The Role of Ras and its Downstream Targets on Cell Cycle Control

Bryony S. Wiseman

A thesis submitted in partial fulfillment of
the degree of Doctor of Philosophy,
University of London.

March 1999

Growth Control and Development Laboratory
Imperial Cancer Research Fund
44, Lincoln’s Inn Fields
London
Abstract

The oncogene Ras can both stimulate and inhibit cell proliferation depending on the cellular context in which it is activated. Ras is believed to mediate its effects through activation of downstream effector proteins. The best characterised of these is the serine/threonine kinase, Raf. Through conditional activation of Ras or Raf we have shown that depending on the intensity of signal activation, these proteins are able to both stimulate and inhibit proliferation in murine fibroblasts. Strong signals are inhibitory to growth which is shown to be dependent upon an induction of the cell cycle inhibitor p21\(^{Cip-1}\) which prevents upregulated G1 cyclins from forming active complexes. Weaker signals can induce DNA synthesis in low mitogen conditions since they can upregulate cyclin D1 expression, yet are unable to stimulate p21\(^{Cip-1}\) expression. Thus we demonstrate that Raf controls opposing cellular responses through differential activation of cell cycle regulatory proteins. Ras however demonstrates a greater propensity to stimulate growth than Raf which correlates with an ability to downregulate Raf induction of p21\(^{Cip-1}\). The Ras effectors PI 3-kinase and Rif are unable to reproduce this effect and furthermore, RhoA, which has been proposed to downregulate p21\(^{Cip-1}\), is also unable to attenuate induction of p21\(^{Cip-1}\) by Raf. Indeed we show that RhoA and PI 3-kinase co-operate with Raf to induce p21\(^{Cip-1}\). Furthermore Raf and PI 3-kinase co-operate to induce DNA synthesis in the presence of high levels of p21\(^{Cip-1}\). In addition we investigate the ability of activated Rac, which has been implicated as a downstream target of Ras, to control the cell cycle. It is demonstrated that Rac\(^{V12}\) is able to stimulate anchorage-independent growth and upregulate cell cycle regulatory proteins, yet is unable to stimulate mitogen-independent growth. Thus cell growth control by Ras depends both on signal strength and a panel of downstream targets.
Acknowledgements

Firstly, I would like to thank Hucky for giving me the opportunity to work in his laboratory and for his guidance during my time there. I would like to acknowledge the work of Andreas Sewing which is included in this thesis. Furthermore I would like to thank J.Bos, P.Brennan, D.Cantrell, C. Dickson, J.Downward, M.Fried, M.Gossen, T.Jacks, C.Marshall, G.Peters, S.Reeves, A.Ridley, P.Rodriguez-Viciana, R.Treisman and E.Sahai for reagents and/or helpful discussions. The FACS laboratory and the photography department at ICRF have also given invaluable assistance. I would especially like to thank Alan Hall and Fiona Watt for interesting conversations, support and perspective.

In the lab I would like to thank Alison whose irrepressible and unrelenting questioning taught me scientific method but also for support, proof-reading and friendship. Thanks also to Sharon whose sharp wit and words of comfort kept my feet on the ground; Susan, the Bolton boys still remember; David P., for calmness in chaos and for knowing who Little Jimmy was; to Andreas for all his help and much silliness; David S. for his interesting points of view; Liz whose presence continuously reassures me that life can be fun and who has it all to come; Soo for, amongst other things, pointing out that this sentence is too long; Sally for head-banging reminiscences; Betina for vibrancy in a dull place; Laurent for showing me that after it all you can still be chilled; Beatrice who is just wonderful; Nacho, with whom I have been through so many tears, smiles and the odd scowl - thank you for everything and finally Frank-I understand!

Outside of the lab, thank you everyone who has made this time enjoyable, but especially to Nicky for being there through it all; Jo for trying to make me look good and for being such an understanding friend these past few months; Shugs for supporting me even when my ambitions seemed to come first; to Matt-man for the dog with pants, the dropped pints and for endlessly supporting me down Marlboro Road; Robby for friendship that I can’t pin down to anything, but may have something to do with an appreciation of the virtues of shallowness; Ann for helping me split up sentences over the past few months; the Boys for making our house a real-life soap opera and I would like to give a special thank you to Romana and Fiona without whose advice and honesty I may not have got this far.

Finally I would like to thank my family for their help, support and reassurance through out the past few years. This thesis is dedicated to C.H.Peters and T.G.Dupe without whom none of this would have been possible.

'Long is the way and hard, that out of hell leads up to light.'
-John Milton Paradise Lost
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<td>&quot;</td>
<td>inch</td>
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<tr>
<td>(d)NTP</td>
<td>(2'-deoxy)ribonucleoside 5' triphosphate</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid residue</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampere</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5' triphosphate</td>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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<tr>
<td>BHI</td>
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<td>bp</td>
<td>base pair</td>
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<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>Ci</td>
<td>Curie</td>
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<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
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<tr>
<td>CKI</td>
<td>cyclin dependent kinase inhibitor</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CR</td>
<td>conserved region</td>
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<td>CRD</td>
<td>cysteine rich domain</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles media</td>
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<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<td>deoxyribonucleic acid</td>
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<td>doxycycline</td>
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<td>DTAF</td>
<td>dichlorotriazinyl amino fluorescein</td>
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<td>E.coli.</td>
<td><em>Escherichia coli</em></td>
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<td>ECL</td>
<td>enhanced chemi-luminescence</td>
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<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetraacetic acid (disodium salt)</td>
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<tr>
<td>ER</td>
<td>oestrogen receptor</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>guanine dissociation inhibitor</td>
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<td>guanine exchange factor</td>
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<td>HBD</td>
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<td>HCl</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>Definition</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
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<td>kD</td>
<td>kilo Dalton</td>
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<td>l</td>
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<td>RBD</td>
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1. - Introduction
Pathological and epidemiological evidence point to tumorigenesis as a multi-step process in which several genetic lesions are required to cause cellular malignancy. Epidemiological investigations have shown that familial cancer generally develops much earlier than an equivalent sporadic tumour which implies that accumulation of genetic lesions promote tumorigenesis. This view is supported by the correlation between cancer and age (Peto et al., 1975). Most tumours also display gross chromosomal defects which indicate that many genetic changes have occurred. A multistep nature for tumour development is implied from investigations into the development of colorectal tumours. These have shown a correlation between the number of genetic lesions and the stage of the tumour. The inheritance of an altered copy of the gene associated with familial adenomatous polyposis (FAP), adenomatous polyposis coli (APC) (a tumour suppressor gene) predisposes patients to the development of colorectal cancer. Analysis of tumours at different stages of progression have led to a model (Figure 1.1) suggesting that colorectal tumours acquire specific mutations in a sequential manner which allow the tumour to progress to a malignant state. The genetic changes associated with colorectal tumorigenesis include activation of Ki-Ras and loss of the $p53$ tumour suppressor gene.

The requirement for multiple genetic lesions in cellular transformation is demonstrated by

![Figure 1.1 Genetic changes associated with colorectal tumorigenesis](image)

Patients with FAP inherit APC mutations which initiate the neoplastic process. Later they develop numerous dysplastic aberrant crypt foci (ACF), some of which progress as they acquire the other mutations indicated in the figure. Mis-match repair (MMR) deficiency speeds up this process. K-Ras requires only one genetic event to become activated. Inactivation of $p53$ function may also only require one genetic event since its mutant proteins are able to inhibit the function of the wild type protein. The other genes indicated are tumour suppressor genes that require two genetic events (one in each allele) for their inactivation. $DCC$, $DPC4$ and $JV18$ genes are proposed as candidates in colorectal neoplasia from a region on Chromosome 18q21. A variety of other genetic alterations have been described in a small fraction of advanced colorectal cancers.

The response of primary rodent cells to oncogenes in tissue culture. A single oncogene is insufficient to cause malignant cellular transformation of non-established primary fibroblasts, focus formation requires two or more cellular oncogenes (Land et al., 1983; Ruley, 1983) or an oncogene and the loss of a tumour suppressor gene (Tanaka et al., 1994). The increased oncogenicity of multiple genetic lesions has been demonstrated in vivo using mouse tumour models. For example, co-expression of $v$-Ha ras and
constitutive c-myc in the mammary gland of transgenic mice causes much faster and more dramatic tumour formation than the expression of either of these oncogenes alone (Sinn et al., 1987). Furthermore, expression of activated Ras and c-myc in a reconstituted prostate gland demonstrated that each oncogene makes different contributions to tumour development and that co-expression has additional effects (Thompson et al., 1989). However, it must be noted that rodent cells are much more easily transformed in vitro than human cells. For example, co-expression of oncogenic Ras, the viral oncoproteins E6 and E7 (that inactivate p53 and pRb) and overexpression of telomerase does not permit human fibroblasts to grow in the absence of adhesion to substratum; a characteristic of transformed cells that correlates most closely with malignancy (Morales et al., 1999).

The work described in this thesis derives from investigations into the mechanisms of oncogene co-operation. It is now clear that cellular responses to the oncogene Ras are different depending on cell-type, the presence of other oncogenes and cellular environment. Ras can induce a variety of cellular growth responses, such as proliferation, cell cycle arrest and differentiation by differential regulation of the cell cycle. This introduction will begin with an overview of the current understanding of the molecular mechanisms that regulate the cell cycle. Ras and its downstream targets will then be introduced and discussed in the context of their regulation of cell proliferation. Finally the small GTPase Rac and its known role in cell cycle regulation will be discussed.

1.1 Cell Cycle Control

The somatic cell cycle can be divided into the period of DNA replication (S-phase) and the period of chromosome segregation (M-phase), these phases are separated by two gap phases (G1 and G2) in which the cell increases in size and prepares for DNA synthesis or mitosis by accumulating the necessary components for the next phase. The cell can also exit the cell cycle to differentiate or it can withdraw from the cell cycle and enter a state referred to as quiescence (or the G0 phase). Cells can be induced to enter this phase through starvation. The cell cycle is controlled by multiple mechanisms and tumorigenesis often involves disruption of these controls through interference with pathways that are necessary for cell cycle control. The work in this thesis is mainly concerned with the G1 to S-phase transition (which is summarised in Figure 1.2) and the current understanding of the mechanisms controlling this shall be discussed below.

