9. Soluble EphrinA1Fc Activates the EphA2 Receptor in EpH4 Cells
Conditioned medium from ephrinA1Fc- or mock- transfected COS-1 cells was added to EpH4 cells for 10 minutes. EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to western blot analysis using (a) an anti-EphA2 antibody (Santa Cruz), or (b) an anti-phosphotyrosine antibody.

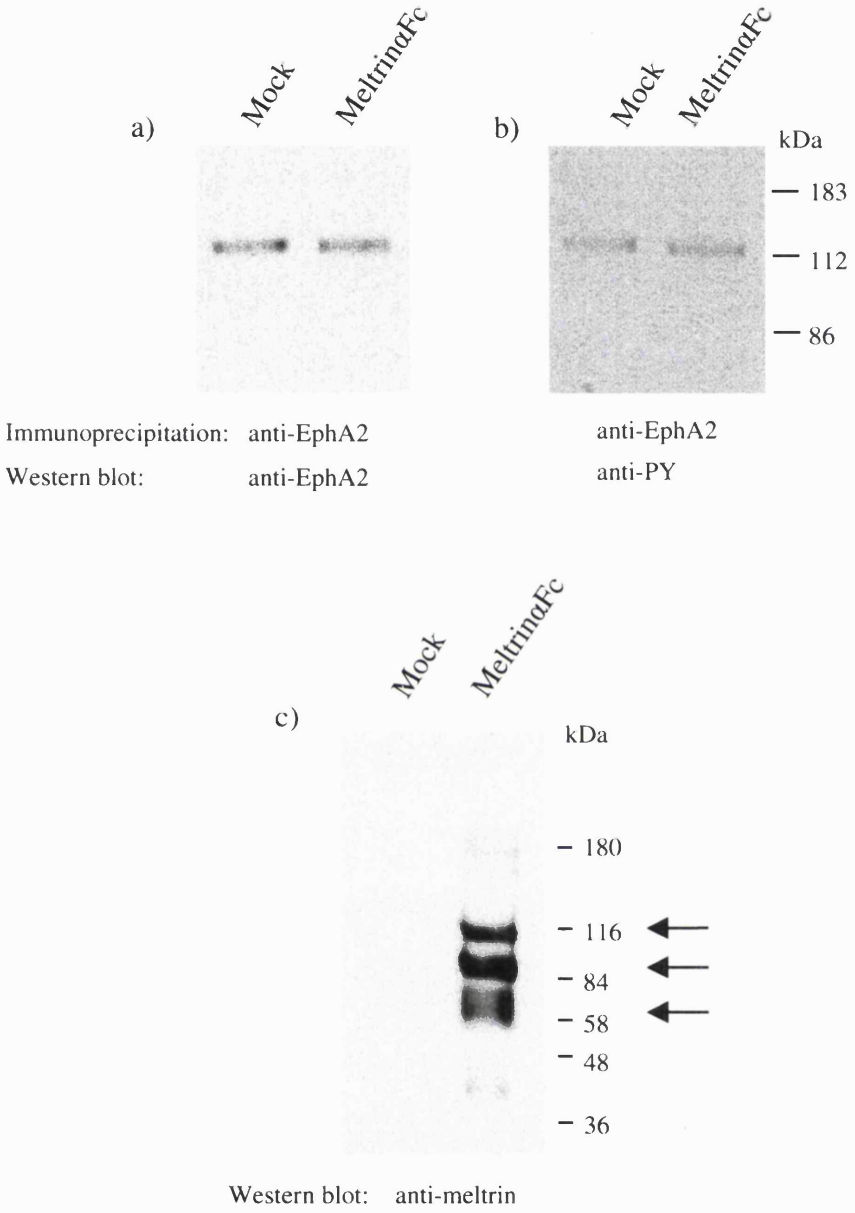


Figure 6.10. Conditioned Medium from MeltrinαFc-Transfected COS Cells Fails to Activate EphA2

Conditioned medium from meltrinαFc- or mock- transfected COS-7 cells was added to EpH4 cells for 10 minutes. EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to western blot analysis using (a) an anti-EphA2 antibody (Santa Cruz) or (b) an anti-phosphotyrosine antibody. (c) Conditioned medium from meltrinαFc transfected COS-7 cells was incubated with protein A-Sepharose and analysed by western blotting using an anti-meltrinα antibody (Table 2.3). Arrows correspond to the differentially processed forms of meltrinα.

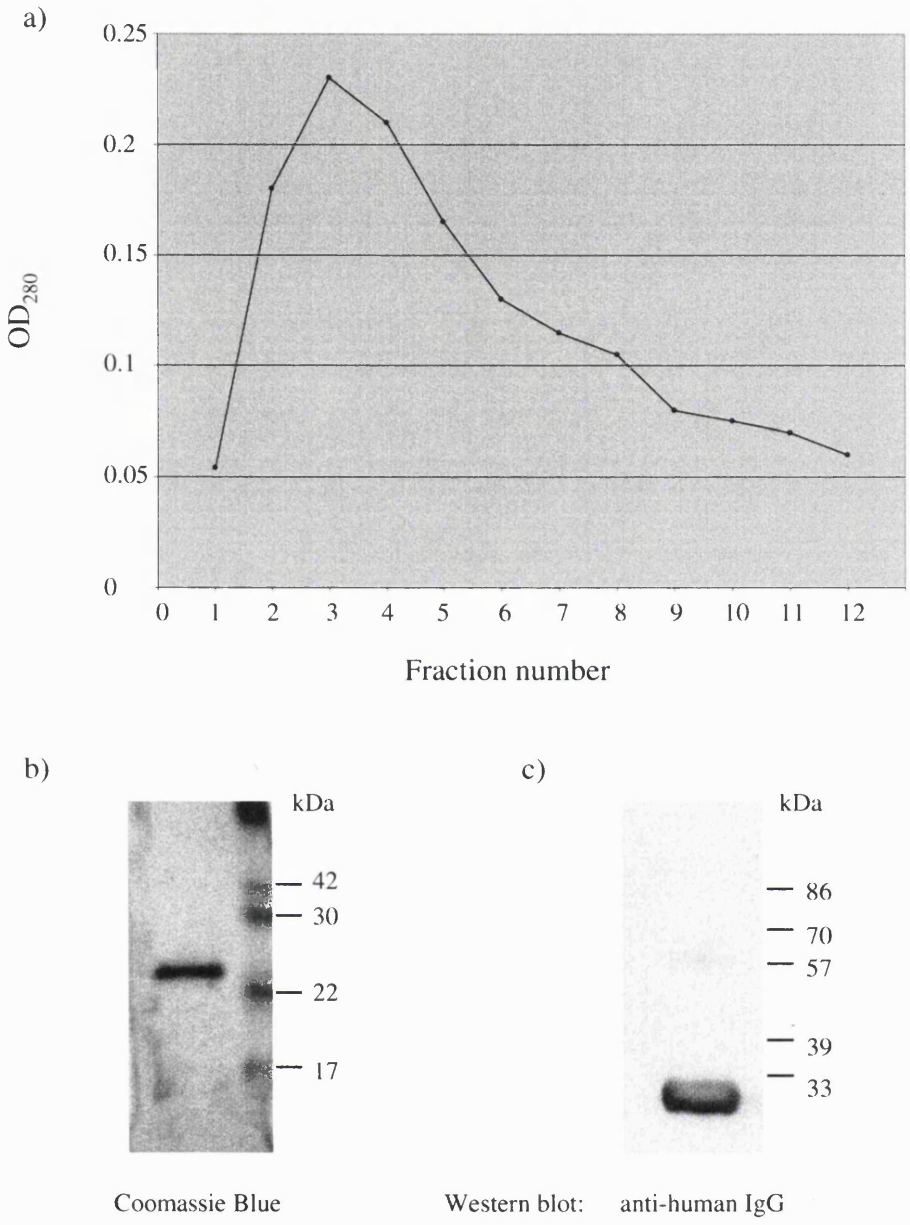
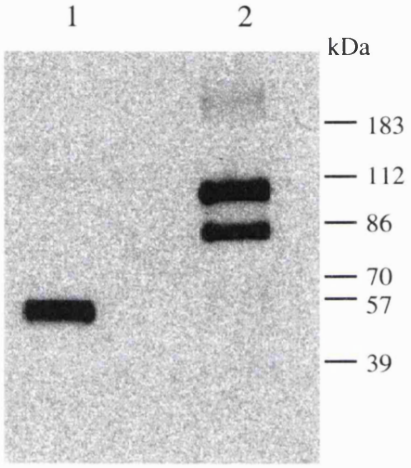


Figure 6.11. Large-Scale Production of Purified, Soluble Human Fc
Conditioned medium from COS-7 cells transfected with pIgsig$^+$ by DEAE-Dextran was harvested and recombinant human Fc was isolated on protein A-Sepharose. Human Fc was eluted from the protein A-Sepharose using 0.1 M glycine-HCl pH2.7. (a) the OD_{280} of fractions 1-12 was measured. Fractions were concentrated and subjected to SDS-PAGE followed by either (b) Coomassie staining, or (c) western blotting with an anti-human IgG (Fc specific) antibody.

The pIgsig$^+$ vector could therefore have been used as a vector in which to subclone the sequence for soluble ephrinA1. However, a commercial source of ephrinA1Fc became available by this time, comprising the extracellular domain of mouse ephrinA1 fused via a 6 amino acid polypeptide ligand to a 6 x histidine-tagged Fc region of human IgG1. This was therefore used as an alternative source of ephrinA1Fc protein.

To directly address the issue of whether purified ephrinA1Fc can self-dimerise, 1 μg of this recombinant ephrinA1Fc protein was subjected to SDS-PAGE under both reducing and non-reducing conditions and then western blotted using the anti-human ephrinA1 antibody. In the presence of β-mercaptoethanol, the fusion protein migrated as a single band of ~ 55 kDa (Figure 6.12, (1)), corresponding to the glycosylated form of the protein. However, under non-reducing conditions (2), two ephrinA1-immunoreactive bands of ~80 and 110 kDa were observed. These bands most likely correspond to a dimeric form of glycosylated ephrinA1Fc (110 kDa), and to monomers migrating differently under non-reducing conditions due to presence of highly conserved cysteine residues in the ephrin sequence (Davis *et al.*, 1994).

To confirm that the commercial source of recombinant ephrinA1Fc was bioactive, it was added at a concentration of 1 μg/ml to a number of mammary epithelial cell lines for 10 minutes. In addition to the previously studied EpH4, EpRas and MCF-7 cell lines, an additional mammary cell line, MDA-MB-231, was also used. Cells were lysed and the EphA2 receptor was immunoprecipitated using an anti-EphA2 antibody. Immunoprecipitates were then subjected to SDS-PAGE and western blotted with an anti-EphA2 antibody to analyse steady-state levels of the protein, and an anti-phosphotyrosine antibody to assess tyrosine phosphorylation. EphrinA1Fc was able to induce EphA2 receptor phosphorylation in all mammary cells lines tested at a concentration of 1 μg/ml, again in the absence of a clustering agent (Figure 6.13).



Western blot: anti-human ephrinA1

Figure 6.12. Purified Soluble EphrinA1Fc Forms Dimers
1 μg of purified, soluble recombinant ephrinA1Fc was subjected to SDS-PAGE under (1) reducing or (2) non-reducing conditions. Western blotting was performed using an anti-human ephrinA1 antibody.

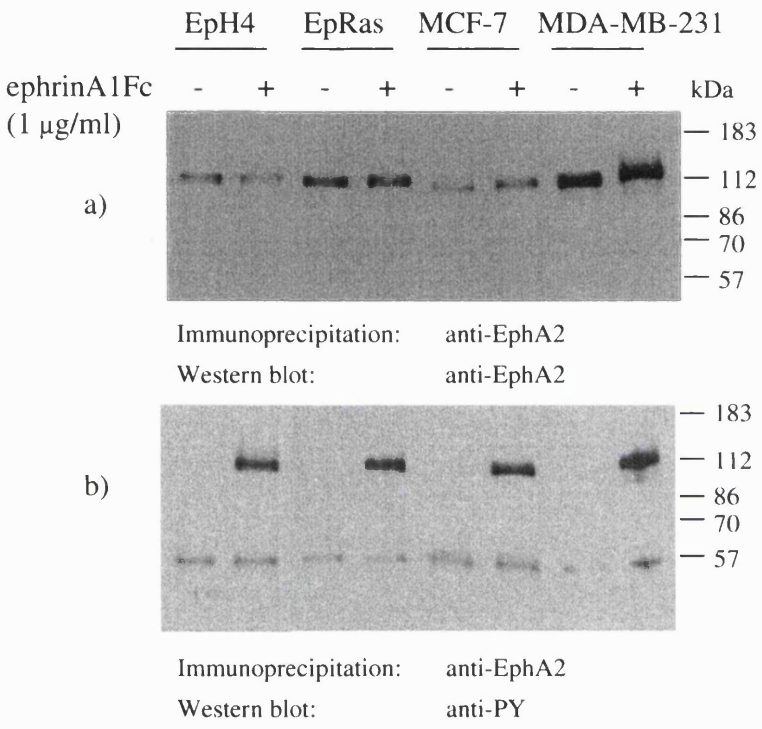


Figure 6.13. Purified Soluble EphrinA1Fc Activates EphA2 in a Variety of Mammary Cell Lines

EpH4, EpRas, MCF-7 and MDA-MB-231 cells were stimulated for 10 minutes with 1 µg/ml of human IgG (-) or 1 µg/ml of purified soluble ephrinA1Fc (+). EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blot analysis using (a) an anti-EphA2 antibody (UBI), or (b) an anti-phosphotyrosine antibody.

6.2.7. Full Length EphrinA1 Induces EphA2 Tyrosine Phosphorylation in EpH4 Cells

Although ephrinA1 was initially identified as a soluble ligand, it was later found to be expressed as a full length, GPI-linked protein. To verify that full length ephrinA1 was also bioactive, COS-7 cells were either transfected with ephrinA1FL, or mock-transfected. 72 hours post-transfection, cells were detached using 1 mM EDTA in PBS, washed three times in PBS and then added at a density of 3 x 10^6 cells in 1 ml of PBS to an 80% confluent 60 mm dish of EpH4 cells (seeded 24 hours earlier in 0.5% FCS-containing medium). Following a 10 minute co-culture at 37°C, the EpH4 cell monolayer was washed extensively in PBS to remove the COS cells. EpH4 cells were then lysed and subjected to immunoprecipitation with an anti-EphA2 antibody. Western blotting was then performed using an anti-EphA2 antibody and an anti-phosphotyrosine antibody. The EphA2 receptor became tyrosine phosphorylated at low levels by the ephrinA1FL-transfected COS cells but not by the mock transfectants (Figure 6.14). Soluble ephrinA1Fc, at a concentration of 1 μg/ml, again induced a significant amount of EphA2 tyrosine phosphorylation.

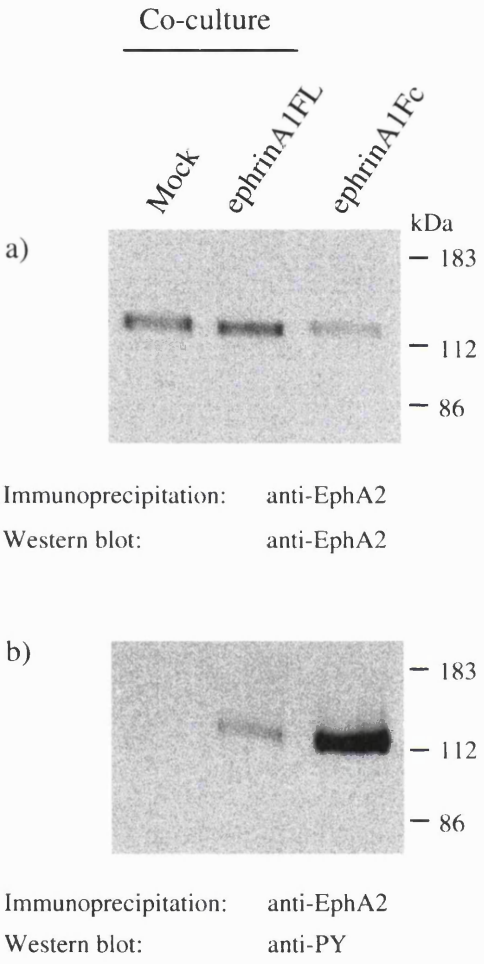


Figure 6.14. Membrane-Bound Full Length EphrinA1 Activates the EphA2 Receptor in EpH4 Cells

COS-7 cells were transfected with ephrinA1FL, or mock-transfected. 72 hours post-transfection, the cells were detached from dishes using 1 mM EDTA in PBS, resuspended in 1 ml PBS and overlayed onto EpH4 cells for 10 minutes before being extensively washed off with PBS. EphA2 was immunoprecipitated from cell lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blot analysis using (a) an anti-EphA2 antibody (UBI), or (b) an anti-phosphotyrosine antibody.