The proteins that are responsible for directly regulating cell cycle progression are the cyclins and cyclin-dependent kinases (CDKs) (Lees, 1995; Morgan, 1995; Nigg, 1995; Pines, 1994; Sherr, 1993). Cyclin binding to its CDK partner is necessary for CDK activation. Cyclins have specific CDK partners. D-type cyclins form active complexes with CDK4 and CDK6 (Matsushime et al., 1992; Meyerson and Harlow, 1994), they can
also form complexes with CDK2, however these complexes are generally not thought to be active (Higashi et al., 1996), although there is a recent report of active Cyclin D2/CDK2 complexes in human breast cells (Sweeney et al., 1997). Cyclin E forms active complexes only with CDK2 (Dulic et al., 1992; Koff et al., 1992). Cyclin A is required for both entry into S-phase and onset of mitosis (Girard et al., 1991; Pagano et al., 1992). It complexes with and activates both CDK2 and CDK1 (also known as Cdc2). Cyclin A/CDK2 complexes predominate in late G1 and S-phase whereas Cyclin A/CDK1 appear in G2. It is thought that CDK2 containing Cyclin A complexes are most important in G1 to S-phase transition (Pagano et al., 1992).

Figure 1.2 G1 to S-phase Transition

The D-type cyclins (1, 2 & 3), Cyclin E and Cyclin A and their partners CDK2, CDK4 and CDK6 control G1 to S-phase transition. The CDKs inactivate the retinoblastoma gene product, pRb through phosphorylation. The cyclins are regulated in a cell cycle specific manner and bind to CDK partners and direct them to appropriate substrates during particular phases of the cell cycle. Thus determining the time and location of substrate phosphorylation. Hypophosphorylated pRb represses transcription of genes required for G1/S transition, amongst others, the E2F family. This control is relinquished upon gradual hyperphosphorylation. In addition to being regulated by their cyclin partner, CDKs are also controlled in part by activating and inhibitory phosphorylation events. Moreover, CDK activity is controlled by the CDK inhibitors (CKIs). The Cip/Kip family of CKIs contains, p21^{CIP1}, p27^{KIP1} and p57^{KIP2}, which specifically inhibit G1 cyclin/CDK activity by binding to both sub-units. The Ink4 family of p15, p16, p18 and p19 specifically block CDK4 and CDK6 interaction with their cyclin partner by altering the conformation of the CDK. This is a simplified view of cell cycle control and will be dealt with more thoroughly in the text.

Another important factor in Cyclin-CDK regulation is the role of the cyclin-dependent kinase inhibitors (CKIs). Currently these proteins fall into two distinct classes. One of these, the specific polypeptide inhibitors of the CDK4 (INK4) family consists of four members: p15^{INK4B} (Hannon and Beach, 1994); p16^{INK4A} (Serrano et al., 1993) (also called multiple tumour supressor 1 (MTS1)(Kamb et al., 1994)); p18^{INK4C} and p19^{INK4D} (Chan et al., 1995; Guan et al., 1994; Guan et al., 1996; Hirai et al., 1995). These proteins bind to CDK4 and CDK6 and prevent interaction with cyclins. They do not bind other CDKs and thus are specific for inactivation of Cyclin D-associated kinase activity (Brotherton et al., 1998; Hannon and Beach, 1994; Russo et al., 1998; Serrano et al., 1993).
The Cip/Kip family consists of p21\textsuperscript{Cip1} (also referred to as Waf1, Sdi1, CAP20 and mda-6) (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Jiang et al., 1995a; Noda et al., 1994; Xiong et al., 1993), p27\textsuperscript{Kip1} (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994) and p57\textsuperscript{Kip2} (Lee et al., 1995; Matsuoka et al., 1995). These proteins bind to both cyclin-CDK subunits and inactivate complexes but have a preference for G1 cyclin complexes over others (Fotedar et al., 1996; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). p27\textsuperscript{Kip1} has been demonstrated to bind to both subunits of the CDK2/CyclinA complex. It blocks a substrate binding domain of Cyclin A and restructures the upper lobe of the CDK2 sub-unit as well as blocking ATP binding (Morgan, 1996; Russo et al., 1996a). An additional mechanism by which Cip/Kip family members may prevent CDK activation is suggested by experiments in which p21\textsuperscript{Cip1} has been shown to block de-phosphorylation of the T14 and Y15 residues of CDK2, and a peptide based on a Cdc25-like motif of p21\textsuperscript{Cip1} is able to compete with Cdc25A for CDK2 binding (Saha et al., 1997). However, although first found to be inhibitory, p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} have been shown by immunoprecipitation to be present in active CDK2, CDK4 and CDK6 complexes respectively in most normal diploid cells (Florenes et al., 1996; Harper et al., 1995; LaBaer et al., 1997; Soos et al., 1996; Zhang et al., 1994). Since the addition of extra p21\textsuperscript{Cip1} is able to inhibit p21\textsuperscript{Cip1} containing complexes, a model has been proposed in which the subunit stoichiometry of the complex determines its activity (Harper et al., 1995; Zhang et al., 1994). This in turn has led to the proposition that the Cip/Kip family has a role in the assembly of active CyclinD/CDK4 complexes (LaBaer et al., 1997). This ability has been shown to be independent of inhibitory function (Welcker et al., 1998). However the subunit stoichiometry model does not concur with the structural data which indicates that a single p27\textsuperscript{Kip1} molecule is sufficient to disrupt the kinase activity of the cyclin/CDK complex. Furthermore, recent data have shown that a single molecule of p21\textsuperscript{Cip1} is sufficient to inhibit the kinase activity of cyclin A/ckd2 complexes using recombinant proteins in vitro (Hengst et al., 1998). At present this issue remains unresolved, however these differences may derive from in vitro and in vivo differences, and additional cellular factors may promote the activation of p21\textsuperscript{Cip}-containing CDK/cyclin complexes.

Active CDKs exert their regulatory function through phosphorylation of key proteins involved in cell cycle progression. These include the retinoblastoma gene product, pRb and related proteins p130 and p107, which constitute a family referred to as the pocket proteins. Active pRb binds to and represses E2F, which itself is bound to E2 elements in promoters of G1/S regulated genes including Cyclin E and Cyclin A (DeGregori et al., 1995a; Nevins, 1992; Ohtani et al., 1995; Schulze et al., 1995; Weintraub et al., 1992). Inhibition of E2F directed transcription is thought to involve pRb-mediated histone deacetylation followed by chromatin condensation in the vicinity of the E2F site which
also results in inhibition of other transcription factors on the promoter (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Weintraub et al., 1995). Furthermore, E2F is thought to have an active role in transcriptional repression through its interaction with pRb. The importance of regulated E2F activity is indicated by overexpression studies in which deregulated E2F in mammalian cells or during Drosophila embryogenesis disrupts normal control of the cell cycle and drives cells into S phase (DeGregori et al., 1995b; Duronio and O'Farrell, 1995; Lukas et al., 1996). E2F proteins are also thought to have an important role in tumour suppression since mice which are null for E2F1 are tumour prone (Field et al., 1996; Yamasaki et al., 1996). This may be related to the ability of E2F to promote apoptosis which can be separated from its ability to induce DNA synthesis (Phillips et al., 1997) or its role as a transcriptional repressor (Helin, 1998). The major role for D-type cyclins in cell cycle progression appears to be the inactivation of pRb through phosphorylation, thus leading to the relief of transcriptional repression. The foundation for this hypothesis comes from several lines of evidence, firstly, RB1/- fibroblasts or cells expressing either viral oncoproteins that sequester pRb or inactivated pRb, no longer require D-type cyclins for S-phase entry (Lukas et al., 1995; Lukas et al., 1994a; Lukas et al., 1994b). Secondly, cells expressing a specific inhibitor of D-type cyclin associated activity, p16INK4, arrest in G1 and that arrest depends on the presence of functional pRb (Koh et al., 1995). Thirdly, the theory that the predominant role of Cyclin D1 is to phosphorylate pRb is supported by the observation that unlike Cyclin E, ectopic expression of Cyclin D1 is unable to overcome cell cycle inhibition caused by a constitutively active mutant of pRb that is immune to inactivating phosphorylation (Lukas et al., 1997). Thus it appears that once pRb is inactivated and E2F transcription can ensue, that D-type cyclins are no longer required. In support of this view, ectopic expression of E2F is able to promote S-phase entry even when D-type cyclin activity is suppressed, either by expression of inhibitor proteins specific for CDK4 and CDK6, or by microinjection of Cyclin D1 neutralising antibodies (Lukas et al., 1996; Mann and Jones, 1996).

Cyclin E and Cyclin A also have important roles in cell cycle progression, and appear to regulate aspects of cell cycle control different from D-type cyclins. One indication of this is their different expression patterns. Upregulation of Cyclin E protein and associated activity occurs later than D-type cyclins, and peak just prior to S-phase entry. Moreover, E2F-mediated activation of Cyclin E is necessary for S-phase entry once Drosophila embryos have exhausted their maternal supplies (Duronio et al., 1996). Cyclin A was originally thought to have its main role in G2 since that is where its protein level and activity peaks, however, accumulation of Cyclin A occurs prior to S-phase entry (Dulic et al., 1994; Dulic et al., 1992) and it has been shown to promote S-phase entry when ectopically expressed (Resnitzky et al., 1995). Unlike the D-type cyclins, Cyclin E and
Cyclin A cannot directly bind pRb (Dowdy et al., 1993; Kato et al., 1993), however they do mediate pRb phosphorylation, but on different sites from those phosphorylated by D-type cyclins (Hinds et al., 1992; Zarkowska and Mittnacht, 1997). In support of this, full phosphorylation of mammalian pRb, expressed in yeast requires the expression of multiple cyclins (Hatakeyama et al., 1994). A possible model for G1 cyclin-mediated phosphorylation of pRb is that D-type cyclins initially partially phosphorylate pRb, inactivating it sufficiently to free enough E2F to transcribe Cyclin E. Cyclin E-associated kinase activity then phosphorylates pRb further, leading to complete inactivation, and thus a higher level of E2F activity which is then sufficient for Cyclin A transcription. Cyclin A then maintains this level of pRb phosphorylation beyond G1. Recently, constitutively active pRb has been shown to prevent completion of DNA replication. This can be relieved by ectopic expression of SV40 LT, adenovirus E1A or high levels of E2F-1, but not Cyclin A or Cyclin E (Chew et al., 1998; Knudsen et al., 1998). Therefore a component in addition to the cyclins is required for completion of DNA synthesis.