6.3 Discussion

The results in this chapter have described the cloning and expression of ephrinA1 as both a full length and a soluble Fc fusion protein using RNA prepared from virgin mouse mammary gland. Andres and colleagues have previously reported the presence of RNA for the EphA2 receptor in the virgin mammary gland (Andres *et al.*, 1994), thus the expression of both receptor and ligand in mammary gland strongly implies that EphA2-mediated signalling occurs in this tissue. Since RNA from whole mammary gland was used for reverse transcription, it is not possible to identify the cell type responsible for ephrinA1 RNA expression from this study. However, this issue could be resolved by performing *in situ* hybridisation or immunohistochemistry. It would be interesting to see if ephrinA1 exists in the same cell type as EphA2, as this would imply the presence of an autocrine loop. It has recently been reported that ephrinA1 protein is co-expressed with EphA2 in both tumour cells and endothelial cells of mammary tumours (Ogawa *et al.*, 2000), suggesting that both autocrine and paracrine signalling may exist in the transformed mammary gland. Furthermore, ephrinA1 expression has been reported in ZR-S human breast carcinoma cells (Shao *et al.*, 1995). However, expression of ephrinA1 in non-neoplastic mammary tissue has not been studied in any detail.

EphrinA1 was expressed as an Fc fusion protein which was secreted into the surrounding conditioning medium over a 72 hour period as detected by both an anti-mouse (Fc specific) antibody and an anti-ephrinA1 antibody. Maximal yield of the protein was obtained by replacing the medium after 48 hours of conditioning and harvesting the second batch a further 24 hours later. The observation that this gave a better yield than a single 72 hour conditioning period could be due to degradation of the fusion protein over this extended time, or it might suggest that an accumulation of recombinant ephrinA1Fc in the conditioned medium was detrimental to the production and/or secretion of more protein. It is possible that in COS cells, which express endogenous EphA2 receptor, prolonged exposure to the exogenously secreted ephrinA1Fc resulted in chronic Eph signalling which adversely affected the cells. However, it was not known at this stage whether the recombinant protein was biologically active. Biological activity was subsequently demonstrated using conditioned medium from ephrinA1Fc-transfected cells, which was seen to markedly increase the phosphotyrosine content of WT38 cells. It was not possible to quantify the concentration of ephrinA1Fc in the conditioned medium, but the failure to produce

significantly large amounts by large-scale transfection and purification suggests that ephrinA1Fc was only present in small concentrations. In accordance with this, Bartley and colleagues demonstrated the ability of unclustered ephrinA1 to induce phosphorylation of the EphA2 receptor at concentrations as low as 16 ng/ml (Bartley *et al.*, 1994).

Conditioned medium containing recombinant ephrinA1Fc was found to induce tyrosine phosphorylation of the EphA2 receptor in the presence of an anti-Fc antibody at both 1 μg/ml and 10 μg/ml. Thus, a 10-fold difference in the amount of clustering reagent failed to affect the amount of EphA2 tyrosine phosphorylation. In addition, the ability of soluble ephrinA1Fc to activate the EphA2 receptor was demonstrated not to be a function of receptor density in WT38 cells, as tyrosine phosphorylation of the endogenous EphA2 receptor was also induced in naïve NIH3T3 cells. In this experiment, conditioned medium was bioactive in the absence of an exogenous clustering agent. Conditioned medium, again lacking a clustering agent, also stimulated tyrosine phosphorylation of the endogenous EphA2 receptor in EpH4 mammary epithelial cells. These observations strongly imply that recombinant ephrinA1Fc was bioactive in the absence of a clustering agent. In support of this, commercially available purified soluble ephrinA1Fc induced tyrosine phosphorylation of the EphA2 receptor in all mammary cell lines tested.

EphrinA1 was initially identified as a soluble protein in HUVEC conditioned medium, and indeed, a membrane-bound form could not be detected in these cells (Holzman *et al.*, 1990). Soluble ephrinA1, again present in conditioned medium, was subsequently identified as the ligand for the EphA2 receptor, as it was able to induce EphA2 tyrosine phosphorylation (Bartley *et al.*, 1994). Davis and colleagues, however, demonstrated that only when a myc-tagged ephrinA1 monomer was clustered with an anti-myc antibody was it able to activate the EphA5 receptor. However, whereas soluble ephrinA1myc was able to induce low level activation of an EphA2/FGF chimeric receptor-expressing reporter cell, monomeric ephrinA3myc failed to do so, suggesting that the ability of soluble ligands to evoke responses may be dependent on the particular receptor-ligand interaction (Davis *et al.*, 1994). Many other groups have taken advantage of the ability of Fc domains to spontaneously dimerise to study Eph receptor activation and receptor-substrate recruitment, with differing results. Pandey and colleagues used ephrinA1Fc to induce endothelial cell migration (Pandey *et al.*, 1995b),

and to demonstrate phosphorylation dependent p85- and SLAP- EphA2 interactions (Pandey *et al.*, 1994; Pandey *et al.*, 1995a). Other groups have also used both ephrinA- and ephrinB-Fc fusion proteins in the absence of further clustering to activate Eph receptors (Gale and Yancopoulos, 1997; Park and Sanchez, 1997; Choi *et al.*, 1999), whereas in several other cases, although high affinity interactions were demonstrated using biochemical techniques, receptor activation was not induced until the ligandFcs were clustered (Sakano *et al.*, 1996; Ohta *et al.*, 1997), and in some instances activation could only be demonstrated using the membrane-bound form (Ciossek and Ullrich, 1997). This may be due to differences between research groups in the preparation of the purified ligand, or it may differ according to which ligand-receptor pair is being studied. In general, ephrinB ligands appear to show greater requirement for higher order clustering, whereas ephrinA ligand interactions appear to be dependent on which ligand-receptor pair is being studied (Gale and Yancopoulos, 1997). In addition, earlier binding studies using Fc-tagged receptor or ligand fusion proteins suggested that there was a great deal of overlap in the repertoire of receptor-ligand interactions (Gale *et al.*, 1996a). However, Lackmann and colleagues employed a number of biochemical techniques to postulate that several of these *in vitro* ligand-receptor interactions may not exist *in vivo*, or may exist only in areas of very high ligand-receptor density (Lackmann *et al.*, 1997). Additionally, other Eph-ephrin interactions may serve to stabilise otherwise weaker interactions, again illustrating the importance of demonstrating receptor activation *in vivo*. Lackmann has proposed a model for receptor activation whereby a high ligand concentration favours receptor clustering which, on reaching a critical level, results in receptor oligomerisation (Lackmann *et al.*, 1998). Accordingly, several groups have proposed that soluble ligands act as receptor antagonists by binding to the receptor but preventing its activation in a dominant negative manner (Winslow *et al.*, 1995; Durbin *et al.*, 1998). However, as previously reported, and as demonstrated within this chapter, this does not appear to hold true in the case of ephrinA1, as soluble ephrinA1 is able to induce tyrosine phosphorylation of the EphA2 receptor (Bartley *et al.*, 1994). Thus, it appears that ephrinA1 is entirely effective in both a soluble and a clustered form at inducing EphA2 phosphorylation.

Furthermore, COS cells expressing recombinant full length ephrinA1 were also able to induce tyrosine phosphorylation of the EphA2 receptor in EpH4 cells. Recombinant full length ephrinA1 protein was expressed as a number of distinct forms in COS cells. In addition to the expression of full length recombinant ephrinA1 of the predicted

molecular size, a number of larger anti-ephrinA1 immunoreactive protein forms were observed. It is likely that these forms are the consequence of differential protein glycosylation, although this was not directly demonstrated. Higher molecular weight forms of both soluble and membrane-bound ephrinA1 have previously been observed (Holzman *et al.*, 1990; Easty *et al.*, 1999) and attributed to glycosylation, although this has not been demonstrated. Interestingly, western blot analysis of mouse mammary gland tissue demonstrated that two molecular weight forms of anti-ephrinA1 immunoreactive protein were expressed at different stages of the reproductive cycle (data not shown). Whereas a ~ 25 kDa anti-ephrinA1 immunoreactive protein was observed in the mammary gland of virgin and pregnant mice, mammary glands undergoing lactation were seen to express a protein of higher molecular weight (approximately 45 kDa). Since it has been reported that the activity of a number of the enzymes involved in glycosylation is influenced by the hormonal status of the mammary gland (Vijay, 1998), this may provide an explanation for this observation. Moreover, differential glycosylation may have important consequences for ephrinA1 function.

In summary, the experiments undertaken in this chapter have shown that both full length recombinant ephrinA1 and soluble recombinant ephrinA1Fc were effective at inducing EphA2 receptor autophosphorylation in all cell lines tested.

Chapter 7

Signalling Through the EphA2 Receptor in Mammary Epithelial Cells

7.1. Introduction

Eph receptors have been proposed to perform roles in diverse processes, from axon fasciculation to the establishment of rhombomere boundaries. The signalling pathways downstream of activated Eph receptors that may mediate such processes have only recently begun to be elucidated. Changes in cell morphology are common to many of the responses evoked *in vitro* by Eph receptor activation, and it has indeed been demonstrated that Eph receptor activation affects regulation of the actin cytoskeleton (Magal *et al.*, 1996; Wahl *et al.*, 2000). In addition to mediating such immediate effects, Eph receptor activation is likely to have pleiotropic effects and be involved in the regulation of longer-term responses, as has been demonstrated for other RTKs, such as the PDGF receptor. These effects may involve changes in gene transcription, enabling cells to adapt to a new environment or to maintain the initial response. Like that of other RTKs, the outcome of Eph receptor signalling will depend on, among a number of factors, the repertoire of signalling molecules that the activated receptor recruits in response to ephrin-induced dimerisation and the duration of the response. With regard to the latter, dramatic differences in the outcome of PC12 cell behaviour (with respect to differentation or proliferation) can occur depending on whether MAPK activation is sustained or transient (Marshall, 1995). A similar scenario operating in Eph signalling could also have significant consequences on the cellular response.

Studies undertaken in this chapter made use of soluble, recombinant ephrinA1Fc to investigate Eph receptor-mediated signalling in EpH4 and EpRas mammary epithelial cell lines, with initial experiments focussing on the interaction of endogenous EphA2 with ephrinA1Fc.

7.2. Results

7.2.1. EphrinA1Fc Elicits a Rapid and Transient Increase in EphA2 Tyrosine Phosphorylation in Mammary Epithelial Cells

In Section 6.2.8, a 10 minute treatment with 1 μg/ml of recombinant ephrinA1Fc was shown to induce EphA2 receptor tyrosine phosphorylation in a number of mammary epithelial cell lines. To gain further information about the dynamics of EphA2 receptor phosphorylation and its potential effects on downstream signalling, the activation profile of the EphA2 receptor in response to ephrinA1Fc stimulation was analysed over a 3 hour time course in both EpH4 and EpRas cells.

Subconfluent EpH4 and EpRas cells were stimulated for 0, 2, 5, 10, 30, 60, 120 and 180 minutes with 1 μg/ml ephrinA1Fc prior to immunoprecipitation of the EphA2 receptor with an anti-EphA2 antibody. Both the steady-state protein levels and the tyrosine phosphorylation status were analysed by western blotting using an anti-EphA2 antibody and an anti-phosphotyrosine antibody.

EphrinA1Fc-dependent phosphorylation of EphA2 in EpH4 cells was transient, with maximal phosphorylation occurring between 2 and 10 minutes, but residual levels were still detectable after 180 minutes (Figure 7.1b). Steady-state receptor levels were seen to decrease after 30 minutes, at which point the phosphotyrosine content also decreased (Figure 7.1a). The rapid induction of EphA2 phosphorylation was reproduced in EpRas cells, although phosphorylated EphA2 could still be detected even after 180 minutes of incubation with ephrinA1Fc, presumably reflecting the higher steady-state levels of EphA2 receptor in the EpRas cells (Figure 7.2b). Again, steady-state receptor levels were seen to decrease after 30 minutes of stimulation with ephrinA1Fc (Figure 7.2a(i)), concomitant with the appearance of a protein of ~ 50 kDa, which could be detected using both the anti-EphA2 antibody (Figure 7.2a(ii)) and the anti-phosphotyrosine antibody (Figure 7.2b). Incubation of EpH4 or EpRas cells with 1 μg/ml of human Fc or human IgG did not induce tyrosine phosphorylation of the EphA2 receptor, and it can be seen that the steady-state levels of EphA2 protein in these samples remained comparable to those seen in unstimulated cells.

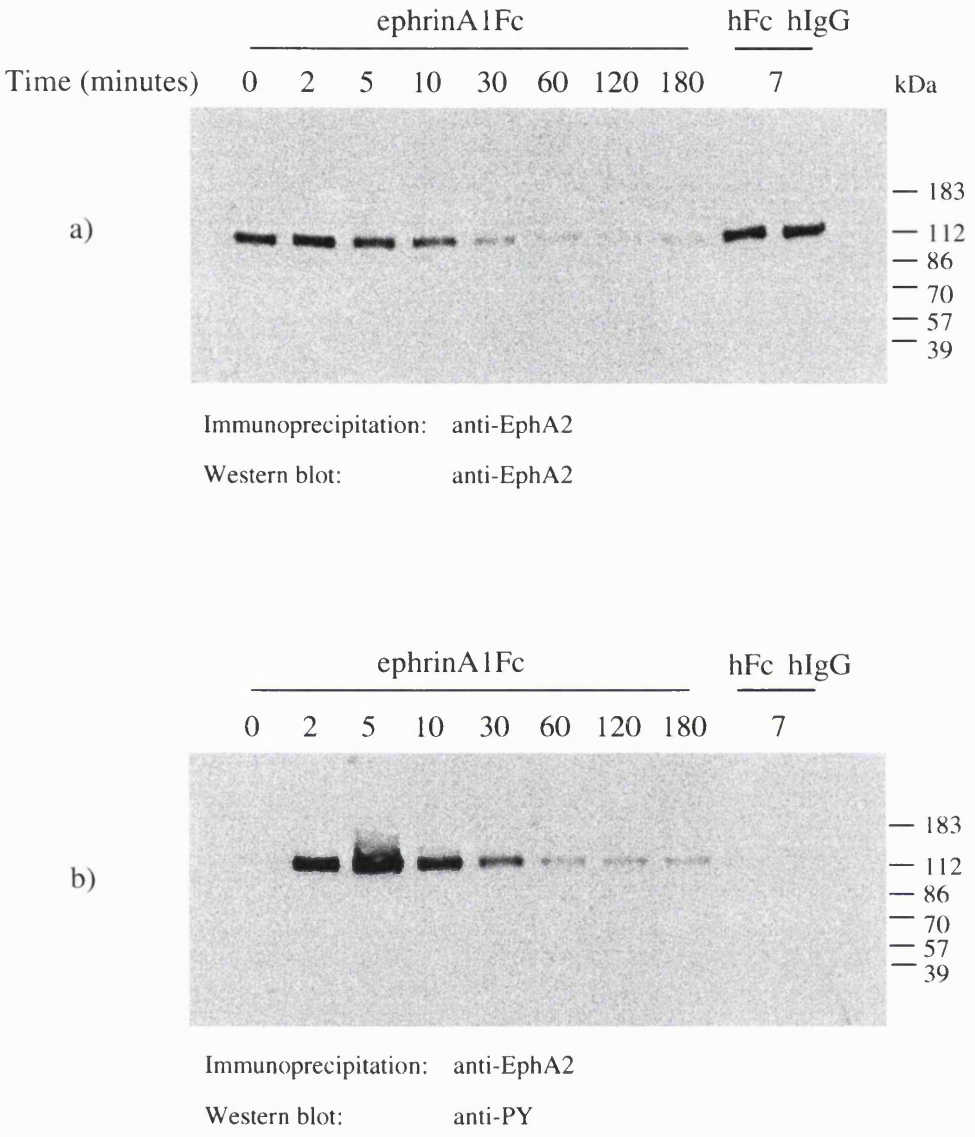


Figure 7.1. EphA2 is Rapidly and Transiently Tyrosine Phosphorylated in Response to EphrinA1Fc in EpH4 Cells

5 x 10^5 EpH4 cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 2, 5, 10, 30, 60, 120 or 180 minutes, or 1 µg/ml of purified human Fc (hFc) or human IgG (hIgG) for 7 minutes. EphA2 was immunoprecipitated from RIPA lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI), or (b) an anti-phosphotyrosine antibody.

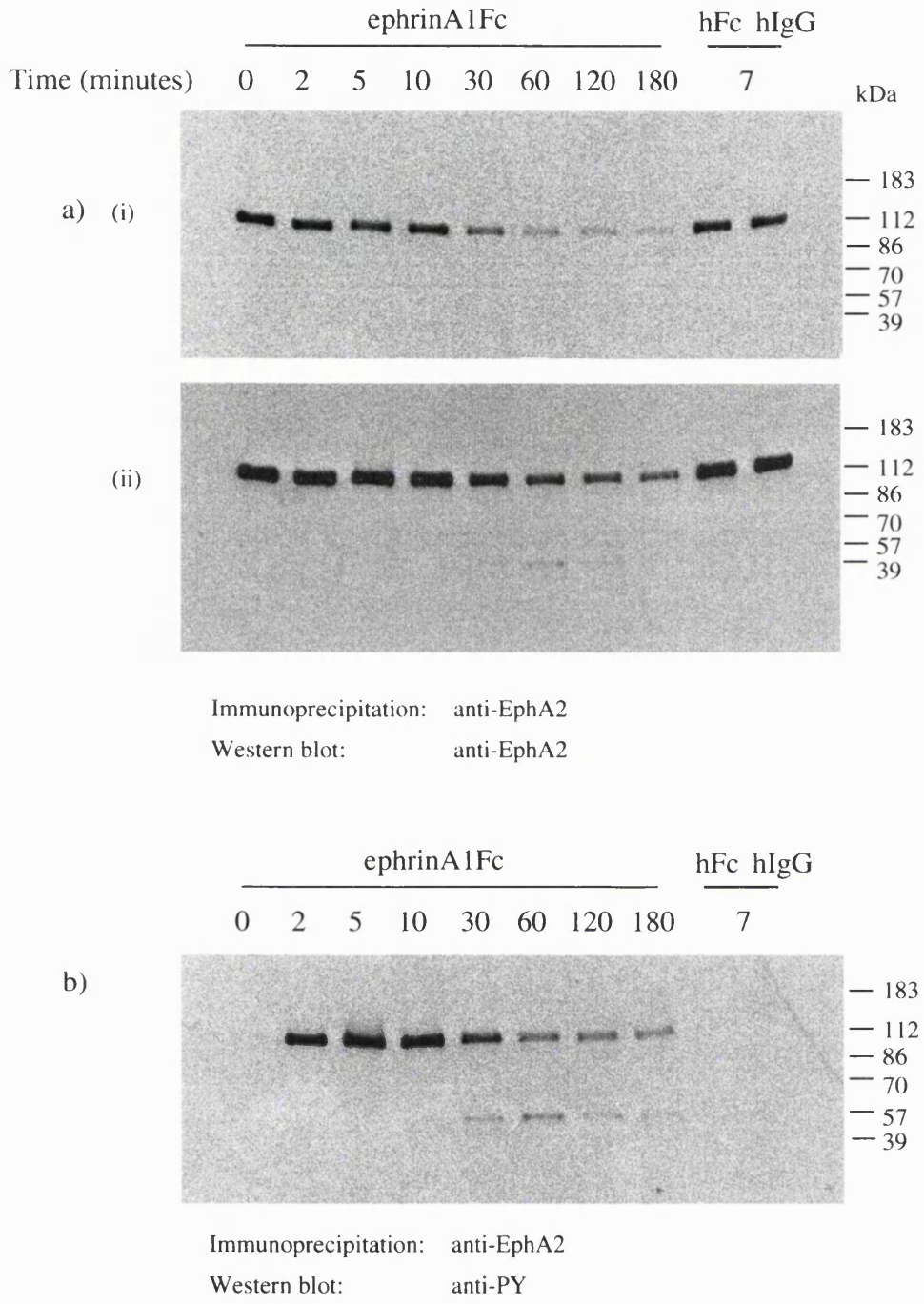


Figure 7.2. EphA2 is Rapidly and Transiently Tyrosine Phosphorylated in Response to EphrinA1Fc in EpRas Cells

5 x 10^5 EpRas cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 2, 5, 10, 30, 60, 120 or 180 minutes, or 1 µg/ml of purified human Fc (hFc) or human IgG (hIgG) for 7 minutes. EphA2 was immunoprecipitated from RIPA lysates using an anti-EphA2 antibody (Santa Cruz) and subjected to SDS-PAGE and western blotting using (a) an anti-EphA2 antibody (UBI) ((ii) represents prolonged exposure of (i)), or (b) an anti-phosphotyrosine antibody.

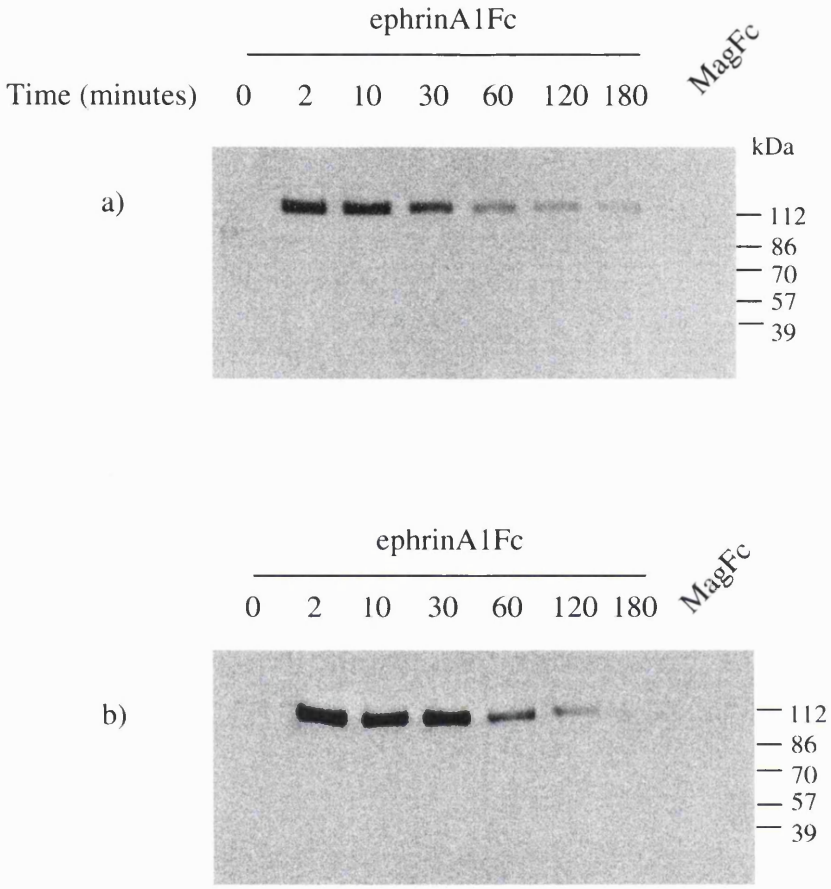


Figure 7.3. EphA2 Associates with EphrinA1Fc in EpH4 and EpRas Cells
5 x 10^5 EpH4 cells (a) or EpRas cells (b) were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 μg/ml ephrinA1Fc for 0, 2, 10, 30, 60, 120 or 180 minutes, or a control Fc fusion protein, MagFc, for 5 minutes. Cells were lysed in MAPK lysis buffer and lysates incubated with protein A-Sepharose. Eluted proteins were then subjected to SDS-PAGE and western blotting using an anti-EphA2 antibody (UBI).

7.2.2. EphrinA1Fc Interacts Stably with the EphA2 Receptor

The rapid increase in EphA2 tyrosine phosphorylation induced by ephrinA1Fc implied that ephrinA1Fc was promoting the formation of EphA2 dimers, thereby causing receptor autophosphorylation by increasing intrinsic kinase activity. To determine whether recombinant ephrinA1Fc was able to interact with the EphA2 receptor in both EpH4 and EpRas cells, cells were stimulated for 0, 2, 10, 30, 60, 120 and 180 minutes with 1 μg/ml ephrinA1Fc before lysates were prepared using a mild lysis buffer (MAPK lysis buffer – Section 2.3.1.4). In order to isolate ephrinA1-associated proteins, lysates were incubated with protein A-Sepharose to capture ephrinA1Fc via the Fc tag. Proteins bound to the Sepharose beads were eluted in sample buffer and subjected to SDS-PAGE. Western blot analysis using an anti-EphA2 antibody demonstrated that EphA2 was present (Figure 7.3). In both EpH4 and EpRas cells, the largest amount of full length EphA2 was found to be associated with the protein A-Sepharose-ephrinA1Fc complex between 2 and 30 minutes, with a moderate amount after 60 minutes, and trace levels between 2 and 3 hours. Anti-EphA2 immunoreactive protein was not pulled down by protein A-Sepharose alone in the absence of ephrinA1Fc, and no EphA2 was seen to associate with a control Fc chimeric protein, MagFc (myelin associated glycoprotein).

7.2.3. EphrinA1Fc Induces Downregulation of MAPK Activity in EpH4 Cells

A number of RTKs are able to influence the activity of the MAPK pathway. In addition to promoting cell proliferation, activation of MAPK is able to enhance cell migration by phosphorylating cytoplasmic proteins and mediating changes in gene transcription.

Thus, to investigate a potential role for MAPK in Eph-mediated signalling in mammary epithelial cells, the effect of exogenous addition of ephrinA1Fc upon MAPK activity was studied in EpH4 cells. Levels of dually phosphorylated MAPK were measured using an anti-phosphoMAPK antibody, whereas the activity of MAPK towards an exogenous substrate, MBP, was measured by an *in vitro* kinase assay (Section 2.3.9.4). EpH4 cells were treated with ephrinA1Fc at 1 μg/ml for 0, 2, 5, 10, 30, 60 and 120 minutes. Two dishes of EpH4 cells were also treated with 1 μg/ml of human Fc or human IgG as negative controls. Lysates were prepared from the cells and subjected to

either SDS-PAGE and western blotting, or immunoprecipitation with an anti-MAPK antibody followed by an *in vitro* kinase assay.

Levels of dually phosphorylated MAPK (p42 and p44) had decreased after the addition of ephrinA1Fc to EpH4 cells for 2 minutes, reaching their lowest between 5 and 10 minutes, and then gradually increasing (Figure 7.4a). After stimulating the cells for 2 hours, levels of phosphorylated MAPK almost resembled those of unstimulated cells. Phosphorylation of MAPK was unaffected by the addition of 1 μg/ml of human Fc or human IgG for a 7 minute period. The addition of ephrinA1Fc did not affect steady-state levels of MAPK protein (Figure 7.4b).

The activity of MAPK, as measured by its ability to phosphorylate MBP, was also decreased by the addition of ephrinA1Fc (Figure 7.4c and d). The specific activity of MAPK decreased transiently, reaching its lowest level (0.09 pmol/mg/min) after treating the cells with ephrinA1Fc for 5-10 minutes, and then almost returning to unstimulated levels (0.25 pmol/mg/min) after 1-2 hours (Figure 7.4d). Accordingly, levels of phosphorylated MBP were approximately 3-fold less following treatment of EpH4 cells with ephrinA1Fc for 5-10 minutes than those of unstimulated cells, but were unaffected by the addition of human Fc or human IgG (Figure 7.4c).

7.2.5. EpRas Cells Partially Overcome the EphrinA1Fc-Induced Downregulation of MAPK Activity

In accordance with upregulation of MAPK phosphorylation in Ras-transformed cells lines (Amundadottir and Leder, 1998; Martinez-Lacaci *et al.*, 2000), and as reported previously in Section 3.2.4, EpRas cells displayed a markedly higher level of phosphorylated MAPK than EpH4 cells (Figure 7.5). The effect of ephrinA1Fc on the level of MAPK phosphorylation and activity in EpRas cells was assessed in the same ways as described for EpH4 cells. EphrinA1Fc was able to induce only a slight decrease in MAPK phosphorylation, in contrast to the decrease observed in EpH4 cells (Figure 7.6a). Steady-state levels of MAPK protein were unaffected by the addition of ephrinA1Fc. Again, addition of human Fc or human IgG to the cells had no affect on levels of phosphorylated MAPK or total protein levels (Figure 7.6b).

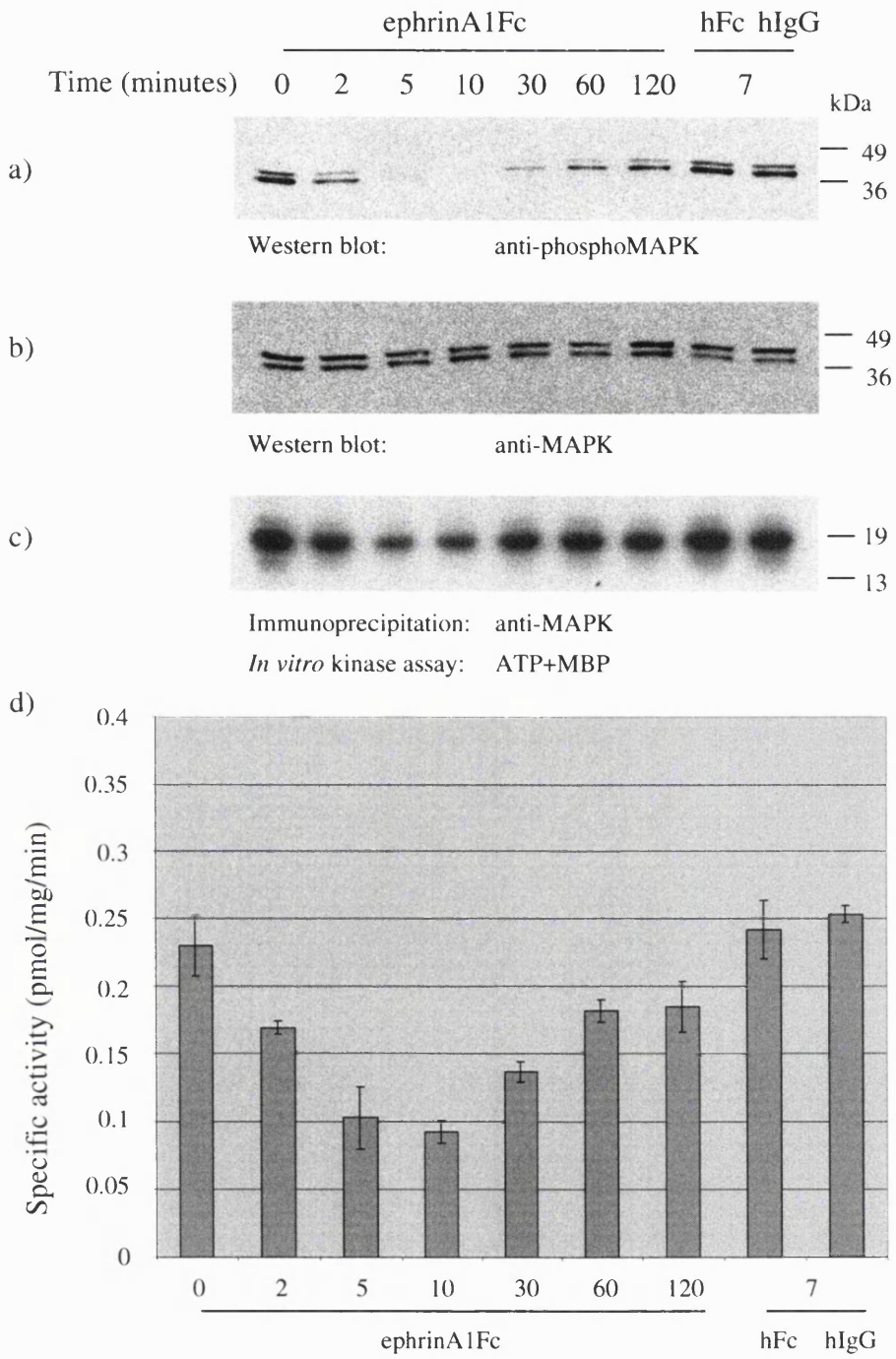


Figure 7.4. EphrinA1Fc Downregulates MAPK Activity in EpH4 Cells
5 x 10^5 EpH4 cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 2, 5, 10, 30, 60 or 120 minutes, or 1 µg/ml of purified human Fc (hFc) or human IgG (hIgG) for 7 minutes prior to lysis. (a) and (b) 25 µg of precleared lysate was subjected to SDS-PAGE and western blotting using either (a) an anti-phosphoMAPK antibody, or (b) an anti-MAPK antibody. (c) and (d) 50 µg of precleared lysate was immunoprecipitated with an anti-MAPK antibody and subjected to an *in vitro* kinase assay using MBP as a substrate. Phosphorylated MBP was then (c) subjected to SDS-PAGE and autoradiography, or (d) spotted onto P81 paper, washed in orthophosphoric acid and counted in a scintillation counter. Error bars denote standard error of the mean of three immunoprecipitations per sample.

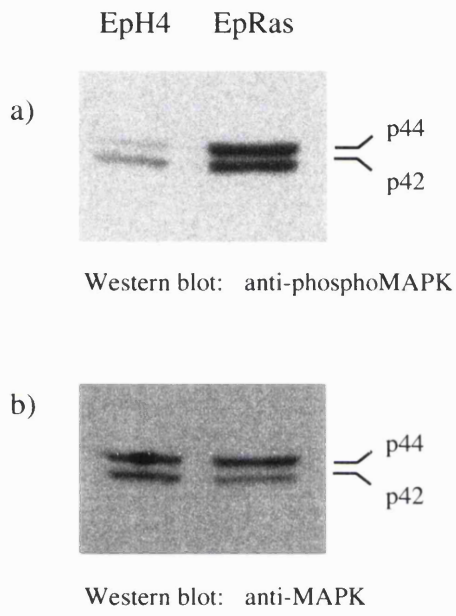


Figure 7.5. Comparison of MAPK Phosphorylation in EpH4 and EpRas Cells
5 x 10^5 EpH4 and EpRas cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. 25 µg of precleared lysate was then subjected to SDS-PAGE and western blotting with (a) an anti-phosphoMAPK antibody, or (b) an anti-MAPK antibody.