A role for Cyclin E and Cyclin A in mediating G1/S transition, that is distinct from the requirement to phosphorylate pRb and thus release E2F transcriptional activity, is suggested by several lines of evidence. Firstly, although overexpression of E2F can overcome a p16\textsuperscript{INK4} induced block, it is unable to overcome a block induced by another CKI family, p21\textsuperscript{Cip1} or p27\textsuperscript{Kip1} which indicates that cyclin-dependent kinase activities other than those associated with D-type cyclins are required for G1/S progression (Mann and Jones, 1996). Secondly, SV40 LT is unable to promote S-phase entry in the presence of a dominant negative CDK2, even though pRb is no longer inhibiting E2F (Hofmann and Livingston, 1996). This indicates that sequestration of pRb is insufficient for cell cycle progression. Thirdly, Cyclin E is able to overcome inhibition of S-phase entry caused by an inactivatable mutant pRb, overexpression of pRb or overexpression of p16\textsuperscript{INK4} which inhibits pRb phosphorylation (Alevizopoulos et al., 1997; Knudsen et al., 1998; Lukas et al., 1997). Moreover, although ectopic expression of E2F can overcome p16\textsuperscript{INK4} inhibition, this is still dependent on Cyclin E activity since it is inhibited by p27\textsuperscript{Kip1} (Alevizopoulos et al., 1997). This indicates that Cyclin E has an important role outside pRb phosphorylation and E2F activation. Moreover these data indicate that a possible purpose of pRb phosphorylation in G1 is to initiate Cyclin E transcription, which is then sufficient to drive G1 to S-phase progression.

Cyclins and CDKs are themselves regulated by many different mechanisms. Cyclin A promotes CDK2 activation by inducing two major structural changes in the kinase that are likely to be conserved in other CDK/cyclin pairs (Jeffrey et al., 1995; Russo et al., 1996b) CDKs also require phosphorylation on Thr 160 or 161 from an activity referred to as CDK activating kinase or CAK (Solomon et al., 1992). There is strong evidence implicating CDK7 in complex with Cyclin H as the prime candidate for CAK (Harper and
Elledge, 1998). Phosphorylation of CDKs can also lead to inactivation. This occurs specifically on Thr 14 and Tyr 15 within their ATP binding domain (Gu et al., 1992; Sebastian et al., 1993). The importance of these phosphorylation events in normal cell regulation, is demonstrated by the inability of cells to arrest in response to ultraviolet (UV) radiation when expressing a mutant of CDK4 which cannot be phosphorylated on these residues (Terada et al., 1995). The phosphatase implicated in this dephosphorylation is Cdc25A. This is related to the mammalian homologue of yeast cdc25, Cdc25C, which is the phosphatase responsible for de-phosphorylation of T14 and Y15 on cdc2 in yeast. Microinjection of Cdc25A neutralising antibodies leads to G1 arrest (Hoffmann et al., 1994; Jinno et al., 1994).

1.1.1 The relationship between p21Cip1 and p53

p21Cip1 is a direct downstream target for the transcription factor p53 which is a tumour suppressor gene that is frequently found mutated in human tumours (el-Deiry et al., 1993). p21Cip1 is causally involved in many p53 mediated cellular responses, such as the DNA damage response. p21Cip1 null cells have a partial deficiency in G1 cell cycle arrest in response to radiation or nucleotide depletion (Brugarolas et al., 1995; Deng et al., 1995). DNA damaging agents cause upregulation of p21Cip1 which enters and inhibits Cyclin E/CDK2 complexes arresting cells in G1. Induction of p21Cip1 and the ensuing cell cycle arrest in response to DNA damage is dependent on p53 since arrest does not occur in cells containing non-functional p53 (Dulic et al., 1994; el-Deiry et al., 1994). p21Cip1 is also involved in mediating growth arrest associated with terminal differentiation (Jiang et al., 1995a; Liu et al., 1996; Zhang et al., 1995b) independently of p53. p21Cip1 can be transcriptionally upregulated in the absence of p53 function, either in p53 null cells or cells expressing a dominant negative p53 mutant in response to specific DNA damaging agents (etopiside), growth factor stimulation (PDGF, FGF, EGF and TGFβ but not insulin), serum deprivation and differentiation agents in a variety of cell types (Elbendary et al., 1994; Michieli et al., 1994; Sheikh et al., 1994; Steinman et al., 1994). The mechanisms by which this occurs have not yet been fully elucidated however there is some evidence that p53 independent upregulation of p21Cip1 involves the transcription factor Sp1 (Biggs et al., 1996; Nakano et al., 1997).

1.1.2 Cell cycle regulatory proteins and tumorigenesis

Many of the genes associated with the cell cycle have been identified as tumour suppressor genes or proto-oncogenes.

The oncogenic potential of Cyclin D1 has been demonstrated repeatedly. Identification of the Cyclin D1 gene, CCND1, occurred through three independent events. It was identified as a gene linked to the parathyroid hormone gene in human parathyroid adenomas containing an inversion of chromosome 11 and designated PRAD1 (Motokura
et al., 1991). It was separately identified as a gene that could complement a deficiency of the yeast G1 cyclins, Cln 1, 2 and 3 (Xiong et al., 1991). Furthermore, three mouse homologues of CCND1 were identified as cellular genes whose expression was stimulated by the colony stimulating factor 1 in macrophages (Matsushime et al., 1991). Cyclin D1 has been found overexpressed in many cancers as a result of gene amplification, chromosomal translocations and increased protein stability (Palmero and Peters, 1996; Welcker et al., 1996). A role in tumorigenesis is supported by transgenic studies in which overexpression of Cyclin D1 in the mammary cells of transgenic mice leads to abnormal cell proliferation and mammary adenocarcinomas (Wang et al., 1994).

The gene locus for one of Cyclin D's partners, CDK4, is also amplified in some sarcomas and gliomas, however this region also contains other cancer associated genes, including the p53 antagonist, MDM2 (He et al., 1994; Reifenberger et al., 1994).

In addition to D-type cyclins, the other genes involved in the control of pRb phosphorylation appear to have an important role in tumorigenesis. Germ-line mutations in the retinoblastoma tumour supressor gene, RB1, leads to retinoblastoma and osteosarcoma in children (Friend et al., 1986; Knudson, 1971). However, sporadic non-familial mutations can also cause tumours for example in small cell lung carcinomas (SCLC). p16INK4 regulates pRb function through inactivation of D-type cyclin-associated kinase activity. It is a tumour supressor gene and is inactivated in many human tumours for example familial melanoma (Palmero and Peters, 1996). The INK4 locus also encodes another putative tumour suppressor gene ARF, which has been linked to p53 mediated growth arrest. Inactivating mutations in pRb and p16INK4 occur in a mutually exclusive fashion in tumorigenesis which is predicted by their occurrence on the same control pathway. For example, pRb is frequently inactivated in small cell lung carcinomas (SCLC), which usually express wild-type p16INK4, and non-SCLCs which are predominantly wild-type for pRb, have frequently lost p16INK4 function (Palmero and Peters, 1996). A similar inverse correlation is seen with p16INK4 expression and CDK4 gene amplification, in sarcomas and gliomas. In addition a mutation of CDK4 found in some melanomas retains its ability to bind Cyclin D1, but p16INK4 binding is disrupted, and thus acts as a dominant oncogene which disrupts the pRb checkpoint (Wolfel et al., 1995). The tumorigenic potential of Cyclin D1 and CDK4 activation or the loss of Rb and p16INK4, which all directly control pRb phosphorylation, emphasises the importance of proper regulation of the pRb checkpoint to prevent uncontrolled cell cycling.

Gene amplification or inactivation of other cell cycle regulators is less common. Even though the human Cyclin A gene was originally identified in a hepatocellular carcinoma, as the integration site of hepatitis B virus (HBV), the evidence for a role of Cyclin A in tumorigenesis is not very strong. In this case the cyclin destruction box of Cyclin A had been replaced with viral sequences, making the protein non-degradable, and therefore
constitutive. However, whether this mutant Cyclin A gene was causally involved in tumour development is not clear (Wang et al., 1990; Wang et al., 1992). Up to now other instances of Cyclin A mutations in human tumours have not been reported. Cyclin A protein has been found upregulated in some tumours, however this could be the result of disruption of events preceding Cyclin A transcription. Transgenic mice ectopically expressing wild type or non-degradable Cyclin A in their mammary glands show some hyperplasia and increased apoptosis, but no tumours (Bortner and Rosenberg, 1995). Since destruction of Cyclin A is required prior to mitosis, this may explain the inability of constitutive Cyclin A to form tumours. This is indicated by reports that NIH 3T3 cells cannot grow with constitutive Cyclin A expression (Guadagno et al., 1993).

Tumour-specific mutations in Cyclin E and p27Kip1, one its regulators, are rare. However, the levels of these proteins can be altered post-transcriptionally, indicating that mutations in their respective genes may not be required for changes in relative abundance. Indeed, upregulation of Cyclin E or downregulation of p27Kip1 protein does occur in several human cancers, including breast cancers, lymphocytes from patients with lymphatic leukaemia and colorectal carcinomas (Catxavelos et al., 1997; Keyomarsi et al., 1994; Loda et al., 1997). Moreover, there are data indicating that the relative protein levels of Cyclin E and p27Kip1 can be correlated with the prognosis of breast cancer patients, which suggests that pathways controlled by these elements are important in tumour progression (Porter et al., 1997; Steeg and Abrams, 1997). In rodents, Cyclin E can be tumorigenic if ectopically expressed in the mammary cells of transgenic mice (Bortner and Rosenberg, 1997) and p27Kip1 nullizygous mice are reported to develop pituitary tumours (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996).

Another CKI, p21Cip1 has been shown to have a very important role in cell cycle regulation. However, analysis into the occurrence of p21Cip1 mutations in human tumours have shown that although polymorphisms occur, in most instances these do not correlate with increased incidence of tumour formation (Chedid et al., 1994; Koopmann et al., 1995; Li et al., 1995b; Mousses et al., 1995; Shiohara et al., 1994; Wan et al., 1996). However somatic mutation in p21Cip1 has been described in some primary prostate tumours (Gao et al., 1995). The absence of p21Cip1 inactivation in human tumours equates with the lack of disposition to tumour formation observed in p21Cip1 null mice. However loss of p21Cip1 may have some oncogenic potential. Expression of activated Ras in p21Cip1 null keratinocytes causes them to become tumorigenic whereas the same is not true of wild type cells (Missero et al., 1996), thus p21Cip1 is able to repress transformation by activated Ras. In addition, inhibition of p21Cip1 protein expression is required for the full transformation of primary rat Schwann cells by Ras (Lloyd et al., 1997) this will be discussed in further detail later. It is currently not clear whether there is a role for p21Cip1 in human tumorigenesis.
1.2 Ras

1.2.1 Background

Ras genes were first identified as the transforming principle of the Harvey and Kirsten strains of rat sarcoma viruses (Harvey, 1964; Kirsten and Mayer, 1967). These are viral homologues of cellular genes that have been constitutively activated by point mutation (DeFeo et al., 1981; Ellis et al., 1981; Parada et al., 1982; Santos et al., 1982) (Balmain and Pragnell, 1983; Der et al., 1982; Eva and Aaronson, 1983; Guerrero et al., 1984; Reddy et al., 1982; Shimizu et al., 1983b; Sukumar et al., 1983; Tabin et al., 1982; Taparowsky et al., 1982; Yuasa et al., 1983). Three human Ras genes have so far been identified; Ha-ras (Parada et al., 1982; Santos et al., 1982), Ki-ras (Der et al., 1982) and N-ras (Hall et al., 1983; Shimizu et al., 1983b). These genes and the pathways they regulate are very well conserved in eukaryotes. Ras has been demonstrated to have a role in multiple aspects of mammalian cell growth and development, including; cell proliferation; apoptosis; differentiation and senescence. It has also been demonstrated to be essential for development of the eye and vulva in Drosophila and C.elegans respectively.

1.2.2 The Ras Superfamily

Ras proteins are members of a much larger Ras-like GTPase superfamily that are separated into sub-groups determined by structure and function. These proteins are listed in their family groups in Table 1.

Families other than Ras, in the Ras subfamily also appear to have roles in regulating cell growth and development. Rap1 was identified as a supressor of Ras transformation (Noda, 1993), and may function by sequestering RasGEFs. Activated R-Ras, TC21 and R-Ras3 are able to transform rodent fibroblasts and R-Ras is able to bind the apoptosis regulator, Bcl-2 at its C-terminus (Chan et al., 1994; Cox et al., 1994; Graham et al., 1994; Kimmelman et al., 1997; Saez et al., 1994). In a similar manner to Rap1, Rheb is able to antagonise Ras transformation and signalling, this may be linked to an interaction between Raf-1 and Rheb, which is potentiated with phosphorylation of Raf-1 on serine 43 by protein kinase A, an event which decreases H-Ras interaction (Clark et al., 1997b; Yee and Worley, 1997). The specific functions of other Ras subfamily members are only just beginning to emerge (Bos, 1997). A major function of the Rho family is actin cytoskeleton organisation, however it also has many other functions which will be discussed in more detail later. The Rab sub-family are involved in the secretory and endocytic pathways (Schimmoller et al., 1998), and comprises the largest family of GTPases. ADP-ribosylation factors (ARFs) are critical for several vesicular trafficking
pathways (Moss and Vaughan, 1998) and for efficient ADP-ribosylation of $G_{\alpha}$ by cholera toxin (Bobak et al., 1989; Sewell and Kahn, 1988). The Ran (Ras-related nuclear protein) sub-family is involved in trafficking proteins and RNA in and out of the nucleus, and unlike most other Ras superfamily members which exist in the cytoplasm, they are found in the nucleus (Mattaj and Englmeier, 1998). Rad proteins are believed to have a role in calcium signalling since they bind Calmodulin (Fischer et al., 1996; Moyers et al., 1997).

Table 1 Members of the human Ras-like GTPase superfamily

<table>
<thead>
<tr>
<th>Ras-like Subfamily</th>
<th>Family/ Class</th>
<th>Members</th>
<th>Review/ References</th>
</tr>
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<tbody>
<tr>
<td>Ras</td>
<td>Ras</td>
<td>Ha-Ras, Ki-Ras, N-Ras 1A, 1B, 2A, 2B A, B R-Ras, TC21 (R-Ras2), R-Ras3 Rheb</td>
<td>(Bos, 1997)</td>
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<tr>
<td></td>
<td>Rap</td>
<td>A, B, C</td>
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<td></td>
<td>R-Ras</td>
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<td>Rheb</td>
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<tr>
<td>Rho</td>
<td>Rho</td>
<td></td>
<td>(Hotchin and Hall, 1996; Van Aelst, 1997)</td>
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<tr>
<td></td>
<td>Rac</td>
<td>A, B, C</td>
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</tr>
<tr>
<td></td>
<td>TC10</td>
<td>1, 2, 3 TC10</td>
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<tr>
<td></td>
<td>Cdc42</td>
<td>Cdc42, G25K, RhoG</td>
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<td></td>
<td>RhoG</td>
<td>RhoG</td>
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<tr>
<td></td>
<td>RhoE</td>
<td>RhoE. Rho8/Rnd3, Rnd1/Rho6, Rnd2/Rho7 RhoD TTF</td>
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<td></td>
<td>RhoD</td>
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<td>TTF</td>
<td>TTF</td>
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<tr>
<td>Rab</td>
<td>Rab</td>
<td>Over 40 members in mammalian family</td>
<td>(Novick and Zerial, 1997)</td>
</tr>
<tr>
<td>ADP Ribosylation Factor (ARF)</td>
<td>Class I</td>
<td>ARF 1, 2, 3</td>
<td>(Moss and Vaughan, 1998)</td>
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<td></td>
<td>Class II</td>
<td>ARF 4, 5</td>
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<tr>
<td></td>
<td>Class III</td>
<td>ARF 6</td>
<td></td>
</tr>
<tr>
<td>Ran</td>
<td>Ran</td>
<td>Ran</td>
<td>(Mattaj and Englmeier, 1998; Rush et al., 1996)</td>
</tr>
<tr>
<td>Rad</td>
<td>Rad</td>
<td>Rad, Gem, Kir, Rem</td>
<td>(Cohen et al., 1994; Finlin and Andres, 1997; Maguire et al., 1994; Reynet and Kahn, 1993)</td>
</tr>
<tr>
<td>Rin</td>
<td>Rin</td>
<td>Rin, Rit</td>
<td>(Lee et al., 1996; Wes et al., 1996)</td>
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</table>

1.2.3 Ras in human tumorigenesis

Mutations in the ras genes are commonly found in human tumours. Their overall incidence is around 10-15%, however this varies strongly amongst tumour types. For example, over 90% of tumours of the pancreas and half of colonic cancers contain
activating ras mutations (Bos, 1989). Individual ras genes are commonly associated with specific tumours; Ki-ras mutations are predominantly found in adeno-carcinoma of the lung and pancreatic and colorectal cancers; N-ras is predominantly found in a subset of acute leukemias and in myelodysplastic syndromes; mutations in Ha-ras are quite rare, although Ha-ras was originally identified in human bladder carcinomas (Bos, 1989; Rodenhuis, 1992). It is clear that activation of Ras is advantageous for tumour formation. However, although the reason for this is not yet known the ability of activated Ras to act as an independent mitogen may be relevant. Furthermore, many components involved in Ras activation are also found mutated in human tumours. For example inactivation of the neurofibromatosis type I gene (NF-1), a Ras GTPase activating protein (RasGAP) is implicated in neurofibromatosis (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a; Xu et al., 1990b). In addition the epidermal growth factor receptor (EGFR) has been found overexpressed in many human tumours (Hesketh, 1997). Therefore although these tumours do not contain Ras mutations, they still contain a constitutively activated Ras pathway.

1.2.4 Activation of Ras

The three human ras genes encode four 188-189 amino acid proteins of 21 kDa in size (H-Ras, N-Ras, K-Ras4A and K-Ras4B). The K-Ras proteins use different exons to encode the last 25 amino acids in their carboxy termini (Capon et al., 1983; McGrath et al., 1983; Shimizu et al., 1983a). Ras proteins localise and become active at the plasma membrane due to post-translational modifications to their carboxy termini via their CAAX motif. Prenylation is the first modification that occurs to Ras proteins, which then become farnesylated or geranylgeranylated by farnesyl transferase or geranylgeranyl transferase type I respectively. After prenylation Ras proteins undergo carboxy-terminal cleavage, carboxymethylation and palmitoylation (Lowy and Willumsen, 1993). However only farnesylation is necessary for the oncogenicity of Ras (Kato et al., 1992) which has made it a major target for drug therapy (Kohl et al., 1995).