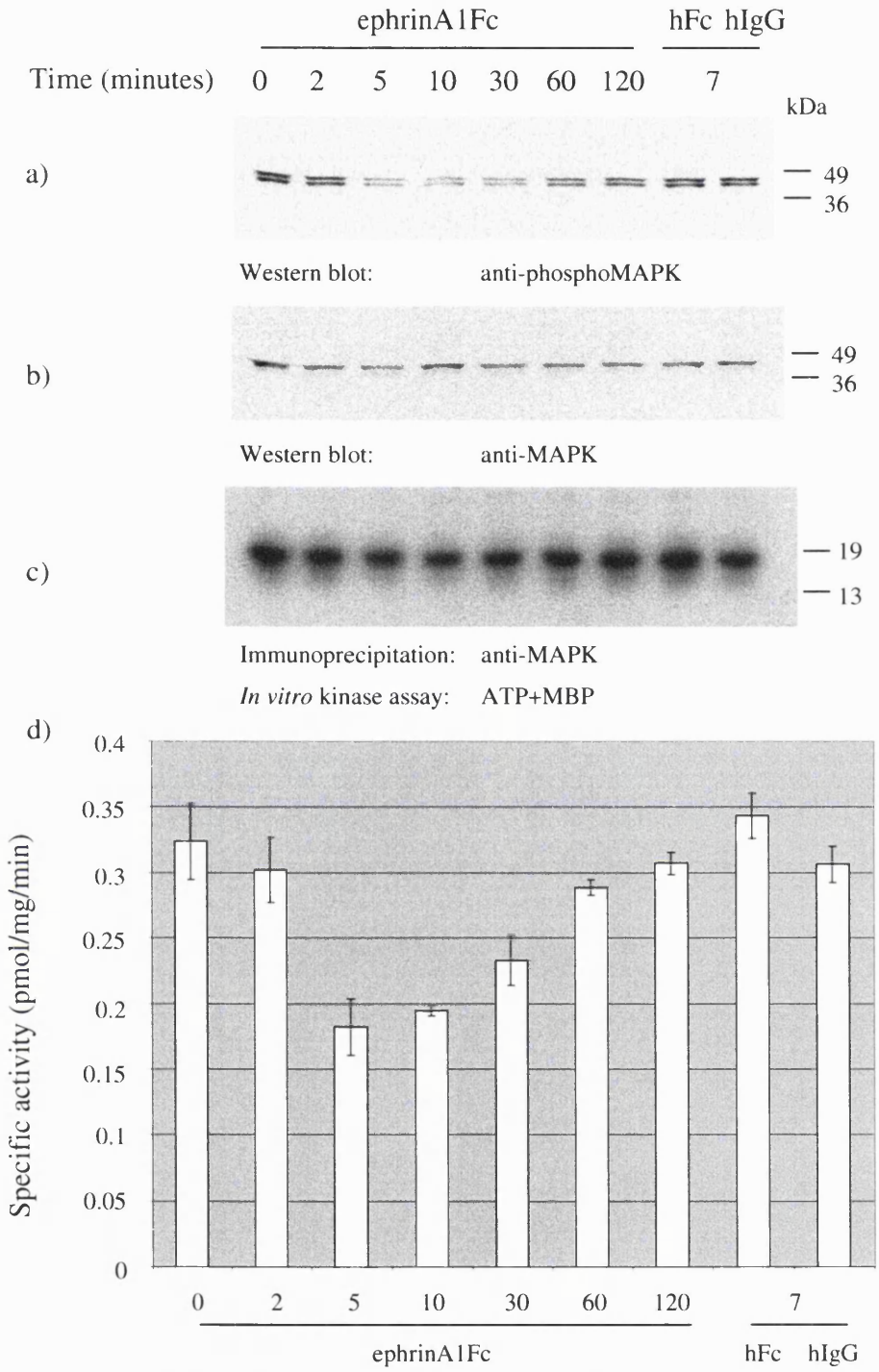


Figure 7.6. EpRas Cells Partially Overcome EphrinA1Fc-Mediated MAPK Inhibition
5 x 10^5 EpRas cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 2, 5, 10, 30, 60 or 120 minutes, or 1 µg/ml of purified human Fc (hFc) or human IgG (hIgG) for 7 minutes prior to lysis. (a) and (b) 25 µg of precleared lysate was subjected to SDS-PAGE and western blotting using either (a) an anti-phosphoMAPK antibody, or (b) an anti-MAPK antibody. (c) and (d) 50 µg of precleared lysate was immunoprecipitated with an anti-MAPK antibody and subjected to an *in vitro* kinase assay using MBP as a substrate. Phosphorylated MBP was then (c) subjected to SDS-PAGE and autoradiography, or (d) spotted onto P81 paper, washed in orthophosphoric acid and counted in a scintillation counter. Error bars denote standard error of the mean of three immunoprecipitations per sample.

The activity of MAPK in EpRas cells towards MBP was also slightly impaired by the addition of ephrinA1Fc (Figure 7.6c and d). The specific activity of MAPK was approximately two-fold less in cells treated with ephrinA1Fc for 5 minutes (0.18 pmol/mg/min) than in unstimulated cells (0.32 pmol/mg/min). Accordingly, a slight transient decrease in phosphorylated MBP was also observed upon treatment of EpRas cells with ephrinA1Fc (Figure 7.6c). Addition of human Fc or human IgG did not decrease the activity of MAPK towards MBP (Figures 7.6c and d).

In agreement with Figure 7.5, where EpRas cells were shown to have increased levels of phosphorylated MAPK over EpH4 cells, the specific activity of MAPK towards MBP in unstimulated EpRas cells was higher (at 0.32 pmol/mg/min) than in unstimulated EpH4 cells (0.23 pmol/mg/min).

7.2.6. Downregulation of MEK Activity by EphrinA1Fc

The decrease in MAPK activity observed in EpH4 cells, and to a lesser extent in EpRas cells, upon addition of ephrinA1Fc could be due to the decreased activity of MEK, or the increased activity of a MAPK phosphatase. To analyse the role of MEK in the observed downregulation of MAPK, western blotting using phospho-specific antibodies directed against dually phosphorylated MEK (anti-phosphoMEK) and MEK activity assays, measuring the activity of MEK towards recombinant GST-p42 MAPK (Section 2.3.9.3), were performed. EpH4 cells and EpRas cells were treated with 1 μg/ml of ephrinA1Fc for 0, 7, 25 or 40 minutes, or human IgG at 1 μg/ml for 7 minutes, before lysates were prepared and subjected to either SDS-PAGE and western blotting, or immunoprecipitation with an anti-MEK antibody followed by an *in vitro* kinase assay.

Western blot analysis with anti-phosphoMEK revealed that addition of ephrinA1Fc to EpH4 cells resulted in a marked downregulation of levels of phosphorylated MEK, which was apparent at the 7 and 25 minute time points (Figure 7.7a). By 40 minutes, the level of phosphorylated MEK appeared to be increasing slightly. The addition of human IgG had no effect on the phosphorylation of MEK. Steady-state levels of MEK were not affected by the addition of either ephrinA1 or human IgG (Figure 7.7b).

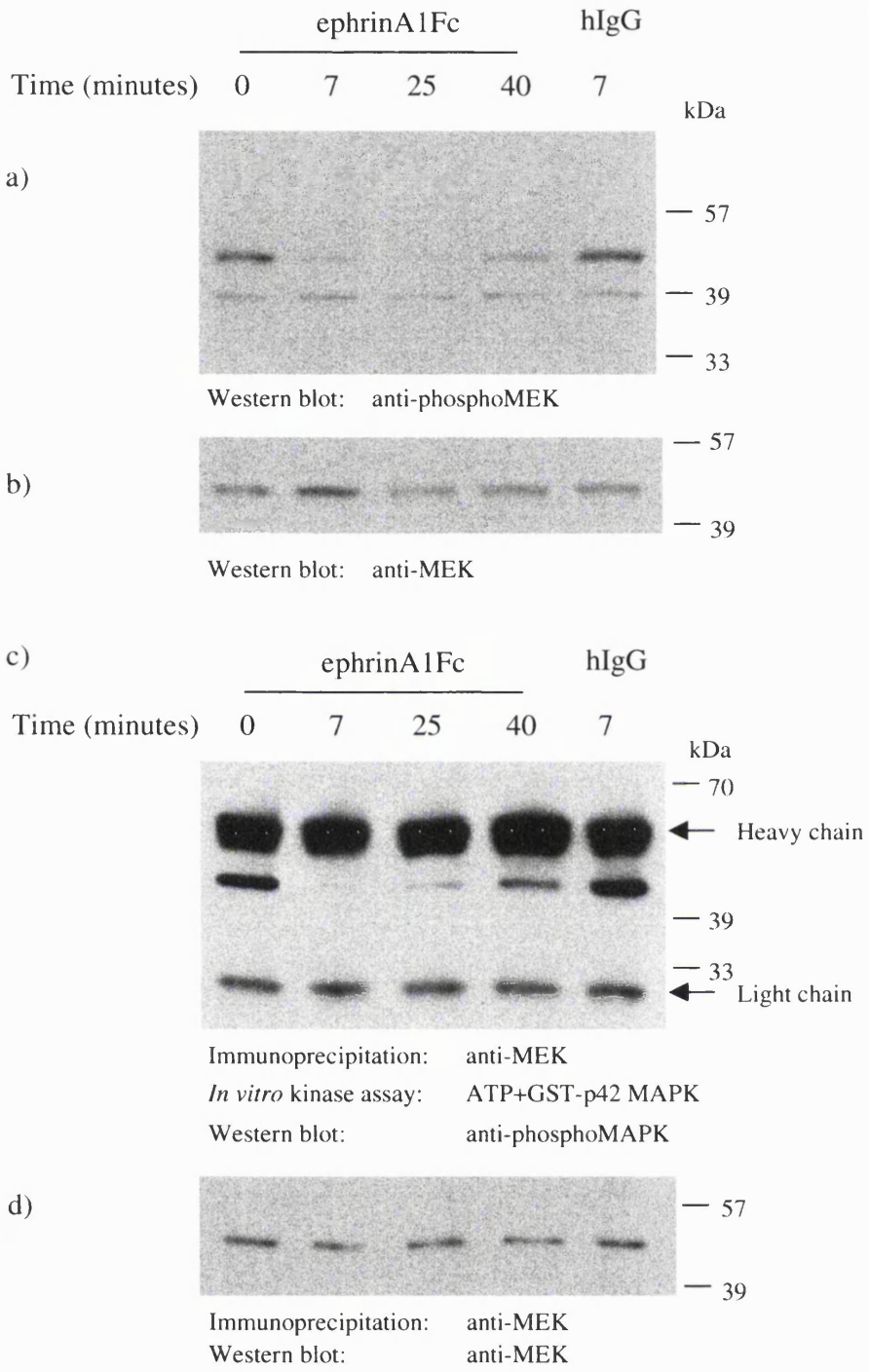


Figure 7.7. EphrinA1Fc Downregulates MEK Activity in EpH4 Cells
5 x 10^5 EpH4 cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 7, 25 or 40 minutes, or 1 µg/ml of human IgG (hIgG) for 7 minutes prior to lysis. (a) and (b) 25 µg of precleared lysate was subjected to SDS-PAGE and western blotting using (a) an anti-phosphoMEK antibody or (b) an anti-MEK antibody (NEB). (c) and (d) 75 µg of precleared lysate was immunoprecipitated with an anti-MEK antibody (Transduction Labs) and subjected to an *in vitro* kinase assay using GST-p42 MAPK as a substrate. Samples were then subjected to SDS-PAGE and western blotting using (c) an anti-phosphoMAPK antibody, or d) an anti-MEK antibody (NEB).

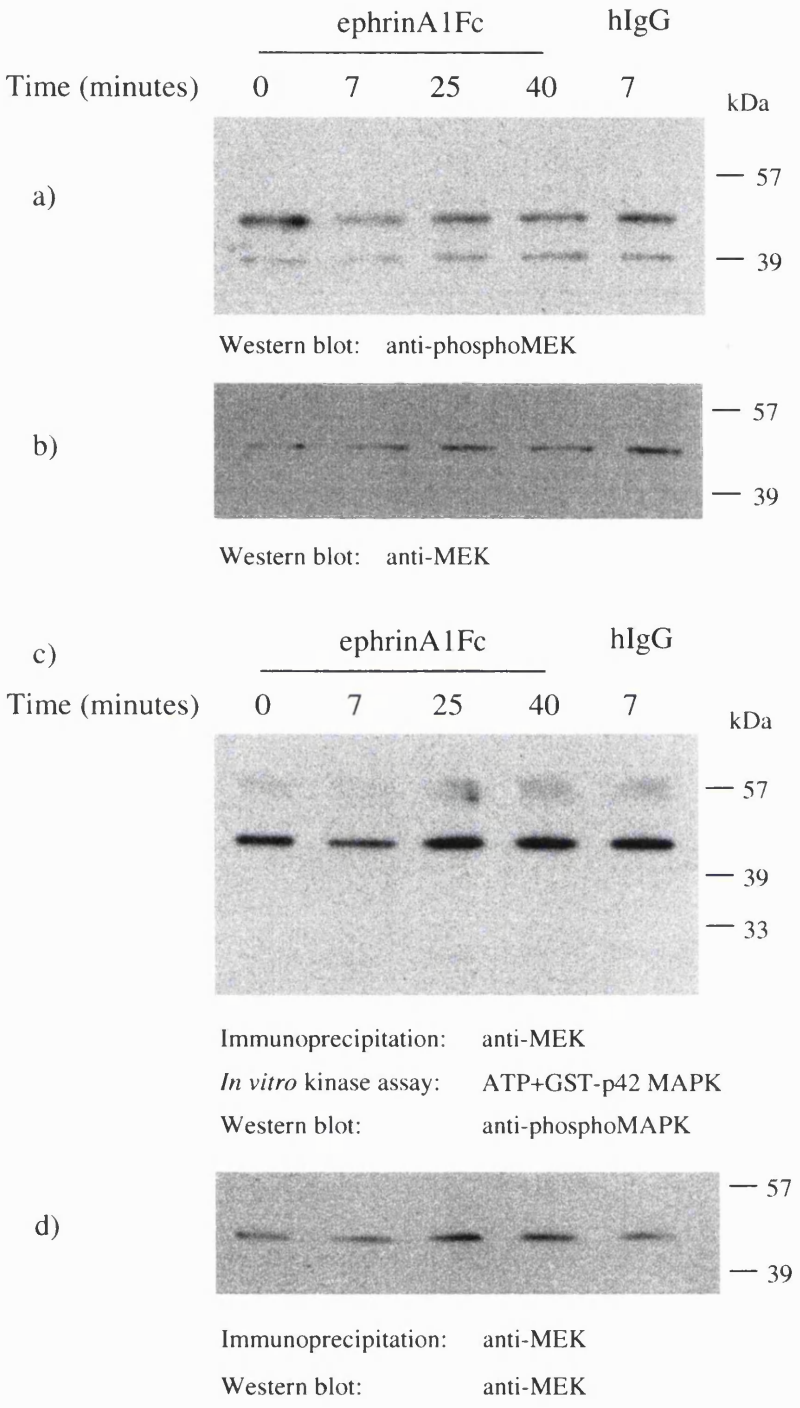


Figure 7.8. EphrinA1Fc-Mediated MEK Inhibition is Partially Overcome in EpRas Cells
5 x 10^5 EpRas cells were plated in 60 mm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 7, 25 or 40 minutes, or 1 µg/ml of human IgG (hIgG) for 7 minutes prior to lysis. (a) and (b) 25 µg of precleared lysate was subjected to SDS-PAGE and western blotting using (a) an anti-phosphoMEK antibody or (b) an anti-MEK antibody (NEB). (c) and (d) 75 µg of precleared lysate was immunoprecipitated with an anti-MEK antibody (Transduction Labs) and subjected to an *in vitro* kinase assay using GST-p42 MAPK as a substrate. Samples were then subjected to SDS-PAGE and western blotting using (c) an anti-phosphoMAPK antibody or (d) an anti-MEK antibody (NEB).

The activity of MEK towards exogenous GST-p42 MAPK was also strongly, but transiently, inhibited by the addition of ephrinA1Fc to EpH4 cells (Figure 7.7c). MEK activity was most compromised at the 7 minute time point but appeared to be increasing again following treatment with ephrinA1Fc for 40 minutes. Equal amounts of MEK were immunoprecipitated from the cell lysates (Figure 7.7d).

MEK phosphorylation and activity towards GST-p42 MAPK was only slightly impaired in EpRas cells in response to ephrinA1Fc. The level of phosphorylated MEK was only marginally reduced after treatment with ephrinA1Fc for 7 minutes, after which levels were almost unaffected (Figure 7.8a). MEK activity towards GST-p42 MAPK was similarly affected (Figure 7.8c and d).