Ras proteins are similar to the two other families of GTP binding proteins, the heterotrimeric G proteins and the elongation and initiation factors of protein synthesis. All act as molecular switches whose signalling ability is activated upon association with guanosine triphosphate (GTP). The GTP is then hydrolysed into guanosine diphosphate (GDP), by intrinsic GTPase activity and the protein becomes deactivated (Bourne et al., 1990). Two regions of Ras change conformation upon GTP hydrolysis, Switch I and Switch II, which comprise amino acids 30-37 and 59-76 respectively. These regions contain amino acid residues that interact with the γ-phosphate of GTP (Wittinghofer and Nassar, 1996).
The intrinsic GTPase activity and GDP exchange rate of Ras is quite low and therefore two types of regulatory proteins assist Ras GTP/GDP cycling, this is depicted in figure 1.3. GTPase activating proteins (GAPs) are negative regulators of Ras signalling (Trahey and McCormick, 1987). They accelerate the intrinsic GTPase activity of Ras, possibly by stabilising the transition state for GTP hydrolysis (Prive et al., 1992; Scheffzek et al., 1997).

Two GAPs which show catalytic activity towards Ras are p120GAP (Adari et al., 1988; Trahey and McCormick, 1987; Vogel et al., 1988) and NFl (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a). Guanosine nucleotide exchange factors (GEFs) (also called GDP dissociation stimulators (GDSs) and guanine nucleotide releasing proteins (GNRPs)) promote exchange of GDP for GTP and thus activate Ras signalling (Quilliam et al., 1995). The major mammalian GEFs in Ras signal transduction are thought to be Sos 1 and 2, (Bowtell et al., 1992; Chardin et al., 1993) which are mammalian homologues of the Drosophila melanogaster GEF, Son Of Sevenless (Simon et al., 1991). Additional mammalian RasGEFs are Cdc25Sm/RasGRF1 and RasGRF2 (Fam et al., 1995). The role of other putative Ras GEFs is more controversial. It has been suggested that Vav, a GEF with sequence homology to Dbl, acts as a RasGEF (Gulbins et al., 1993; Gulbins et al., 1994), however other groups disagree and believe it is a GEF for the Rho family (Bustelo et al., 1994; Khosravi-Far et al., 1994). Smg-GDS is able to act as a GEF for K-Ras4B and other members of the Ras superfamily, but not for the other Ras proteins (Mizuno et al., 1991; Takai et al., 1993). Mutants that act in a dominant negative fashion towards Ras family members such as Ras617 have a very low affinity for guanosine nucleotides and may act by sequestering RasGEFs and preventing normal Ras molecules from utilising GEF exchange activity (van den Berghe et al., 1997).
Activation of normal Ras occurs in response to a wide variety of stimuli, such as growth factors, cytokines, hormones, and neurotransmitters. These stimuli signal to transmembrane receptors such as receptor tyrosine kinases (RTKs), non-receptor tyrosine kinase-associated receptors and G-protein coupled receptors. The best characterised Ras-mediated signal transduction pathway is the activation of the epidermal growth factor receptor. This is depicted in A. This RTK undergoes autophosphorylation on cytoplasmic tyrosine residues in response to epidermal growth factor (EGF) stimulation. These phosphorylated residues then provide binding sites for the Src-homology 2 (SH2) domains of growth factor receptor-bound protein 2 (Grb2) (Lowenstein et al., 1992; Matuoka et al., 1992). Grb2 consists almost entirely of one SH2 domain and two proline binding SH3 domains (Clark et al., 1992). The SH3 domains bind to the proline rich carboxy terminus of Sos which is in complex with Ras at the plasma membrane (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). This proposed mechanism derives from several observations including the potentiation of EGFR activation of Ras upon over expression of Grb2, and also the activation of Ras by an artificially membrane localised Sos (Aronheim et al., 1994; Gale et al., 1993; Quilliam et al., 1994). Other growth factor receptors use similar mechanisms, for example the Sos-Grb2 complex binds to the insulin receptor via another adaptor protein, Shc and the insulin receptor substrate (IRS-1) (Skolnik et al., 1993). A similar, though more complex mechanism is used by integrins for Ras activation of the mitogen activated protein (MAP) kinase pathway in keratinocytes (see B). Caveolin has been shown to recruit the tyrosine kinase Fyn to the α integrin which binds Shc. She then binds Grb2 and Sos complexes and this ultimately leads to Ras activation (Wary et al., 1996; Wary et al., 1998). Increase in GEF activity may just be one mechanism by which Ras is activated. Phorbol ester treatment in T cells which leads to Ras activation, occurs with no change in guanine nucleotide exchange activity and instead coincides with downregulation of RasGAP activity (Downward et al., 1990).
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Furthermore, Ras activity is regulated by PI 3-kinase-mediated inhibition of GAP activity in adipocytes (DePaolo et al., 1996).

Ras genes are proto-oncogenes and become constitutively activated when point mutated in specific amino acid residues. The most common mutations in human tumours are 12, 13 and 61 (Lowy and Willumsen, 1993). Ras proteins activated by these mutation become immune to the effects of GAPs since they have lost their intrinsic GTPase activity and so become GTP bound (Adari et al., 1988; Scheffzek et al., 1997; Trahey and McCormick, 1987; Vogel et al., 1988). Expression of these mutants in rodent fibroblasts leads to cellular transformation and constitutive upregulation of Ras targets (Chang et al., 1982; Parada et al., 1982; Shih and Weinberg, 1982; Stacey and Kung, 1984).

1.2.5 Cellular Effects of Ras

Ras is involved in many cellular processes and inappropriate activation of Ras signals can stimulate a variety of responses including changes in growth control, morphological and structural changes to cells and alterations in gene expression.

Ras was initially isolated because of its ability to overcome contact inhibition in rodent fibroblast cell-lines and the subsequent ability of those cells to promote tumour formation in nude mice (Chang et al., 1982; Shih and Weinberg, 1982). Microinjection experiments then demonstrated that oncogenic Ras increased the proliferation and induced DNA synthesis of serum-deprived cells (Feramisco et al., 1984; Stacey and Kung, 1984). Ras was found to be an essential component of growth factor signalling because Ras inhibitory antibodies or dominant negative, Ras\textsuperscript{N17} prevented induction of DNA synthesis by serum and growth factors (Cai et al., 1990; Feig and Cooper, 1988). Ras activation by growth factors is required at multiple points during transition from quiescence through G1 and is no longer necessary after the cells have initiated DNA synthesis (Dobrowolski et al., 1994; Mulcahy et al., 1985). Ras is able to induce Cyclin D1 expression (Aktas et al., 1997), and as discussed earlier, in some cells ectopic expression of Cyclin D1 is sufficient to induce cell cycle progression. Moreover, the resulting effect of Cyclin D1/Cdk4/6 activation is the phosphorylation of pRb. Inactivation of Ras, by dominant negative mutants or inhibitory antibodies has less inhibitory effect on mitogen stimulated proliferation in \textit{Rb} null cells, than \textit{Rb} positive cells (Mittnacht et al., 1997; Peeper et al., 1997). This work shows Ras partly stimulates DNA synthesis by promoting hyperphosphorylation of pRb, but that it also has other functions.

Oncogenic Ras also relieves the requirement for cells to be adhered to a substratum when cultured \textit{in vitro}. This is a characteristic of most neoplastic cells, in contrast to non-transformed cells which require specific signals from their adhesion receptors to
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proliferate (Giancotti and Mainiero, 1994). For example, F2408 rat fibroblasts require adhesion to substratum for 4-8 h after serum stimulation to progress into S-phase (Inoue et al., 1996), however on expression of viral oncogenes, cells no longer require adhesion signals to be stimulated by mitogens. As described earlier, β1 integrins and also α4β6 integrins are able to directly activate Ras and the Extracellular regulated kinase (ERK) MAP kinase pathway, via the adaptor molecule Shc (Mainiero et al., 1997; Wary et al., 1996; Wary et al., 1998). Moreover only integrin signalling mediated through Shc can co-operate with mitogens to stimulate cell cycle progression (Wary et al., 1996). Adhesion and mitogenic signals have been shown to synergise in their activation of the Ras-ERK MAP kinase pathway. This is demonstrated by the synergistic upregulation of ERK activity in human fibroblasts by the addition of EGF, PDGF or bFGF coupled with integrin receptor occupation, by fibronectin-coated beads or an RGD peptide (Miyamoto et al., 1996). However, this is not always the case, attachment to fibronectin-coated dishes did not alter the ability of PDGF to activate ERK in fibroblasts maintained in suspension (Hotchin and Hall, 1995; Zhu and Assoian, 1995). In contrast to this another group has reported that serum is unable to stimulate ERK activity in suspended Swiss 3T3 cells (Inoue et al., 1996). It has however been demonstrated that constitutive activation of MEK1 appears to be sufficient for both mitogen and anchorage independent growth, since fibroblasts expressing constitutive MEK1 are able to form colonies in soft agar, and proliferate in the absence of mitogens (Cowley et al., 1994; Mansour et al., 1994). Thus integrin-mediated and growth factor-mediated proliferative signals may converge on the ERK MAP kinase pathway, possibly through activation of Ras.

Oncogenic Ras also enables cells to overcome the constraints of growth inhibition by cell-cell contact. This is demonstrated by the occurrence of foci of transformed RasV12 transfected cells on an untransfected monolayer. P27Kipl is induced in fibroblasts upon cell-cell contact and downregulates Cyclin E-associated kinase activity (Polyak et al., 1994a). However p27Kipl null MEFs are still contact-inhibited (Nakayama et al., 1996). Alternatively, in epithelial cells Ras has been shown to downregulate the expression of cadherins which mediate cell-cell interactions and are also important signalling molecules (Ben Ze'ev, 1997).

Although Ras was originally discovered as a transforming and growth promoting oncogene, these properties only arise in established cell-lines or in co-operation with another oncogene in primary cells. It is assumed that established cell-lines have acquired genetic lesions as part of the immortalisation process. Indeed immortalised mouse embryo fibroblasts have lost function of either of the tumour supressor genes p53 or p19ARF (Zindy et al., 1998). Activated Ras is unable to transform cells of primary origin. It requires the presence of a second co-operating oncogene to achieve full transformation. Indeed Ras actively inhibits proliferation of some cell types. For example, v-Ha ras or
activated Raf was shown to directly inhibit cell proliferation in primary rat Schwann cells, although full transformation could be achieved if co-expressed with a viral oncogene, simian virus 40 large T antigen (SV40 LT) (Lloyd et al., 1997; Ridley et al., 1988). This arrest occurs in the G1 phase of the cell cycle and is associated with a p53-dependent upregulation of the cell cycle inhibitor, p21\(^{Cip-1}\) (Lloyd et al., 1997). In addition, Ras is able to mimic the effects of nerve growth factor (NGF) on a rat phaeochromocytoma cell-line, PC12, and induce cessation of cell growth and neurite outgrowth, a differentiated phenotype (Noda et al., 1985). More recently, it has been shown that primary human and rodent fibroblasts respond to activated Ras with a growth arrest. This arrest is associated with the upregulation of the CKIs p16\(^{INK4A}\) and p21\(^{Cip-1}\) and the cells exhibit characteristics associated with senescence such as changes in morphology and expression of senescence markers (Serrano et al., 1997).

Therefore it appears that the default response to activated Ras signalling is to stop growing and this must be overcome in order for Ras to stimulate mitogenesis. The mechanisms by which this is achieved are starting to be understood. For example, the G1 arrest caused by activation of the Ras/Raf/ERK pathway in primary rat Schwann cells can be prevented by interfering with p53-dependent upregulation of p21\(^{Cip-1}\) using a dominant negatively acting p53 mutant, antisense RNA to block p21\(^{Cip-1}\) translation, or SV40 LT which sequesters p53 (Lloyd et al., 1997). In a similar manner, Ras is tumorigenic in p21\(^{Cip-1}\) null keratinocytes, but not in wild type cells (Missero et al., 1996). Likewise, p16\(^{INK4A}\) null MEFs no longer exhibit growth arrest in response to Ras (Serrano et al., 1997). However this may also involve the tumour suppressor gene, p19\(^{ARF}\), which is also affected in this knock out. Thus it appears that in order for Ras to be growth stimulatory or tumorigenic, cell cycle inhibitory proteins must first be inactivated.

### 1.2.6 Ras signal transduction through Raf

#### 1.2.6.1 Raf as a Ras effector

Upon activation, Ras relays its signals through its downstream targets. The most established of these is Raf-1. Raf-1 is a serine threonine protein kinase, and is part of a family also containing A-Raf and B-Raf (Bonner et al., 1984; Huleihel et al., 1986; Ikawa et al., 1988). Raf was originally placed downstream of Ras because genetic evidence from Drosophila and C.elegans showed that it is essential for Ras signalling in eye and vulval development, respectively (Dickson et al., 1992; Han et al., 1993). Raf is also necessary for Ras signalling in mammalian cells since dominant negative Raf molecules block Ras-induced gene transcription (Bruder et al., 1992) and proliferation (Kolch et al., 1991). Moreover, Raf can reproduce many of the cellular responses of Ras in mammalian cells. For example, both Ras and Raf can activate ERK, and dominant negative versions of both can block growth factor induced ERK activation (de Vries-
Direct binding of Raf to Ras was demonstrated using yeast two hybrid analysis and direct in vitro binding assays. Specifically, the N-terminal portion of Raf-1 binds directly to the effector domain of Ras-GTP (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Furthermore, the Ras\(^{V12G37}\) effector domain mutant which can no longer bind to Raf-1 is defective in ERK activation and this impairment can be complemented by mutations in Raf-1 which restore an interaction with Ras\(^{V12G37}\) (White et al., 1995). These observations and similar ones (reviewed in Katz and McCormick, 1997; Marshall, 1996; Marshall, 1993) provide strong evidence that Raf-1 is a genuine effector of Ras signalling responsible for the activation of the ERK MAP kinase pathway.

1.2.6.2 Raf activation

Although Raf-1 has been established as an effector of Ras, purified recombinant Ras is not sufficient to activate Raf-1 in vitro (Traverse et al., 1993; Zhang et al., 1993). Raf-1 activation appears to be a complex, multi-step process involving many components. A current model of Raf activation is depicted in Figure 1.5. Raf-1 is composed of two functional domains, the amino terminal regulatory domain (conserved regions, CR1 (aa 62-194) and CR2 (aa 254-269)) and the carboxy terminal kinase domain (CR3 (aa 330-627)) which interacts with downstream targets of Raf-1. The amino terminal domain suppresses Raf-1 catalytic activity and deletion of part of its regulatory amino-terminal domain results in a constitutively activated Raf-1 mutant (Beck et al., 1987; Heidecker et al., 1990; Ikawa et al., 1988; Stanton and Cooper, 1987; Stanton et al., 1989).

All the components necessary for Raf-1 activation are located at the plasma membrane. Raf-1, purified from an insect expression system can be activated by the addition of plasma membranes from Ras\(^{V12}\) transformed cells (Dent and Sturgill, 1994). Moreover, when plasma membranes which have been treated to remove integral proteins were mixed with recombinant and post-translationally modified Ki-Ras it was sufficient to activate Raf-1 added as a component of cytosol, but not purified recombinant Raf-1 protein. This did not occur in the absence of membranes (Stokoe and McCormick, 1997). Ras is known to translocate Raf-1 from the cytoplasm to the plasma membrane (Traverse et al., 1993). Membrane localisation of Ras can be prevented by blocking its post-translational modification using peptide inhibitors. Unmodified Ras is still able to complex with Raf-1, but these cytosolic complexes are inactive (Kikuchi and Williams, 1994; Lerner et al., 1995; Okada et al., 1996). Raf-1 can be constitutively activated by fusion of the membrane localisation sequence of Ki-Ras to its carboxy terminus which permits membrane binding and activation of Raf-1 independently of Ras (Leevers et al., 1994; Stokoe et al., 1994).
Two separate domains of Raf-1, the Ras binding domain (RBD) αα 2-140 and the
Cysteine rich domain (CRD), αα 139-186, have been found to bind to Ras in its switch I
and switch II regions, respectively. Concurrent binding of both domains is required for
Raf-1 activation. Mutation of Ras in either its Switch I or II region which can disrupt
binding of polypeptides encoding the CRD or the RBD can prevent Raf-1 activation in vitro (Brerva et al., 1995; Drugan et al., 1996; Hu et al., 1995a). Furthermore, Raf-1
fragments corresponding to the CRD are able to inhibit the transforming ability of
activated Ras, indicating that this association is important in Ras transformation (Brerva et al., 1995). This implies that Ras has additional functions important in activation of Raf-
1, in addition to translocation. Ras mutants that are defective in binding the RBD of Raf-1
are unable to bind full length Raf-1 indicating that RBD binding is required prior to CRD
binding. The CRD is possibly unmasked by a conformational change in Raf-1 upon RBD
binding, since removal of CR3 or mutations in CR2 of Raf-1 allow CRD binding in the
absence of prior RBD binding (Drugan et al., 1996).

**Figure 1.5 Activation of Raf-1**

The CRD may be involved in binding other molecules such as 14-3-3 proteins (Michaud
et al., 1995). 14-3-3 proteins are thought to have a regulatory role in Raf-1 activation
(Fantl et al., 1994; Fu et al., 1994), however the details of this remain unclear. Raf-1 has
three putative 14-3-3 binding sites; RxSxSxSxP (single letter code for amino acids where x
represents any amino acid and S* represents phosphorylated serine) (Morrison and
Cutler, 1997). 14-3-3 proteins may be involved in the masking of the amino terminal
CRD of Raf-1, regulating its activation since disruption of 14-3-3 binding by mutation of
a 14-3-3 binding site within the CRD of Raf-1 increases the transforming potential of Raf-
1 (Clark et al., 1997a; Rommel et al., 1997). Ras also interferes with the interaction
between amino terminal of Raf-1 and 14-3-3 (Rommel et al., 1996). 14-3-3 proteins
binding to the carboxy terminal kinase domain of Raf-1 may be required to stabilise an
active conformation of Raf produced during activation, since inhibition of 14-3-3 binding
can deactivate Raf-1 \textit{in vitro} and dimerised 14-3-3 proteins can reactivate Raf-1 (Tzivion \textit{et al.}, 1998). 14-3-3 interaction with Raf-1 can also protect Raf-1 from phosphatases (Dent \textit{et al.}, 1995).

A role for phospholipids in Raf-1 regulation has also been demonstrated. The CRD of Raf-1 binds to phosphatidyl serine (PS) (Ghosh \textit{et al.}, 1994) and addition of PS or reconstituted plasma membranes can enhance activation of Raf-1 by Ras \textit{in vitro} (Kuroda \textit{et al.}, 1996b; Stokoe and McCormick, 1997). Ceramide, which acts as a lipid co-factor induced by interleukin 1\beta, has also been demonstrated to bind and activate Raf-1 (Huwiler \textit{et al.}, 1996). However, ceramides activated by TNF, have been shown to bind Raf-1 and increase its association with Ras but do not increase Raf-1 kinase activity and actually downregulate activation of Raf-1 by EGF and v-Src (Muller \textit{et al.}, 1998). These different effects may be due to cell type specific differences, however, they may well be propagated by kinase supressor of Ras (Ksr).