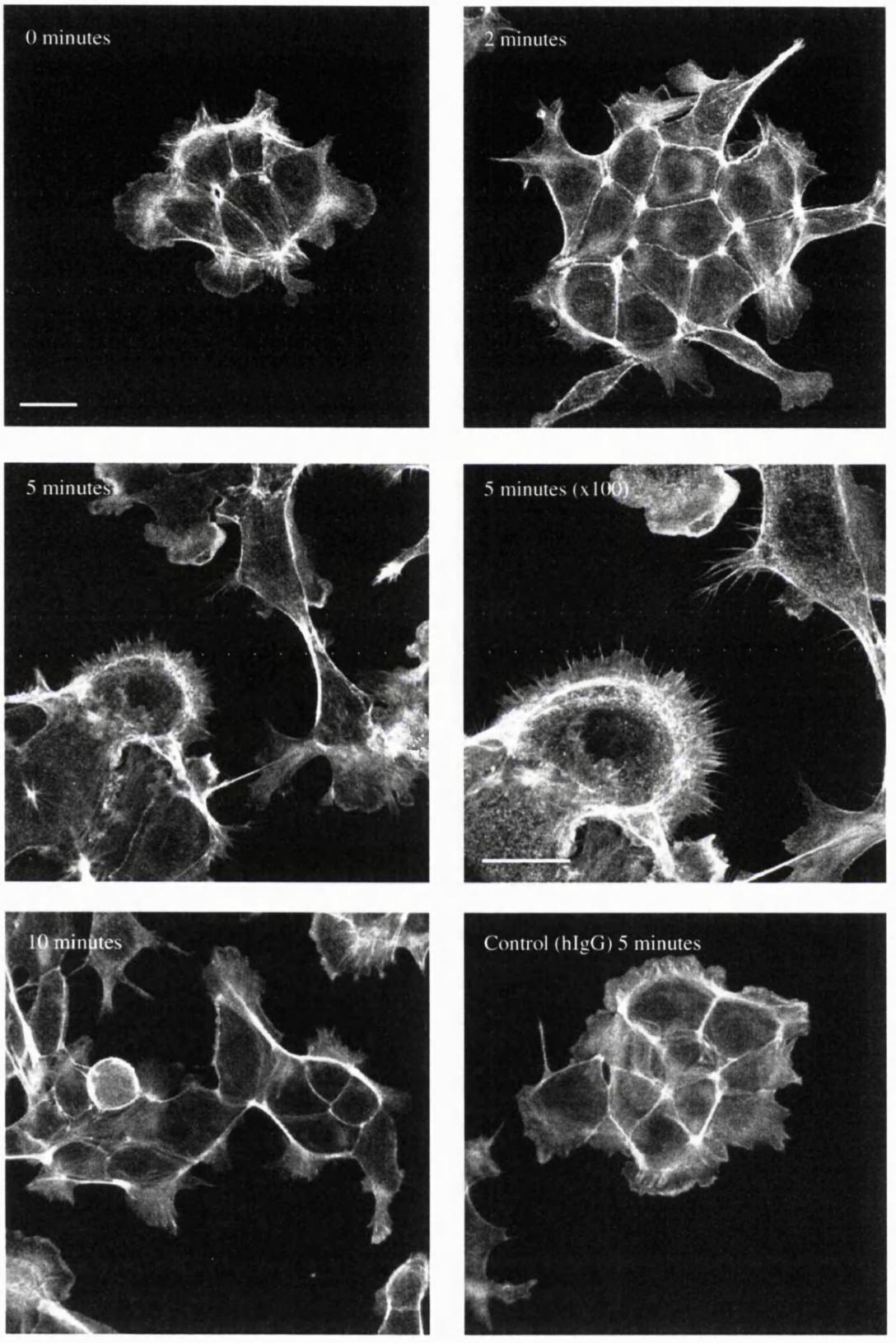
7.2.7. EphrinA1Fc Induces Cytoskeletal Changes in Mammary Epithelial Cells

Changes in the actin cytoskeleton are known to affect cell morphology and motility. To determine whether ephrinA1 was able to induce cytoskeletal changes in mammary epithelial cells, EpH4, EpRas and MDA-MB-231 cells were treated with 1 μg/ml of ephrinA1Fc over a 2 hour period, and filamentous actin (F-actin) was visualised using Alexafluor-conjugated phalloidin (Section 2.3.13). Prior to the addition of ephrinA1Fc, lamellae, fine actin bundles, and F-actin associated with cell junctions were observed in EpH4 cells. Lamellae, actin bundles, and cell junction-associated F-actin were also apparent in EpRas cells. Lamellae were absent in MDA-MB-231 cells, however, punctate staining was apparent within the cells. Treatment with ephrinA1Fc induced the formation, around the periphery of both individual cells and cells within colonies, of hair-like projections of considerable length, resembling filopodia (Figure 7.9a-c). Such structures were rarely observed in unstimulated cells, or control cells treated with hIgG. EphrinA1Fc-induced microspike formation was rapid, occurring within 2-5 minutes of ephrinA1Fc treatment. However, the nature of the response was transient, with the number of microspikes gradually decreasing over a 2 hour period of ephrinA1Fc-treatment (data not shown).

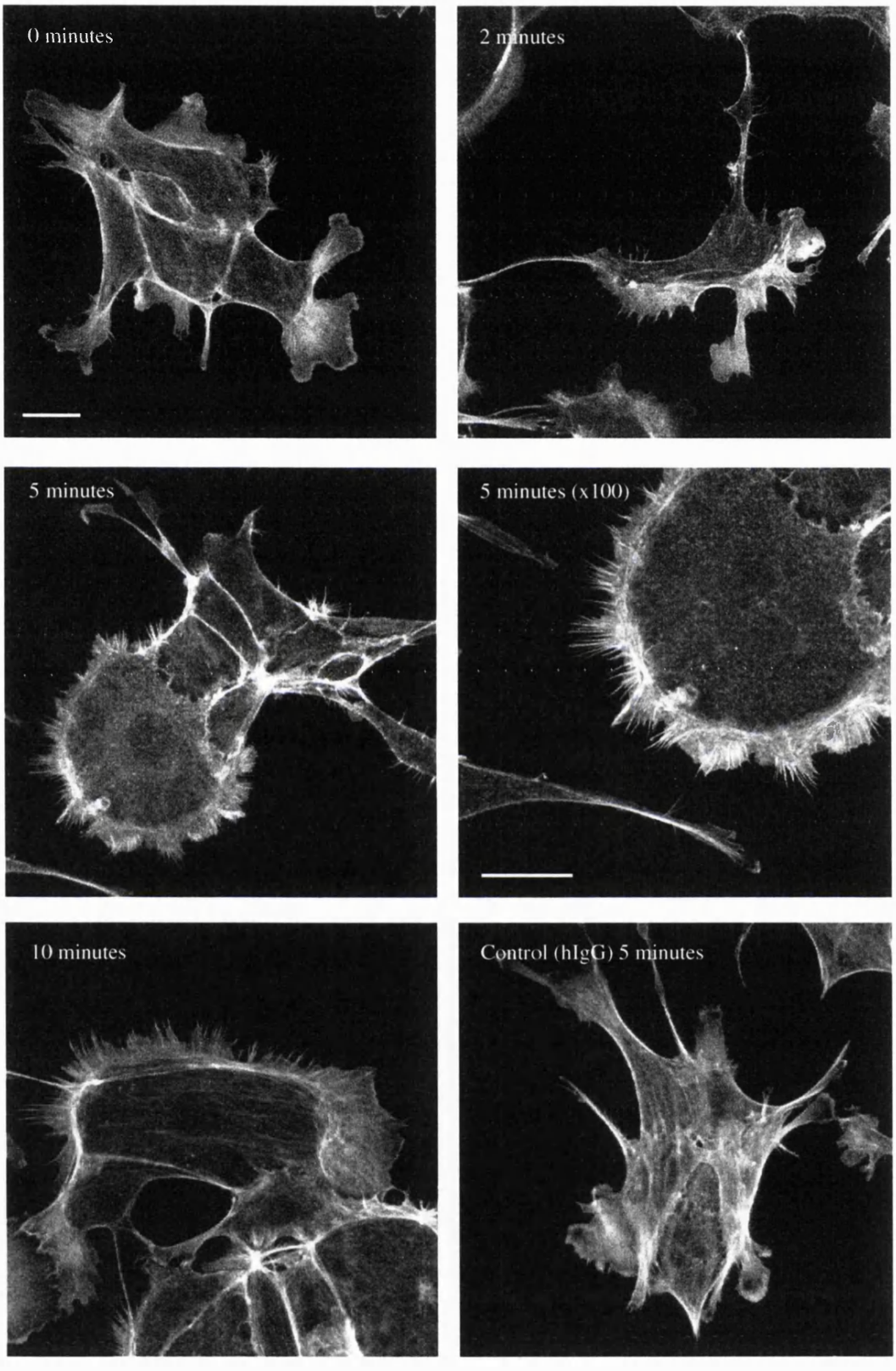
Figure 7.9. EphrinA1Fc Induces the Formation of Filopodia in Mammary Epithelial Cells

1 x 10^5 (a) EpH4, (b) EpRas or (c) MDA-MB-231 cells were plated onto coverslips in 60 mm dishes and cultured for 16 hours. Cell were then treated with 1 μg/ml ephrinA1Fc for 0, 2, 5 and 10 minutes, or 1 μg/ml of human IgG (hIgG) for 2 or 5 minutes, prior to fixation with 4% paraformaldehyde. Filamentous actin was then stained using Alexafluor-conjugated phalloidin and visualised by confocal microscopy. (x100) indicates that cells were viewed using the 100x objective. For all other images, cells were viewed using the 63x objective. Scale bars represent 20 μm.

a)


0 minutes
2 minutes
5 minutes
5 minutes (x100)
10 minutes
Control (hIgG) 5 minutes

b)


0 minutes
2 minutes
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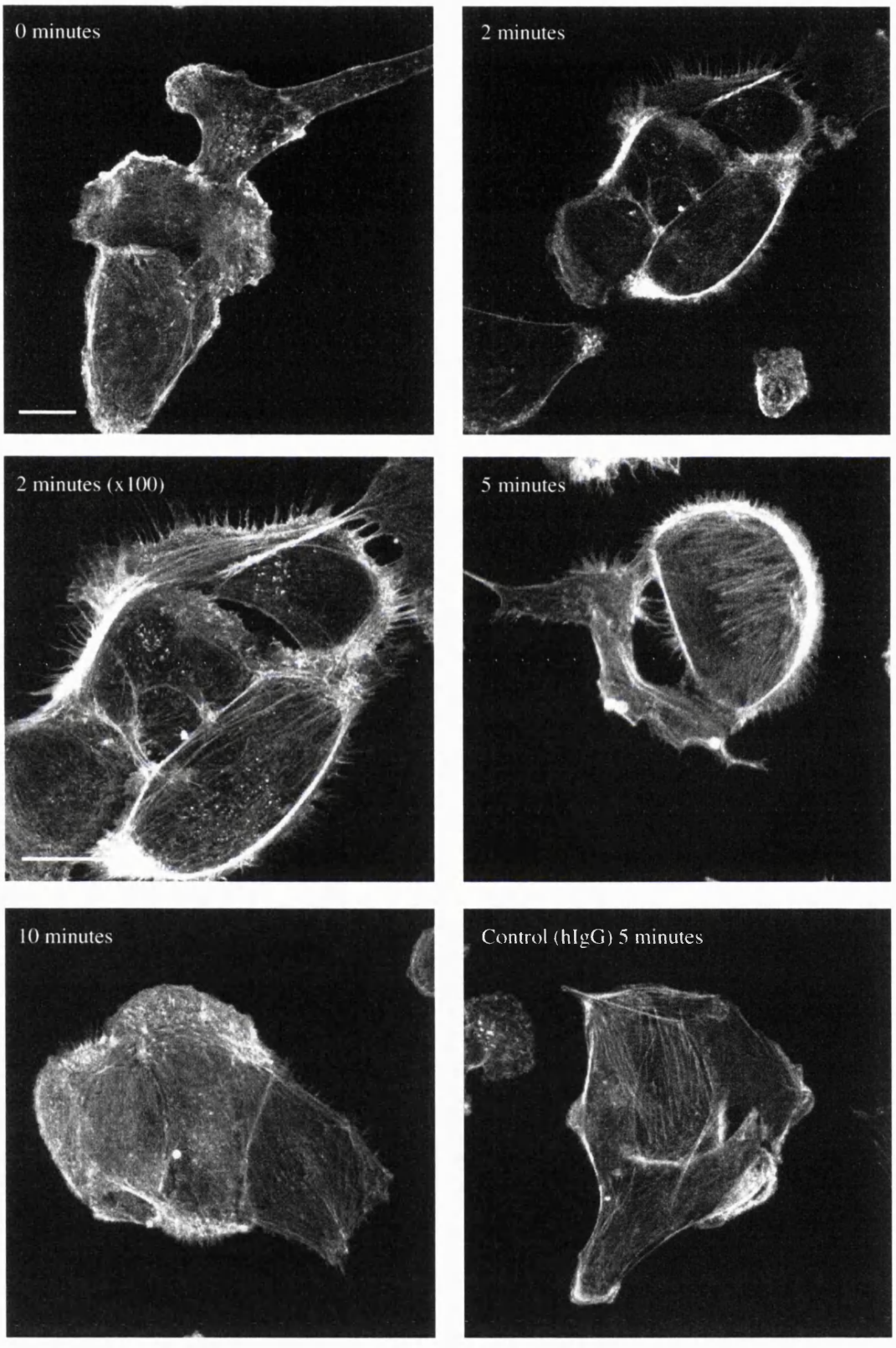
5 minutes (x100)
10 minutes
Control (hIgG) 5 minutes

c)


0 minutes
2 minutes
2 minutes (x100)
5 minutes
10 minutes
Control (hIgG) 5 minutes

7.2.9. EphrinA1Fc does not Activate JNK in EpH4 Cells

Previous reports have suggested that the small GTPases Rac and Cdc42 can act as upstream activators of JNK (Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995). To investigate whether treatment of EpH4 cells with ephrinA1Fc influenced the activity of JNK, three different approaches were taken. EpH4 cells were treated with 1 μg/ml of ephrinA1Fc for 0, 5, 10, 25, 40 and 60 minutes, or with 1 μg/ml of human IgG as a negative control. As a positive control for JNK activation, EpH4 cells were treated with 10 μg/ml of anisomycin for 30 minutes (Kyriakis *et al.*, 1994). Cell lysates were prepared and JNK activity was measured by i) western blotting with an antibody directed against the dually phosphorylated JNK (anti-phosphoJNK), ii) immunoprecipitating JNK and assaying its activity towards an exogenous substrate, GST-ATF2 (Section 2.3.9.5), or iii) incubating lysates with GST-c-Jun and assaying for associated kinases *in vitro* (Section 2.3.9.4).

Only in EpH4 cells treated with anisomycin was phosphorylated JNK detected using the anti-phosphoJNK antibody (Figure 7.10a). Unstimulated cells, or cells treated with ephrinA1Fc or human IgG showed no detectable levels of phosphorylated JNK. Equal amounts of total JNK protein were present in the cell lysates, as detected by an anti-JNK antibody (Figure 7.10b). In accordance with previous results, levels of MAPK phosphorylation were transiently downregulated by ephrinA1Fc (Figure 7.10c), whilst steady-state MAPK levels were unaffected (Figure 7.10d), confirming the bioactivity of the ligand.

The activity of immunoprecipitated JNK towards GST-ATF2 was measured in response to ephrinA1Fc treatment. GST-ATF2 was phosphorylated at a basal level by JNK immunoprecipitated from lysates of both human IgG- and ephrinA1Fc -treated cells (Figure 7.10e). Again, only treatment of cells with anisomycin resulted in an increase in the activity of JNK. Equal levels of JNK were immunoprecipitated from the cells (Figure 7.10f).

Finally, there was no detectable increase in the level of c-Jun-N-terminal kinase activity associated with recombinant GST-c-Jun in lysates prepared from cells treated with ephrinA1Fc (Figure 7.10g). EpH4 cells treated with anisomycin showed a marked increase in the amount of phosphorylated GST-c-Jun, as expected.

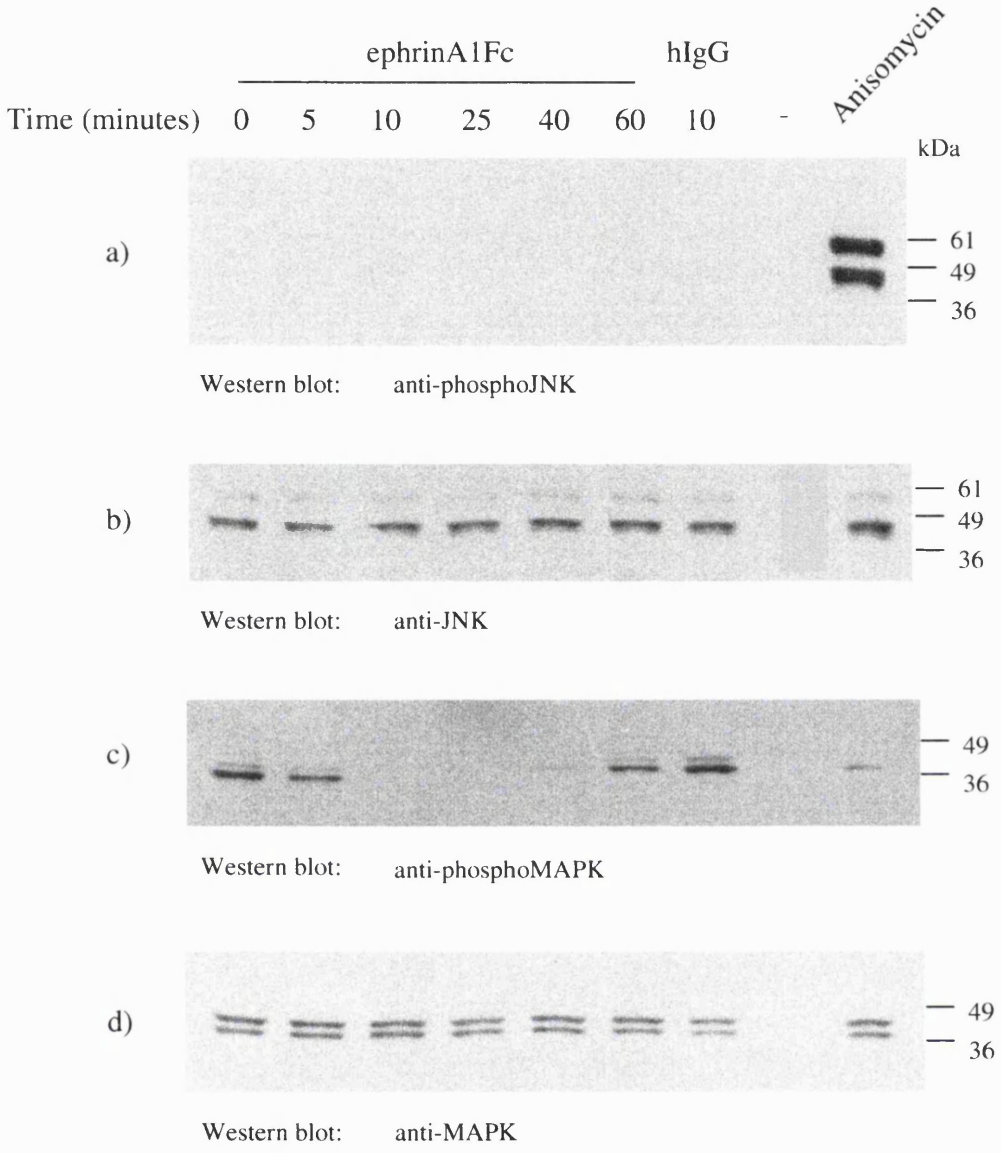
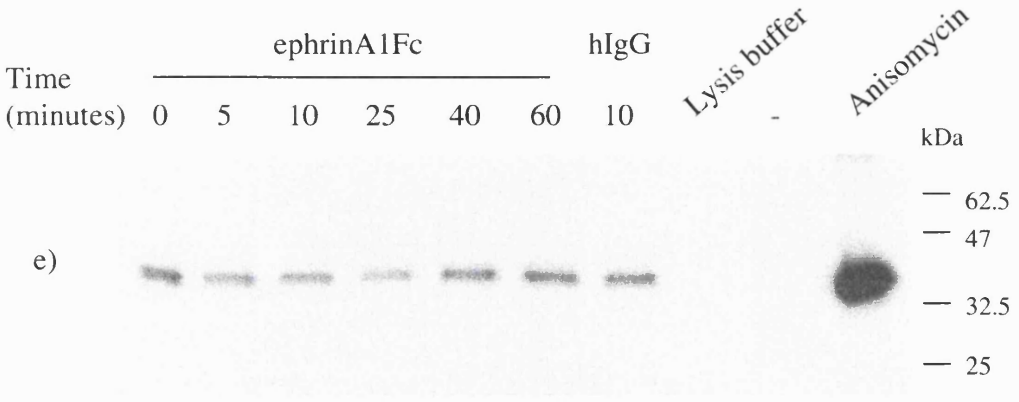


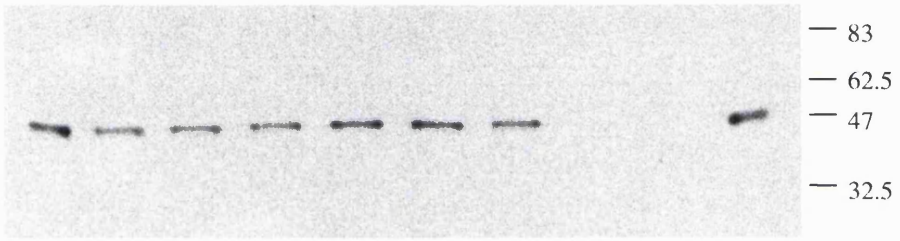
Figure 7.10. EphrinA1Fc does not Increase JNK Activity in EpH4 Cells
1.5 x 10^6 EpH4 cells were plated in 10 cm dishes and cultured for 16 hours in growth medium containing 0.5% FCS. Cells were treated with 1 µg/ml ephrinA1Fc for 0, 5, 10, 25, 40 or 60 minutes, 1 µg/ml of human IgG (hIgG) for 10 minutes, or 10 µg/ml anisomycin for 30 minutes prior to lysis. (a)-(d) 25 µg of precleared lysate was subjected to SDS-PAGE and western blotting using (a) an anti-phosphoJNK antibody, (b) an anti-JNK1 (FL) antibody, (c) an anti-phosphoMAPK antibody or (d) an anti-MAPK antibody. (e) and (f) 250 µg of precleared lysate was immunoprecipitated using a rabbit anti-JNK (C-17) antibody and subjected to an *in vitro* kinase assay using GST-ATF2 as a substrate. Samples were then subjected to SDS-PAGE and (e) autoradiography or (f) western blotting using a goat anti-JNK (C-17) antibody. (g) 250 µg of precleared lysate was incubated with 4 µg of GST-c-Jun (1-79) and precipitated on glutathione beads. Samples were then subjected to an *in vitro* kinase assay followed by SDS-PAGE and autoradiography.



e)

Immunoprecipitation: anti-JNK-1

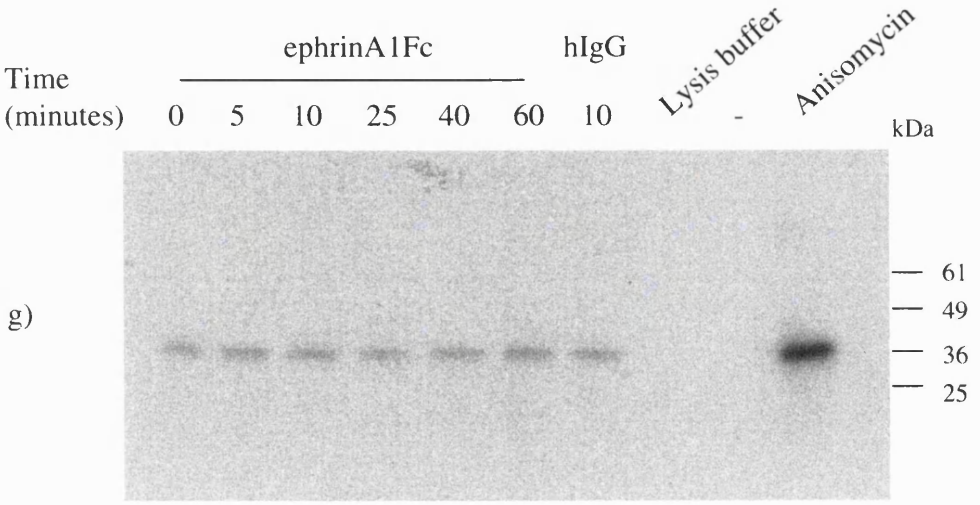
In vitro kinase assay: ATP+GST-ATF2



f)

Immunoprecipitation: anti-JNK-1

Western blot: anti-JNK-1



g)

Pulldown: Glutathione Sepharose

In vitro kinase assay

7.3. Discussion

7.3.1. Analysis of EphA2 Activation

Levels of tyrosine phosphorylation of the EphA2 receptor induced by soluble ephrinA1Fc in both EpH4 and EpRas cells were seen to peak at 5-10 minutes, and then fall again. This is similar to the activation profiles of other tyrosine kinase receptors such as Trk and EGF receptors. Furthermore, in other independent studies, EphA2 activation by ephrinA1Fc was also rapid (Dodge-Zantek *et al.*, 1999; Miao *et al.*, 2000). Slower induction of receptor phosphorylation has been reported for other Eph receptors, for instance tyrosine phosphorylation of EphB2 by ephrinB1 was still seen to be increasing after 10 minutes, reaching its maximum only after 30-40 minutes of stimulation (Gale and Yancopoulos, 1997). However, in addition to reflecting differences in the affinity of the particular ligand-receptor interaction, other factors, such as the cell type, are likely to contribute to different Eph receptor activation profiles.

The steady-state levels of full length EphA2 receptor protein were seen to decrease in both EpH4 cells and EpRas cells following induction of high levels of tyrosine phosphorylation by treatment with ephrinA1Fc. This decrease is most likely a result of agonist-induced receptor downregulation, a common occurrence in receptor tyrosine kinases (reviewed in Sorkin and Waters, 1993). Ligand binding causes receptors to be clustered and internalised into clathrin-coated vesicles, whereupon the ligand-receptor complex is delivered to an early endosome. Here, the decision is made on whether to recycle the receptor to the membrane or whether to target it for degradation, processes which are thought to be the subject of receptor-specific regulation. In the case of the EGF receptor, the decision on whether to recycle or degrade is dependent upon recruitment of the Cbl adaptor protein to the autophosphorylated receptor. Cbl then becomes phosphorylated, resulting in the recruitment of, and subsequent ubiquitination by, E2 ubiquitin ligase, (Levkowitz *et al.*, 1999). This targets the receptor for degradation in the late endosome/lysosome by both proteasomal and lysosomal mechanisms. Similar mechanisms are thought to operate in other receptors, such as PDGF and CSF-1 (Levkowitz *et al.*, 1999). A recent study (Bao *et al.*, 2000) suggests that phosphorylation by PKC can prevent EGF entering the degradative pathway and instead target it for recycling. There is also growing evidence that internalisation may serve to target activated receptors to an appropriate location within the cell, in order to

interact with different signalling complexes to those present at the cell membrane (reviewed in Leof, 2000).

The process of Eph receptor downregulation has not been studied, but the rapid decrease in levels of full length EphA2 protein as a result of ephrinA1Fc stimulation suggests that the response to receptor activation is tightly controlled in mammary epithelial cells. In the case of EphA2, a ~50 kDa protein, which was recognised by both the anti-phosphotyrosine and the anti-EphA2 antibody, was observed soon after activation of EphA2 by ephrinA1Fc in EpRas cells. The presence of this immunoreactive protein has been noted previously on several occasions (e.g. Figure 3.1b, Figure 5.10, Figure 5.11, Figure 7.2 and data not shown), and appears to be correlated with EphA2 receptor activation either as a result of ligand-induced dimerisation or high levels of protein expression. This protein was immunoprecipitated by antibodies directed against the carboxy terminus of EphA2, and is therefore likely to represent the intracellular domain. A similar sized band was also observed following immunoprecipitation of EphA2 from A431 cell lysates using carboxy terminal antisera (Lindberg and Hunter, 1990). Since the 50 kDa protein was also recognised by anti-phosphotyrosine antibodies and its presence correlated with receptor activation, it seems likely that it corresponds to the intracellular domain of EphA2 that has been proteolytically cleaved following autophosphorylation. Whether this has the capacity to signal within the cell is unknown.

Significantly, an interaction between recombinant ephrinA1Fc and EphA2 was demonstrated. A high affinity receptor-ligand interaction (K_d = 1.33 nM) has previously been reported by measuring the binding of an EphA2Fc fusion protein to transiently expressed ephrinA1 (Gale *et al.*, 1996a). Furthermore, the time point at which levels of EphA2 protein isolated by ephrinA1Fc bound to protein A-Sepharose began to diminish appears to correlate with the time at which the levels of full length immunoprecipitable EphA2 decreased.

7.3.2. EphrinA1Fc-Mediated Downregulation of MAPK

EphrinA1Fc was demonstrated to transiently downregulate MAPK phosphorylation and activity in EpH4 cells, and to a lesser extent in EpRas cells. EphA2 is a strong candidate for mediating this downregulation of MAPK based on its rapid activation in response to ephrinA1Fc and the demonstration that it is able to physically associate with

ephrinA1Fc. Also relevant is the transient nature of the decrease in MAPK activity, which strongly correlates with the transient activation of the EphA2 receptor. Indeed, MAPK activity begins to return to pre-stimulated levels concomitant with the previously discussed downregulation of EphA2.

7.3.2.1. Possible Causes of the Downregulation of MAPK Activity

Further investigation of upstream components of the MAPK pathway revealed that the activity of MEK was also inhibited in EpH4 cells by ephrinA1Fc, but to a much lesser extent in EpRas cells. Attempts to study the effect of ephrinA1Fc on Raf phosphorylation and activity were unsuccessful due to problems with assay reproducibility. Despite carrying out Raf immunoprecipitations from relatively large amounts of total cell lysate (500 μg – 1 mg), basal Raf activity was only just detectable (data not shown). Since only a minor proportion of total Raf becomes activated in response to MAPK pathway agonists (Hallberg *et al.*, 1994), small changes in Raf activity in response to ephrinA1Fc would be difficult to detect, particularly if they resulted in decreased activity. The activation of Raf is a complex process (Morrison and Cutler, 1997), which is not fully understood. EphrinA1Fc may negatively influence any of several factors that are involved in regulating aspects of Raf activation, such as its recruitment to the plasma membrane (Leevers *et al.*, 1994), its interaction with 14-3-3 proteins (Tzivion *et al.*, 1998) or its phosphorylation (Mason *et al.*, 1999), and thus negatively impact on MAPK activation. Interestingly, p21-activated kinase (PAK) has recently been shown to phosphorylate Raf (Chaudhary *et al.*, 2000) upon integrin activation in a PI3K-dependent manner, therefore there may be a requirement for cell-ECM interactions in PAK-mediated Raf activation and, as discussed below, these have been shown to be compromised by ephrinA1Fc.

Attempts were also made to investigate any potential changes in the activity of either Ha-Ras or R-Ras, using the Ras binding domain of Raf coupled to a GST-tag (GST-RBD) as a means of isolating active Ras (de Rooij and Bos, 1997), but the assay did not prove sufficiently robust for any conclusions to be made. Previous studies have shown that p120 GAP is capable of binding EphB2 and EphB3 (Holland *et al.*, 1997; Hock *et al.*, 1998). As a GTPase activating protein, p120 GAP negatively regulates Ras signalling pathways (Boguski and McCormick, 1993) and might therefore downregulate MEK and MAPK activities. However, analysis of the juxtamembrane motif of EphA2 and other members of the EphA subfamily reveals a non-conservative substitution in the

sequence motif to which p120 GAP binds (Hock *et al.*, 1998) (from YXXPXE in EphB2 and EphB3 to YXXPXQ in EphA2).

The small GTPase R-Ras has recently been demonstrated to become tyrosine phosphorylated in response to activation of EphB2 (Zou *et al.*, 1999). Phosphorylation occurred within the effector domain of R-Ras and, consequently, binding to GST-RBD was inhibited. Although R-Ras is able to interact with Raf, the issue of whether R-Ras is able to activate the MAPK pathway is controversial (Cox *et al.*, 1994; Rey *et al.*, 1994; Marte *et al.*, 1997; Osada *et al.*, 1999). Thus, whether Eph receptor-mediated tyrosine phosphorylation of R-Ras could function to downregulate MAPK activity is unclear.

EphrinA1Fc has recently been found to suppress integrin function and cause FAK dephosphorylation by recruiting the tyrosine phosphatase Shp2 to the activated EphA2 receptor (Miao *et al.*, 2000). Integrin-mediated 'outside-in' signalling is known to regulate cell growth (Howe *et al.*, 1998). Tyrosine phosphorylation of FAK, and its activity, increase upon engagement of integrins, and a number of signalling molecules such as Grb2 and Shc are recruited to focal adhesions (reviewed in Cary *et al.*, 1999). Signalling via FAK is therefore likely to be responsible for the increase in MAPK activity observed upon cell adhesion to the ECM (Chen *et al.*, 1994; Morino *et al.*, 1995; Schlaepfer *et al.*, 1998). Thus, suppression of integrin function, and accompanying dephosphorylation of FAK may be responsible for the ephrinA1Fc-induced decrease in MAPK activity observed in EpH4 cells.

In EpRas cells, the ephrinA1Fc-induced decrease in MAPK phosphorylation and activation was partially overcome compared to that observed in EpH4 cells. At the level of MEK, the effect of ephrinA1Fc was barely detectable in EpRas cells. This diminished response to ephrinA1Fc by EpRas cells is not unexpected in view of the increased levels of MAPK phosphorylation and activity observed in these cells. Furthermore, it suggests that in cells with a higher basal activity of MAPK, such as in some fully transformed cells, ephrinA1Fc-mediated downregulation of MAPK activity may be overridden.

Inhibition of MAPK activity in response to Eph receptor activation has not previously been reported. Indeed, a recent report suggests that a chimera comprising the

extracellular domain of EphA5 fused to human Fc caused an increase in MAPK activity in ephrinA5-expressing NIH3T3 cells through 'reverse'-signalling (Davy and Robbins, 2000). Significantly, engagement of ephrinA5 also induced increased cell attachment by activating β1 integrin (Davy and Robbins, 2000; Huai and Drescher, 2001). Thus, although these results appear to contradict the findings of Miao and colleagues (Miao *et al.*, 2000) in terms of promotion of integrin function, they do confirm that integrin activation and MAPK activation are positively linked. The difference in integrin function observed by the two groups may be due to the use of different cell types, or to the difference in response between receptor-induced ligand activation and ligand-mediated receptor activation. A second group have also reported an increase in MAPK activity in response to Eph receptor activation. Zisch and colleagues observed an increase in the activity of recombinant MAPK towards MBP when it was co-transfected into 293 cells with wild-type EphB2, but not when it was co-transfected with a kinase-inactivated form of EphB2 (Zisch *et al.*, 2000). Although the discrepancy between the increase in MAPK activity reported by Zisch and colleagues and the ephrinA1Fc-mediated downregulation of MAPK activity presented in this chapter may reflect a genuine difference in the response between EphA and EphB receptor subgroups, it may also be due to experimental artefacts caused by the high expression levels of the recombinant kinases attained in transient transfections. In contrast, experiments carried out in this chapter were shown to influence the activity of endogenous components of signalling pathways, thereby avoiding such problems.