There also appears to be a role for Ksr in Raf-1 regulation downstream of Ras (Therrien \textit{et al.}, 1996). Loss of function ksr alleles act as dominant suppressors to the rough eye phenotype caused by activated Ras in \textit{Drosophila} (Therrien \textit{et al.}, 1995) indicating a requirement for Ksr in Ras signal transduction. Ksr had been previously identified as ceramide activated protein (CAP) kinase (Zhang \textit{et al.}, 1997). It appears to have a role in facilitating signalling via the MAP kinase cascade. Several groups suggest it interacts (possibly via 14-3-3 proteins) with and activates Raf-1 in a membrane bound multi-protein signalling complex (Michaud \textit{et al.}, 1997; Xing \textit{et al.}, 1997; Zhang \textit{et al.}, 1997). However other groups show that it does not interact with Raf-1, but does interact with Mitogen/Extracellular regulated kinase kinase (MEK) and ERK in yeast two hybrid screens and immunoprecipitation of endogenous proteins from PC12 cells (Denouel-Galy \textit{et al.}, 1998; Yu \textit{et al.}, 1998). A novel gene, connector enhancer of ksr (cnk) is also required for efficient signal transmission within the Ras/ERK cascade and was found as a mutation that modified a dominant negative ksr and activated Ras dependent phenotype in \textit{Drosophila}. CNK is a possible target of tyrosine phosphorylation and physically interacts with D-Raf (Therrien \textit{et al.}, 1998). There are also indications that Ksr is a substrate for ERK which may be an event connected to a regulatory feedback loop (Cacace \textit{et al.}, 1999).

Other Raf-1 binding proteins such as the molecular chaperone heat shock proteins, HSP 90 and HSP 50, may have a role in maintaining Raf-1 protein stability and its proper localisation. Their binding to Raf-1 is irrespective of its activation state (Schulte \textit{et al.}, 1995; Schulte \textit{et al.}, 1996).

Raf-1 is also regulated through tyrosine phosphorylation at residues Y340 and Y341. This is dependent upon Ras activity in mammalian cells and is performed by tyrosine
kinases such as the Src family or the Janus activated kinases (JAKs). The presence of v-Src synergistically increases Ras activation of Raf-1 indicating the importance of tyrosine phosphorylation in Raf activation (Fabian et al., 1994; Marais et al., 1995; Xia et al., 1996).

Serine/threonine phosphorylation may also have a role in Raf-1 regulation. The p21 activated protein kinase, PAK3, has been demonstrated to directly phosphorylate serine 338 of Raf-1. Phosphorylation of this site is necessary for Ras activation of ERK, via Raf-1 (King et al., 1998). (This will be discussed in more detail later). In addition, 14-3-3 binding requires a phosphorylated serine residue.

The cyclic AMP dependent kinase (PKA), is able to inhibit Raf-1 activity (Cook and McCormick, 1993). PKA phosphorylates Raf-1 at Ser43, and this phosphorylation inhibits Ras-Raf interaction (Wu et al., 1993a). Furthermore, PKA has an ability to directly inhibit Raf-1 kinase activity that is distinct from its ability to interfere with Ras-Raf interaction, since it is able to inhibit the kinase activity of a truncated Raf-1 mutant, missing its Ras binding domains, or v-Raf which is independent of Ras. This ability may involve phosphorylation of residues within Raf’s kinase domain (Hafner et al., 1994).

Finally, forced dimerization of Raf proteins has been demonstrated by two groups to activate Raf signalling, however one group demonstrates this in the absence of membrane components, i.e. independently of Ras (Farrar et al., 1996), while the other study found that the Ras-Raf interaction was still required (Luo et al., 1996). This may indicate an alternative mechanism for Raf activation, which may be independent of Ras.

1.2.7 Activation of MAP kinase cascades

Raf controls a cascade of dual specificity kinases, in which a MAP kinase kinase kinase, such as Raf-1, phosphorylates and activates a MAP kinase kinase, such as MEK1, which then phosphorylates and activates a MAP kinase, which then goes on to phosphorylate specific targets (see Figure 1.6). This sequence of events is generally termed a MAP kinase cascade. MEK (also called M KK1 and MAPKK1) and ERK are activated upon mitogen stimulation or in the presence of oncogenic Ras or Raf-1. Bacterially expressed oncogenic Raf-1, or wild type Raf-1 immunoprecipitated from insect or mammalian cells that co-express v-Src or v-Ras, is able to directly phosphorylate and activate MEK1, that has been previously subjected to dephosphorylation by phosphatase 2A (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992; Macdonald et al., 1993). Activated MEK can phosphorylate and activate p44EKR1 and p42EKR2 (also referred to as p42 and p44 MAP kinases (or MAPKs)) in an in vitro reconstitution assay (Crews et al., 1992; Wu et al., 1992) and when over expressed in Cos cells (Wu et al., 1993b). ERKs are activated by phosphorylation on both threonine and tyrosine residues (Boulton et al., 1991; Payne et al., 1991). Removal of either of these phosphorylations through the action of specific
phosphatases or site-directed mutagenesis leads to inactivation (Anderson et al., 1990; Cowley et al., 1994).

Other MAP kinase cascades occur in mammalian cells in addition to the Raf-MEK-ERK cascade. These are depicted in Figure 1.1. The JNK and p38 pathways are activated by inflammatory cytokines, TNFα, IL1β and cellular stresses such as UV light, anisomycin, heat shock, sodium arsenite and γ-irradiation (Kyriakis and Avruch, 1996). They can also be activated by Ras, independently of Raf (Minden et al., 1994). These pathways are also targets of the Rho family of GTPases and will be discussed in more detail later. Similar MAP kinase cascades have been identified in yeast, C.elegans and Drosophila.

Different MAP kinase modules in yeast share components and specificity of signalling in response to stimuli is thought to be achieved by the use of scaffold or adaptor proteins. Such an example is Ste5 in S.cerevisiae, which form multi-protein complexes with specific MAP kinase modules. Recently, two non-enzymatic proteins with similar functions have been identified in mammalian cells, JNK-interacting protein-1 (JIP-1) and MEK partner 1 (MPI). JIP-1 is thought to channel signals through a specific set of kinases that activate JNK. It specifically binds the MAP kinase kinases, MLK3 and DLK, the MAP kinase, MKK7 and the MAP kinases, JNK1 and JNK2 (Whitmarsh et al., 1998). MPI does not appear to route an entire pathway, but specifically stabilises MEK1 and p44ERK interaction such that overexpression of MPI is able to increase the ability of MEK1 and p44ERK to co-activate Elk-1-mediated transcription (Schaeffer et al., 1998). It is probable that more proteins with similar functions will be found in mammalian cells, and that such proteins will provide a mechanism for retaining that specificity of MAP kinase modules in response to specific signals, even when the components are shared between pathways. Abuse of scaffolding proteins may account for inappropriate activation of MAP kinase cascades upon overexpression of specific module components. For example, overexpression of MEKK1 has been shown to activate MEK1 (Lange-Carter et al., 1993; Yan et al., 1994).

1.2.7.1 ERK targets

ERKs are activated in the cytoplasm and directly or indirectly activate transcription. MAP kinases can translocate into the nucleus and phosphorylate nuclear transcription factors including those in the Ets family and bZIP and MADS box containing transcriptional regulators. For example, Elk1; Ets transrepressors e.g. ERF; ATFs; c-fos; c-myc and the oestrogen receptor (Lewis et al., 1998). In addition, ERKs can activate protein kinases referred to as MAPK activated protein kinases (MAPKAPK) 1, 2 and 3 and Mnks 1 and 2 (MAPKAPK1α and β were first identified as p90Rsk1 and p190Rsk2). Some MAPKAPK1 substrates are involved in transcription and include cAMP response-element binding protein (CREB), CREB binding protein (CBP), c-fos, Nurr 77 and serum response factor.
MAPKAPK2 and MAPKAPK3 phosphorylate HSP27 which is believed to be an actin capping protein (Clifton et al., 1996; Engel et al., 1995; McLaughlin et al., 1996; Stokoe et al., 1992). Mnk1 and 2 phosphorylate Elongation Initiation Factor 4E (eif4E), implying that they have a role in translational control (Waskiewicz et al., 1997). ERKs also activate phospholipase A2 which suggests a role for ERKs in agonist stimulated arachidonic acid release (Lin et al., 1993; Nemenoff et al., 1993).

A mechanism for activation of MAP kinase directed transcription has recently been proposed in S. cerevisiae which also contain MAP kinase cascades. The MAP kinase homologue Kss1, when unphosphorylated, directly binds to and represses the transcription factor Ste12 which prevents invasive growth. This was demonstrated by the inability of Kss1 mutants that are specifically defective in Ste12 binding (but are able to bind other regulatory molecules, including Ste7) to inhibit invasive growth. Phosphorylation of Kss1 by Ste7, a MEK homologue, weakens the Kss1-Ste12 interaction and thereby relieves Kss1 induced transcriptional repression. Kss1 is then thought to further activate Ste12 by phosphorylation (Bardwell et al., 1998). It remains to be seen whether this mechanism in which MAP kinases have both a repressive and activating role in gene transcription will be found in mammalian MAP kinase cascades.

1.2.7.2 ERK inactivation

Inactivation of MAP kinases in mammalian cells occurs by dephosphorylation involving phosphatases of two different families. Dual specificity phosphatases such as the MAP kinase phosphatases (MKP) 1-4 are able to remove serine, threonine and tyrosine phosphorylation concomitantly in vitro, (Alessi et al., 1993; Charles et al., 1993) and also inactivate ERKs in vivo (Duff et al., 1995; Sun et al., 1993). Serine/threonine protein phosphatases, such as PP1 and PP2A also inactivate ERKs as well as MAP kinase kinases. Treatment of cells with okadaic acid (which is inhibitory for PP1 and PP2A) activates ERK activity, which implies a role for these phosphatases in ERK regulation.(Casillas et al., 1993; Gotoh et al., 1990; Haystead et al., 1990).
Stimulus Pheromones Growth Factors Stress signals TNFα Proinflammatory cytokines Growth Factors Growth Factors?

Small GTPase Cdc42 Sc Ras1 Ras Rac, Cdc42 ?

PAK GCK,HPK1 GCK HPK1

STE20

MAPKKK STE11 Byr2 Raf MEKK1, MEKK4 TAK1,TAO?