7.3.2.2. Possible Consequences of the Downregulation of MAPK Activity

As described in Section 1.2.4.2, MAPK can influence the activity of a number of cytoplasmic and nuclear proteins. Among the cytoplasmic targets of MAPK is MLCK, the activity of which is increased by MAPK phosphorylation (Klemke *et al.*, 1997). As a result, myosin light chains are phosphorylated, and myosin ATPase activity and actin cable polymerisation ensue, causing cell contraction and thus migration. Studies by Klemke (Klemke *et al.*, 1997), and more recently by Nguyen (Nguyen *et al.*, 1999) and Fincham (Fincham *et al.*, 2000) provide evidence that MLCK is involved in MAPK-mediated spreading and migration. A number of studies have reported a decrease in migration upon treatment of cells with the MEK inhibitor, PD 98059 (Klemke *et al.*, 1997; Nguyen *et al.*, 1998; McCawley *et al.*, 1999). Interestingly, a decrease in cell migration in response to ephrinA1Fc was reported by Miao and colleagues (Miao *et al.*, 2000). It can be envisaged that decreased cell spreading and migration might underlie

some of the 'repulsive' processes in which Eph receptor signalling has been implicated, such as inhibiting cell mixing across rhombomere boundaries.

Previous studies demonstrated that MAPK-induced cell migration was independent of changes in gene transcription (Nguyen *et al.*, 1999). Whether or not the ephrinA1Fc-mediated decrease in MAPK activity impinges on aspects of gene transcription remains to be studied. MAPK positively regulates the expression of the cyclin D1 protein, the accumulation of which is necessary for passing the G1 checkpoint (Lavoie *et al.*, 1996). Interestingly, addition of ephrinA1Fc to mammary epithelial cells for 4 hours resulted in a decrease in cell proliferation following decreased cell-ECM interactions (Dodge-Zantek *et al.*, 1999), implying that the decrease in MAPK activity does indeed affect nuclear activities.

The transient nature of the ephrinA1Fc-induced downregulation of MAPK activity appears to correlate strongly with the rapid, but transient, activation of EphA2. The co-incidence of the restoration of MAPK activity with the downregulation of EphA2 receptor levels suggests that the ephrinA1Fc-mediated decrease in MAPK levels must be tightly regulated. It would be possible to study this effect by downregulating the activity of MEK with a compound such as PD 98059 or U0126. However, as this approach will only target the MAPK pathway, it is unlikely to provide an accurate reflection of the physiological situation in which multiple signalling components downstream of EphA2 may be involved. An alternative approach would be to inhibit the downregulation of EphA2 whilst continuing to treat the cells with ephrinA1Fc, which may mimic the effects of chronic ephrinA1Fc stimulation.

7.3.3. EphrinA1Fc-Mediated Cytoskeletal Changes

EphrinA1Fc was seen to induce the formation of microspikes resembling filopodia in mammary epithelial cells. Filopodia, which are thought to play a role in helping the cell to sense its environment, are regulated by the activity of the GTPase Cdc42, whereas Rac and Rho, the other key mediators of cytoskeletal organisation, regulate the formation of lamellipodia and stress fibres, respectively (Nobes and Hall, 1995). However, attempts to demonstrate that ephrinA1Fc treatment increased the activity of Cdc42 using the Cdc42-GTP binding domain of PAK coupled to GST-Sepharose in a pull-down assay were unsuccessful. An alternative approach to demonstrate that activation of Cdc42 was responsible for the formation of filopodia would make use of

Cdc42 constructs bearing the N17 mutation which should act in a dominant negative manner to block the ephrinA1Fc-induced response. In some cases, a hierachical activation of the Rho GTPases has been reported, such that Cdc42 is able to activate Rac, while Rac is able to activate Rho (Ridley *et al.*, 1992; Nobes and Hall, 1995; Peppelenbosch *et al.*, 1995). However, this appears not to be the case in the mammary epithelial cells used in these studies, as the formation of lamellipodia and stress fibres was not induced following treatment with ephrinA1Fc. More recently, ephrinA1Fc has been demonstrated to induce neuronal growth cone collapse by activating Rho (Wahl *et al.*, 2000) and, in this case, the activity of Rac was reduced.

The activity of Rho GTPases, like that of Ras, is controlled by the balance of GEFs and GAPs (reviewed in Kaibuchi *et al.*, 1999), therefore it is likely that the induction of filopodia in mammary epithelial cells in response to ephrinA1Fc occurs as a result of Eph-mediated change(s) in the activity of one or more of these factors. The mechanism by which upstream activators influence RhoGEF activity has been most well-studied in the Vav family of proteins, whose activity is stimulated by tyrosine phosphorylation (Crespo *et al.*, 1997; Han *et al.*, 1997; reviewed in Bustelo, 2000). Although the ability of Cdc42 activity to be stimulated by members of the Vav family is still a contentious issue (Olson *et al.*, 1996; Han *et al.*, 1997; Abe *et al.*, 2000; Bustelo, 2000) tyrosine phosphorylation of Vav, either directly by EphA2, or through recruitment of an adaptor protein, provides an attractive mechanism by which treatment with ephrinA1Fc causes the induction of filopodia in mammary epithelial cells. Formation of filopodia in response to Met receptor activation by HGF has recently been reported as the first instance of Cdc42 activation downstream of a receptor tyrosine kinase (Royal *et al.*, 2000).

PI3K activation by PDGF is known to regulate the activity of Rac (Hawkins *et al.*, 1995). More recently, the PDGF-induced formation of filopodia has been reported to be mediated by the p85 subunit of PI3K independently of PI3K catalytic activity (Jimenez *et al.*, 2000). Since EphA2 activation has been shown to both recruit the p85 subunit of PI3K, and to stimulate PI3K activity (Pandey *et al.*, 1994), this may also provide a means by which filopodia are induced in response to ephrinA1Fc.

7.3.3.1. Possible Consequences of EphrinA1Fc-induced Cdc42 Activation

A number of Cdc42 effectors have been reported, including PAK, activated Cdc42-associated tyrosine kinase (ACK), IQGAP, Wiscott-Aldrich syndrome protein (WASP)/N-WASP and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (reviewed in Kaibuchi *et al.*, 1999; Bishop and Hall, 2000), but recent evidence implicates WASP/N-WASP in the formation of filopodia (Miki *et al.*, 1998). In addition to directly binding to actin monomers, both WASP and N-WASP can bind and activate the Arp2/3 complex (Machesky and Insall, 1998; Rohatgi *et al.*, 1999), which functions as a site of nucleation for polymerisation of new actin filaments (Machesky and Gould, 1999; Welch, 1999).

Addition of ephrinA1Fc to mammary epithelial cells was seen to induce filopodia around the entire periphery of individual cells, or colonies of cells. This may occur in order that these cells 'sense' their immediate environment before a migratory response is initiated. Filopodia are more frequently observed, together with lamellipodia, at the so-called 'leading edge' of migrating cells in order that movement is directional, and a recent study found that Cdc42 activity was necessary to maintain this cell polarisation in migrating fibroblasts (Nobes and Hall, 1999). An active role for Cdc42 in cell migration and invasion has been proposed by a number of groups (Keely *et al.*, 1997; Allen *et al.*, 1998; Banyard *et al.*, 2000; Royal *et al.*, 2000). Significantly, Keely and colleagues demonstrated that a constitutively activated form of Cdc42 (and Rac) disrupted cell polarisation in mammary epithelial cells and enhanced their motility and invasion on collagen (Keely *et al.*, 1997). Although in the case of Rac, downstream activation of PAK was excluded as a means by which this occurred, this was not demonstrated for Cdc42, and interestingly, increased activation of PAK has been implicated in breast cancer cell invasiveness (Adam *et al.*, 1998; Adam *et al.*, 2000). However, increased activity of PI3K was implicated by Keely and colleagues as being sufficient for the Cdc42 and Rac-mediated increase in migration (Keely *et al.*, 1997).

7.3.3.2. EphrinA1-Mediated Cytoskeletal Changes Occur Independently of JNK

Treatment of EpH4 cells with ephrinA1Fc was not found to influence the activity of JNK, as measured by three different assays. As yet, there have been no reports that activation of EphA receptors can activate the JNK pathway, although increased JNK activity in response to activation of EphB1 and EphB2 has been reported (Stein *et al.*,

1998b; Becker *et al.*, 2000; Zisch *et al.*, 2000). This may reflect differences in signalling pathways activated by the EphAs and EphBs, since recruitment of the adaptor protein Nck was found to be required for the EphB1- and EphB2- mediated increase in JNK activity, and a previous study reported that EphA4 was incapable of binding Nck (Ellis *et al.*, 1996).

A number of groups have reported that Cdc42 and Rac are able to activate JNK and p38 pathways (Coso *et al.*, 1995; Minden *et al.*, 1995), although these findings were the result of overexpression of constitutively active forms of Rac and Cdc42 in transient assays, whereas experiments carried out in this chapter relied on activation or repression of endogenous signalling pathway components. Furthermore, it has also been demonstrated that Cdc42 is able to induce the formation of filopodia independently of changes in JNK activity (Lamarche *et al.*, 1996; Puls *et al.*, 1999). Moreover, $Cdc42^{-/-}$ ES cells show comparable levels of JNK and p38 phosphorylation to wild-type ES cells (Chen *et al.*, 2000). However, although no activation of JNK was detected in response to ephrinA1Fc treatment, it would certainly be worth investigating the effect of ephrinA1Fc upon the activity of p38.

The work undertaken in this chapter has resulted in the identification of ephrinA1Fc-induced changes in signalling pathways and the cytoskeleton, which may work separately or in parallel to direct the behaviour of ligand stimulated cells, with respect to both immediate responses and possible longer term consequences resulting from changes in gene transcription.

Chapter 8

General Discussion

Receptor tyrosine kinases are key mediators of intercellular communication, perceiving extracellular signals and transducing them by means of intracellular cascades to affect various aspects of cellular behaviour. The Eph family of receptor tyrosine kinases is known to perform roles in several aspects of development, being implicated in diverse processes from rhombomere boundary formation to limb development. More recently, a role for Eph receptors and their ephrin ligands in vasculogenesis and angiogenesis has been proposed. Overexpression of several Eph receptors in a number of different cancers has also been reported. In particular, EphA2 mRNA expression is upregulated in tumours derived from transgenic mice expressing *ras* in a mammary-specific manner (Andres *et al.*, 1994). Overexpression of EphA2 protein has also been reported in non-transgenic mammary tumours, prostatic tumours and advanced melanomas (Easty *et al.*, 1995; Dodge-Zantek *et al.*, 1999; Walker-Daniels *et al.*, 1999; Ogawa *et al.*, 2000). A key feature of these tumours is their invasiveness and metastatic potential. Indeed, upregulation of EphA2 mRNA was not observed in non-metastatic mammary tumours from transgenic mice overexpressing *myc* (Andres *et al.*, 1994).

Initial experiments carried out in this thesis demonstrated that EphA2 protein was upregulated in mammary tumours of WAP*ras* transgenic mice, and characterised an *in vitro* cell culture model in which the upregulation of EphA2 in response to oncogenic *ras* could be recapitulated. The use of selective kinase inhibitors provided evidence that signalling via the MAPK pathway was necessary for the observed increase in EphA2 protein in Ras-transformed mammary epithelial cells. Experiments designed to investigate whether the MAPK pathway alone is sufficient to upregulate EphA2 protein levels using Ras effector pathway mutants were inconclusive. This was most likely a result of the impaired activity of the mutants towards their appropriate effector compared with the wild-type Ras oncoprotein (Rodriguez-Viciana *et al.*, 1997). The use of constitutively active MEK or MAPK constructs would circumvent this problem, however, the involvement of autocrine factors would be difficult to exclude. Since EphA2 is upregulated in mammary epithelial cells transformed by other oncogenes (Dodge-Zantek *et al.*, 1999; Ogawa *et al.*, 2000), it is possible that further investigation would reveal a common method of EphA2 regulation. Experiments making use of the

MAPK pathway inhibitors in such cell lines would indicate the significance of this pathway in the regulation of EphA2 protein expression. Further analysis of the EphA2 gene is also required with a view to gaining more insight into the transcriptional regulation of EphA2, particularly following observations that EphA2 mRNA and protein continued to be upregulated in mammary tumours of WAP*ras* transgenic mice in which the EphA2 locus has been disrupted by a gene trap insertional event.

The precise roles of the Eph receptors in tumourigenesis are unknown, however, early reports demonstrated that Eph receptors are capable of inducing dramatic changes in cell morphology, indicative of a role in modifying cell-cell and cell-ECM interactions. In view of the fact that overexpression of EphA2 is coincident with the metastatic potential of the tumours in which it is expressed, an active role for EphA2 in the process of tumour invasion and metastasis is particularly attractive. Indeed, activation of the EphA2 receptor in mammary and prostate epithelial cell lines derived from tumours has been demonstrated to decrease cell-ECM interactions (Dodge-Zantek *et al.*, 1999; Miao *et al.*, 2000). Consistent with these results, attempts to stably overexpress EphA2 in the non-transformed mammary epithelial cell line, EpH4, gave rise to a number of cells that appeared to lose contact with the substratum. Epithelial cell-ECM attachment is highly regulated in normal mammary gland homeostasis – loss of ECM constituents during the period of involution is thought to be responsible for the localised apoptosis of epithelial cells through the phenomenon of anoikis. Low level expression of EphA2 may therefore be required for changes in cell-ECM interactions involved in the dynamic remodelling of the normal mammary gland during the oestrus and reproductive cycles. In mammary epithelial cells, EphA2 tyrosine phosphorylation induced by a recombinant, soluble form of ephrinA1 was observed to be transient, concomitant with a decrease in the steady-state levels of full length EphA2 protein. This infers the presence of an important mechanism in mammary epithelial cells to regulate the duration of EphA2 signalling responses. Indeed, further analysis revealed that addition of ephrinA1Fc to EpH4 cells resulted in a transient decrease in MEK and MAPK activity. Since Miao and colleagues (Miao *et al.*, 2000) observed a transient decrease in cell-ECM attachment upon EphA2 activation, coincident with FAK dephosphorylation, one possibility is that decreased outside-in signalling from FAK is responsible for the decrease in MAPK activity observed upon treatment with ephrinA1Fc. The consequences of downregulating MAPK activity have not been fully investigated, but may have been a significant factor in the failure to generate stably-overexpressing

EphA2 mammary epithelial cells. If this were the case, it would be interesting to investigate the consequences of overexpressing recombinant MAPK in addition to recombinant EphA2 in EpH4 cells. Results from experiments using ephrinA1Fc to stimulate EphA2 in EpRas cells indicated that the downregulation of MEK and MAPK activity in EpRas cells was less extreme than that observed in EpH4 cells. This may produce a situation whereby proliferative signals continue to be provided in the absence of cell-ECM attachment. Such a phenotype is likely to confer a distinct advantage upon the progression of tumours. Thus, overexpression of EphA2 could potentially be used as a marker of metastatic tumours.

Moreover, the presence of the Ras oncoprotein may overcome a potential regulatory loop that exists in mammary epithelial cells. The observation that EphA2 activation downregulates MAPK activity in EpH4 cells, coupled with the findings that MAPK activity is necessary for the upregulation of EphA2 protein expression, suggests that in non-transformed mammary epithelial cells, the activities of EphA2 and MAPK are closely interrelated. In such a scenario, an increase in steady-state levels of EphA2 above a certain threshold would initiate ligand-independent signalling, thus resulting in the downregulation of MAPK activity. Consequently, there is less EphA2 protein, the threshold is not exceeded and EphA2-mediated signalling becomes dependent on the presence of ephrinA1 or another ephrinA ligand. In this manner, downstream effects of EphA2 signalling, such as loss of cell-ECM attachment, are subject to tight regulation. In contrast, the presence of the Ras oncoprotein would overcome the ephrinA1-mediated downregulation of MAPK to ensure that levels of EphA2 remain high, resulting in decreased cell attachment and other EphA2-mediated effects, yet providing the necessary signals for the tumour cells to survive and thrive.

Among the additional ephrinA1Fc-induced effects is the rapid formation of filopodia in mammary epithelial cells, indicative of a role for Eph-mediated signalling in cell motility. Future experiments will focus on the signalling pathways responsible for bringing about such changes, and whether this observation occurs independently of changes in MAPK activity. Furthermore, it would also be interesting to investigate the consequences, with respect to cell morphology and motility, of inhibiting the activity of the small GTPase responsible for the cytoskeletal change, upon the behaviour of mammary epithelial cells.

Whilst stable recombinant expression has been used to study the consequences of Eph receptor activation in a number of other studies, experiments carried out in this thesis made use of recombinant ephrinA1 to further study the effects of activating endogenous EphA2 in a mammary epithelial cell line. The significance of studying endogenously expressed proteins is often disregarded and it is important to acknowledge the consequences of overexpressing recombinant proteins several-fold above their physiological levels. Nevertheless, establishing stable recombinant protein-overexpressing cells can provide a useful tool with which to confirm or further analyse observations. As such, it would have been particularly useful to create a stable cell line expressing a kinase-defective version of EphA2 to use in conjunction with the inducible EphA2WT and EphA2Neu MCF-7 cell lines. Future experiments using these cell lines would investigate whether recombinant EphA2 overexpression, either alone, or in combination with additional stimuli, could confer any changes upon the adhesive or migratory properties of cells, and would investigate the signalling pathways by which such changes occur.

In conclusion, experiments carried out within this thesis have demonstrated that active EphA2 protein is upregulated in response to the Ras oncoprotein and have implicated the MAPK pathway in mediating this upregulation. Evidence is also provided that activation of EphA2 downregulates MAPK activity in non-transformed mammary epithelial cells, whereas Ras is able to partially overcome this downregulation. Subsequent studies also revealed a role for ephrinA1 in inducing changes in the actin cytoskeleton. A future challenge will be to investigate the importance of EphA2 in tumour metastasis and progression and to explore the possibility of inhibiting its activity as a potential anti-cancer therapy.

Appendix 1 – The Nucleotide and Amino Acid Sequences of EphA2

The nucleotide and predicted amino acid sequences of mouse EphA2 (Ganju *et al.*, 1994) (accession number X78339) are shown. The initiating methionine is denoted as amino acid number 1. The signal peptide sequence is shown in bold, the putative N-glycosylation sites are boxed, and the transmembrane sequence is underlined. The two conserved tyrosine residues in the juxtamembrane domain are shaded in black. Broken arrows represent the subdomains of the kinase domain, according to (Hanks *et al.*, 1988).

```
1                                       31
--g gca cga ggt tgt ctc tgt cgg cgg gcg ggc agg att ggg gca ccg aga ccg gcg tgc

61                                      91
gga cag cag gga tcg cgg ggg agc gag ggg tgc ggc atg gag ctc cgg aca gtc ggt ttc
                                        M   E   L   R   T   V   G   F

121/9                                   151/19
tgc ctg gcg ctg ctg tgg ggt tgc gcg ctg gcg gcc gcg gcg gca cag gga aag gaa gtt
C   L   A   L   L   W   G   C   A   L   A   A   A   A   A   Q   G   K   E   V

181/29                                  211/39
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V   L   L   D   F   A   A   M   K   G   E   L   G   W   L   T   H   P   Y   G

241/49                                  271/59
aaa ggg tgg gac ctg atg cag aac atc atg gac gac atg cct atc tac atg tac tcg gtg
K   G   W   D   L   M   Q   N   I   M   D   D   M   P   I   Y   M   Y   S   V

301/69                                  331/79
tgc aac gtg gta tcc ggc gac cag gac aac tgg ctc cgc acc aac tgg gtg tac cgg gag
C   N   V   V   S   G   D   Q   D   N   W   L   R   T   N   W   V   Y   R   E

361/89                                  391/99
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E   A   E   R   I   F   I   E   L   K   F   T   V   R   D   C   N   S   F   P

421/109                                 451/119
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481/129                                 511/139
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Y   G   T   N   F   Q   K   R   Q   F   T   K   I   D   T   I   A   P   D   E

541/149                                 571/159
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601/169                                 631/179
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661/189                                 691/199
gcg ctg ctc tcc gtt cgc gtc tac tac aag aag tgt ccc gag atg ctg cag agc ttg gct
A   L   L   S   V   R   V   Y   Y   K   K   C   P   E   M   L   Q   S   L   A

721/209                                 751/219
cgc ttc ccc gag acc att gct gtc gct gtc tcc gat aca caa ccc ctg gcc acg gtg gcc
R   F   P   E   T   I   A   V   A   V   S   D   T   Q   P   L   A   T   V   A
```

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G T C V D H A V V P Y G G E G P L M H C

841/249 871/259
acg gtg gat ggc gag tgg ctg gtg ccc atc ggg cag tgc ctg tgc cag gaa ggc tac gag
T V D G E W L V P I G Q C L C Q E G Y E

901/269 931/279
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K V E D A C R A C S P G F F K S E A S E

961/289 991/299
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S P C L E C P E H T L P S T E G A T S C

1021/309 1051/319
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Q C E E G Y F R A P E D P L S M S C T R

1081/329 1111/339
cca ccc tct gcc ccc aac tac ctc acg gcc att ggc atg ggt gcc aaa gta gaa ctg cgt
P P S A P N Y L T A I G M G A K V E L R

1141/349 1171/359
tgg aca gct ccc aag gac act ggt ggc cgc cag gac att gtc tac agt gtc act tgc gaa
W T A P K D T G G R Q D I V Y S V T C E

1201/369 1231/379
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Q C W P E S G E C G P C E A S V R Y S E

1261/389 1291/399
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1441/449 1471/459
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S T T S L S V T W S I P V S Q Q S R V W

1501/469 1531/479
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K Y E V T Y R K K G D A N S Y N V R R T

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E G F S V T L D D L A P D T T Y L V Q V

1621/509 1651/519
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Q A L T Q E G Q G A G S K V H E F Q T L

1681/529 1711/539
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S T E G S A N M A V I G G V A V G V V L

1741/549 1771/559
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L L V L A G V G L F I H R R R R N L R A

1801/569 1831/579
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R Q S S E D V R F S K S E Q L K P L K T

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Y V D P H T Y E D P N Q A V L K F T T E

1921/609 1951/619
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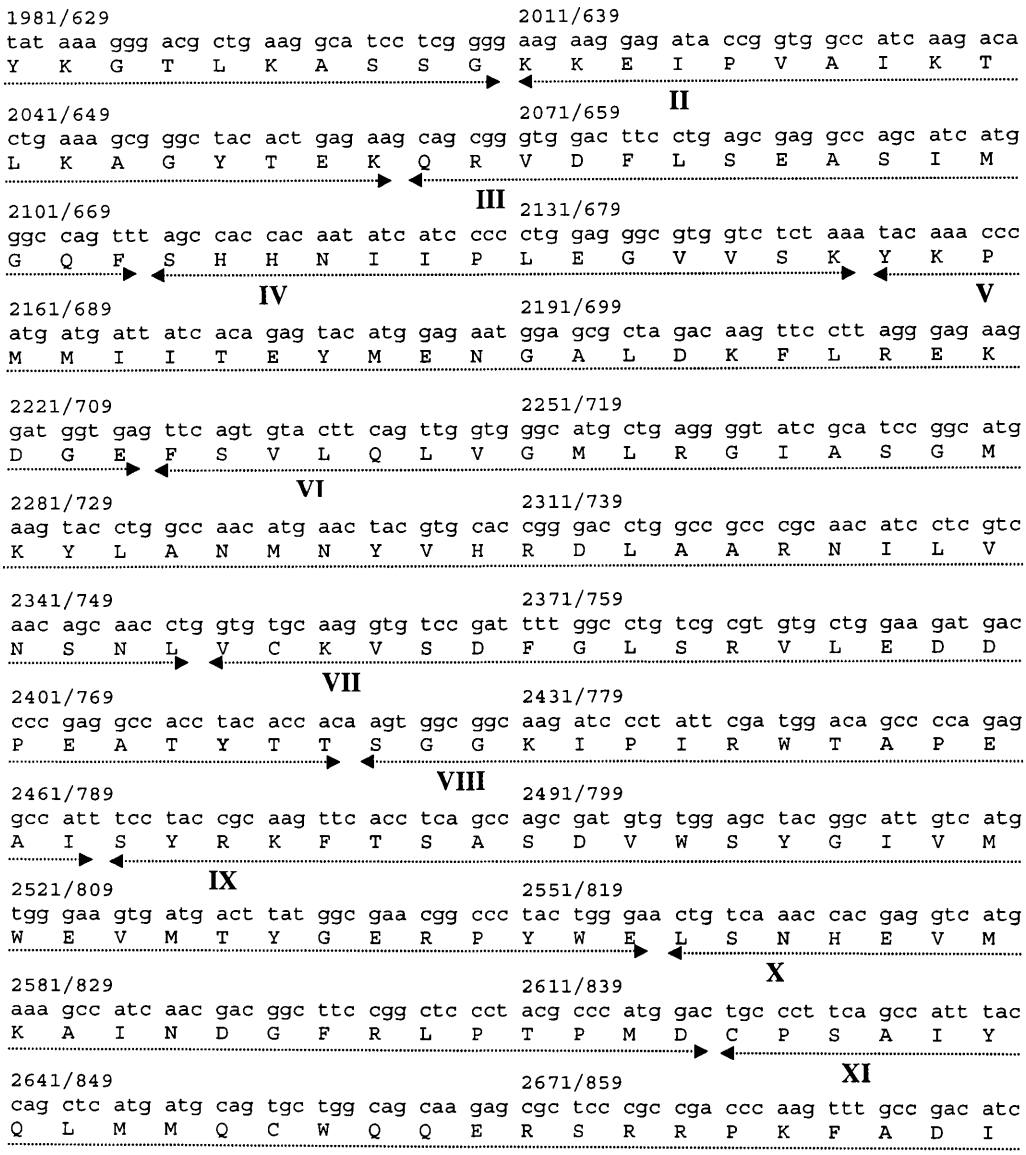
I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II
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3151
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3181 3211
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3241 3271
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3301 3331
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3361 3391
aga agc cac ctc agg agc tga ggc taa gca cta ccc a

Appendix 2 – Previous Names for Eph Receptors and Ephrins

The existing approved nomenclature for Eph receptors and ephrins (Eph Nomenclature Committee, 1997), together with previous names, is outlined below.

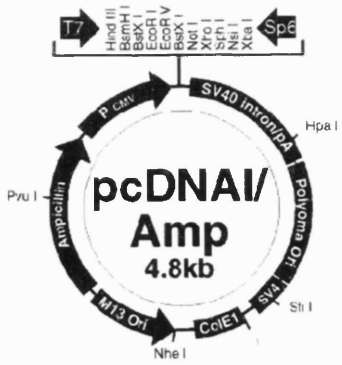
	Previous names
EphA1	Eph, Esk
EphA2	Eck, Sek2, Myk2, XE10, G50, rtk6
EphA3	Hek, Hek4, Tyro4, Mek4, Cek4
EphA4	Hek8, Tyro1, Sek, Sek1, Cek8, Pag, XSek1, rtk1, rtk4, zek1
EphA5	Hek7, Rek7, Ehk1, Bsk, Cek7
EphA6	Hek12, Ehk2
EphA7	Hek11, Mdk1, Ehk3, Ebk, Cek11
EphA8	Hek3, Eek
EphB1	Hek6, Elk, Cek6, Net, Xelk, Xek
EphB2	Hek5, Drt, Erk, Tyro5, Nuk, Sek3, Cek5
EphB3	Hek2, Tyro6, Sek4, Mdk5, Cek10, Tck, rtk3
EphB4	Htk, Tyro11, Myk1, Mdk2, rtk5, rtk8
EphB5	Hek9, Cek9
EphB6	Mep

ephrinA1	B61, LERK-1, EFL-1, XELF-a, L1
ephrinA2	ELF-1, LERK-6, Cek7-L, L3
ephrinA3	Ehk1-L, LERK-3, EFL-2
ephrinA4	LERK-4, EFL-4
ephrinA5	AL-1, RAGS, LERK-7, EFL-5, L2, L4
ephrinB1	LERK-2, Elk-L, Cek5-L, EFL-2, Stra1, XLerk
ephrinB2	Htk-L, ELF-2, LERK-5, NLERK-1
ephrinB3	ElkL-2, LERK-8, NLERK-2, EFL-3

Appendix 3 - Commercial Vectors

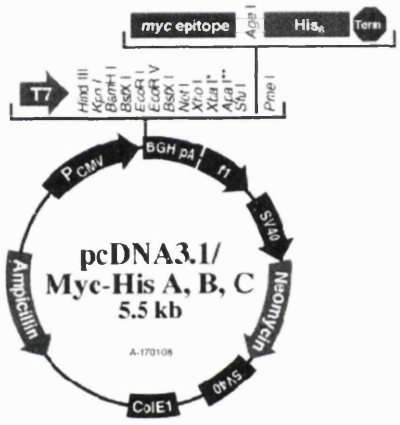
Comments for pcDNA I/Amp
4801 base pairs

ColE1 origin: bases 1-587
M13 origin: bases 588-1180
Ampicillin gene: bases 1360-2203
CMV promoter: bases 2304-2954
T7 primer sequence: bases 2938-2957
Polylinker: bases 2956-3074
Sp6 primer sequence: bases 3075-3093
Splice and PolyA: bases 3094-3792
Polyoma origin: bases 3798-4693
SV40 origin: bases 4634-4797



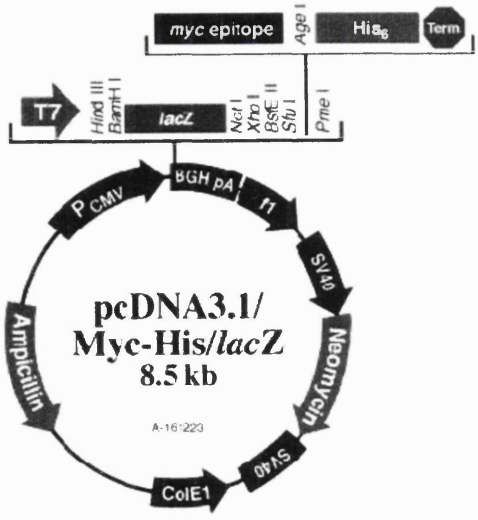
Comments for pcDNA3.1/Myc-His A
5494 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-999
myc epitope: bases 997-1026
Polyhistidine tag: bases 1042-1059
pcDNA3.1/BGH reverse priming site: bases 1082-1099
BGH polyadenylation signal: bases 1081-1295
f1 origin of replication: bases 1358-1771
SV40 promoter and origin: bases 1836-2161
Neomycin resistance gene: bases 2197-2991
SV40 polyadenylation signal: bases 3007-3246
ColE1 origin: bases 3678-4351
Ampicillin resistance gene: bases 4496-5357



Comments for pcDNA3.1/Myc-His/*lacZ*
8541 nucleotides

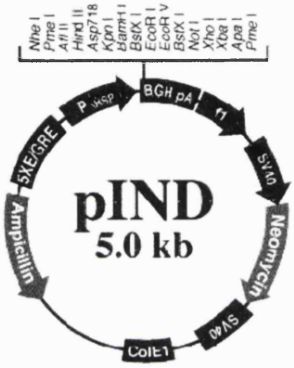
CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
lacZ with C-terminal tag: 963-4106
lacZ ORF: bases 963-4019
myc epitope: bases 4044-4073
Polyhistidine tag: bases 4089-4106
pcDNA3.1/BGH reverse priming site: bases 4129-4146
BGH polyadenylation signal: bases 4128-4342
f1 origin of replication: bases 4405-4818
SV40 promoter and origin: bases 4883-5208
Neomycin resistance gene: bases 5244-6038
SV40 polyadenylation signal: bases 6057-6295
ColE1 origin: bases 6727-7400
Ampicillin resistance gene: bases 7545-8405



Comments for pIND
5021 nucleotides

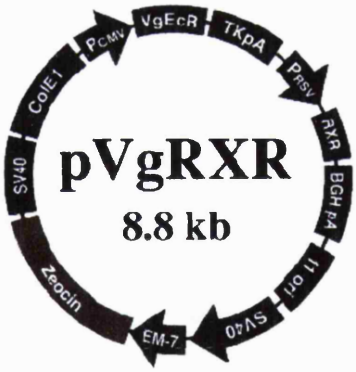
Ecdysone glucocorticoid
Response Elements (5 x EcR/Rxr): bases 1-174
Heat Shock Minimal Promoter: bases 18-475
Ecdysone Forward priming site: bases 429-452
Multiple Cloning Site: bases 476-691
pcDNA3.1/BGH Reverse priming site: bases 603-620

BGH Polyadenylation sequence: bases 602-816
f1 origin: bases 859-1295
SV40 promoter: bases 1357-1682
SV40 origin: bases 1351-1636
Neomycin ORF: bases 1718-2512
SV40 Polyadenylation sequence: bases 2528-2767
ColE1 origin: bases 3199-3872
Ampicillin ORF: bases 4017-4877



Comments for pVgRXR
8803 nucleotides

SV40 early promoter: bases 43-351
EM7 promoter: bases 778-856
Zeocin™ resistance gene (*Sh ble*) ORF: bases 858-1230
SV40 late polyadenylation sequence: base 1294-1424
ColE1 origin: bases 1533-2296
CMV promoter: bases 2285-2883
VP16/EcR f usion protein: bases 3035-5275
VP16: bases 3038-3271
EcR: bases 3302-5275
HSV-1 thymidine kinase polyadenylation sequence: bases 5470-5982
RSV 5' long terminal repeat: bases 6359-6614
RXR ORF: bases 6637-8025
BGH polyadenylation sequence: bases 8108-8332
f1 origin: bases 8502-8809



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