MAPKK STE7 Byr1 MEK1,2 MKK7 MEK4 MEK3 MEK6 MEK5

MAPK Kss1 SPK1 ERK1,2 JNK1,2,3 p38,p38α ERK6, ERK5 SAPK4

Substrate STE12, FAR1 Ets, Elk1, MAPKAP-Ks ATF2, Jun, Elk1 ATF2,CREB, MAPKAP-Ks MCF2C

mating, meiosis Mammalian cascades

S.cerevisiae S.pombe

Figure 1.6 Signal transduction through MAP kinase modules
MAP kinase cascades in mammalian cells and yeast are depicted. Multiple MAP kinase modules exist in yeast and only one is shown. Examples of substrates for the MAPKs are shown, but many more substrates have been identified and the specificity of each MAPK for its substrates has not yet been properly defined. As described in the text, additional interactions between members of cascades occur and are not shown here.
1.2.7.3 Additional regulation of MAP kinase cascades

Each member of MAP kinase cascade also has additional upstream activators and downstream targets. Thus what were once considered linear signalling pathways are now considered part of a much larger signalling network. For example the novel protein kinase C, PKC8, is able to activate ERK in the presence of a dominant negative Ras molecule (Ueda et al., 1996). The same is true of an integrin dependent pathway stimulated by fibronectin binding (Chen et al., 1996b) indicating independence of ERK activation from Ras signalling. The use of specific chemical inhibitors has indicated that p44Erk activation by PDGF has both a MEK1 dependent and a MEK1 independent step that are tempororally distinct. Both phosphatidylinositol 3 kinases (PI 3-kinase) and conventional PKCs are implicated in the MEK1 independent step (Grammer and Blenis, 1997). In addition regulatable oncogenic Raf (and constitutively activated MEK1) has been demonstrated to rapidly activate p70s6 kinase in the presence of the MAP kinase phosphatase, MKP-1 and therefore independently of ERK activity (Lenormand et al., 1996).

Positive feedback loops also occur within the cascade. For instance ERKs can phosphorylate MEK1 (Gotoh et al., 1994; Zheng and Guan, 1993), Raf-1 (Anderson et al., 1991; Kyriakis et al., 1993; Lee et al., 1991) and Ksr, and MEK1 can phosphorylate Raf-1 through an ERK dependent pathway (Zimmermann et al., 1997). However, caution must be used when interpreting this data. For example, MEK was proposed as a target for an alternative MAP kinase kinase kinase MEKK1 because MEKK1 can induce MEK1 activation in vitro and when over expressed in Cos cells (Lange-Carter et al., 1993; Yan et al., 1994). However, lower levels of MEKK1 expression were unable to activate MEK1, but could activate the jun N-terminal kinases or stress activated protein kinases (JNKs or SAPKs). These are now considered to be the genuine physiological targets for MEKK1 (Minden et al., 1994; Yan et al., 1994).

1.2.8 Alternative Ras effectors

A large body of evidence derived from cellular responses to Ras in different organisms suggests that Raf is not the sole effector of Ras. Firstly, adenyl cyclase can be activated in vitro by Ras-GTP and has been demonstrated to be a direct effector of RAS in S.cerevisiae (Toda et al., 1985). However, deletion of the gene does not cause the lethality associated with RAS deletion (Marcus et al., 1993) indicating that Ras controls additional pathways. Secondly, in S.pombe, Byr2 which is similar in structure to Raf-1 and activates a MAP kinase cascade, rescues the sporulation defect but not the conjugation or morphological defects of Ras1 null cells (Wang et al., 1991). Thirdly, Raf is unable to reproduce all the effects of Ras in mammalian cells. For example activated Raf only activates a subset of the genes activated by constitutive Ras in PC12 cells (D'Arcangelo
Similarly activated Raf is able to reproduce MAP kinase activation and gene transcription induced by oncogenic Ras in cardiac myoblasts, but is unable to reproduce the Ras induced changes to the cytoskeleton (Thorburn et al., 1994). Furthermore JNK and p38 MAP kinase pathways can be stimulated by activated Ras, but not directly by activated Raf (Minden et al., 1994) and although activated Ras is able to promote complete transformation of a rat intestinal cell-line (RIE-1), activated Raf is unable to fully reproduce this. These data indicate that Ras must activate additional targets and pathways in addition to Raf to achieve its reported effects.

1.2.8.1 Effector domain mutants of Ras

Some mutations within the effector domain of Ras disrupt Ras signalling but do not compromise GTP binding. This domain comprises amino acids 32-40 and includes part of the Switch I domain. Ras proteins containing these mutations are termed Ras effector domain mutants and each one displays differential abilities to stimulate Ras-mediated effects. These correlate with their abilities to bind or activate specific target or effector proteins. These mutants have been invaluable in identifying the role of specific Ras effectors in Ras-mediated cellular responses. A yeast two hybrid screen designed to identify Ha-Ras mutants which bind Ha-Ras targets differentially led to the isolation of two mutants, RasS35 and RasG37. RasS35 can bind Raf-1 and the S.cerevisiae RasGEF, CDC25, but is unable to bind Byr2 or adenyl cyclase. RasG37 binds Byr2 and adenyl cyclase, but not CDC25 or Raf-1. In mammalian cells, RasV12S35 (an activated version of the mutant) was shown to activate signalling from the serum response element (SRE) of the c-fos promoter and had weak focus forming ability when transfected into NIH 3T3 cells, while RasV12G37 was unable to activate SRE signalling nor form foci. However co-expression of RasV12S35 and RasV12G37 caused a synergistic increase in focus formation (White et al., 1995). This implied that at least two downstream pathways from Ras were required for full transformation. SRE activation by RasV12S35 did not increase upon co-expression of RasV12G37, indicating that Ras-mediated effects can be specific to certain effector pathways. Another effector domain mutant, RasV12C40, is able to activate membrane ruffling (another property of RasV12), but cannot activate ERK. RasV12S35 has reciprocal properties to RasV12C40, however neither is able to induce DNA synthesis. Microinjection of both of these mutants does stimulate DNA synthesis, indicating that Ras needs to activate multiple downstream pathways to induce DNA synthesis (Joneson et al., 1996b). In vitro binding assays showed that RasS35 binds Raf with the greatest efficiency, RasC40 binds the p110 subunit of PI 3-kinase and RasG37 binds Ral-GDS, however interaction with other Ras effectors may also occur. Moreover Ras proteins with these mutations in combination with the V12 mutation co-operate with activated versions of other proposed Ras downstream targets to form foci in NIH 3T3 cells. Specifically
Ras^{V12S35} co-operates with activated Rac1, RhoA and p110, but not Raf-1. Ras^{V12C40} co-operates with activated Raf-1 and RhoA, but not Rac1 or p110; and Ras^{V12G37} co-operates with activated Raf-1 and RhoA and to a lesser extent with p110 and Rac-1. These mutants can also co-operate with each other (Rodriguez-Viciana et al., 1997). However, caution must be used when interpreting the data generated from studies utilising effector domain mutants since these interactions could not be seen in all experimental systems suggesting that effector function may vary between cell types (Stang et al., 1997). Furthermore, the intensity of signals generated by these mutants is much less than activated Ras. We demonstrate later that Ras signal strength is an important factor in Ras-induced cellular responses. However these data add weight to other evidence supporting a role for multiple Ras activated pathways in many Ras-mediated effects.

1.2.9 Ras Effectors

There are a multitude of putative Ras effectors that bind Ras in a GTP-dependent manner, which have been isolated by yeast two hybrid screens, yeast functional screening and biochemical methods (Campbell et al., 1998; Vavvas et al., 1998). Current candidate Ras effectors are included in Figure 1.7. Two main criteria define true Ras effectors. Genuine effectors of Ras should be able to mimic certain Ras responses upon activation. Furthermore, Ras-induced effects should be inhibited by interference with the function of these effectors. So far, only three of these putative effectors as yet meet these criteria; the Raf family, the phosphatidylinositol 3-OH kinase (PI 3-kinase) family and the Ral-guanine nucleotide exchange factor (Ral-GEF) family.
Proteins that have been found to bind to Ras in a GTP-dependent fashion. Rin1 was identified as a gene that interfered with Ras function in yeast. AF-6 (Rbs1) is homologous to the Drosophila protein Canoe. Rin1, Nore1, AF-6 and Canoe have been shown to bind Ras in a yeast two-hybrid system or in in vitro assays, in a GTP-dependent manner and Rin1, AF-6 and Canoe can compete with Raf-1 for Ras binding (Campbell et al., 1998; Vavvus et al., 1998). Their functions have not yet been elucidated, however Rin1 may be involved in linking Abl tyrosine kinase to Ras signalling, since they interact in vitro and Rin1 is a substrate for Abl. PKCε has been shown to be necessary for serum-stimulated mitogenic signalling and forms complexes with Ras in vivo in response to PDGF stimulation. It can bind Ras in a GTP-dependent manner and dominant negative Ras mutants prevent its activation by PDGF. The main function of NF1 is probably as a RasGAP, however it may also have a limited function downstream. MEKK1 has been shown to bind Ras in a GTP-dependent manner and is activated in response to activated Ras, however it is not clear whether it has a role as a Ras effector. The other effectors, highlighted in bold, are discussed in the text.
1.2.10 PI 3-kinase and its role as a Ras effector

PI 3-kinase was first discovered as an activity associated with the viral oncoproteins, v-Src and polyoma middle T antigen. They comprise a family of related proteins and are thought to be important in a wide range of biological activities, including control of proliferation (Vanhaesebroeck et al., 1996). This may involve PI 3-kinase activation of P70^kinase (Chung et al., 1994; Monfar et al., 1995). PI 3-kinases have also been demonstrated to cause cytoskeletal organisation through activation of the Rho family of GTPases (Kotani et al., 1994; Rodriguez-Viciana et al., 1997; Wennstrom et al., 1994a; Wennstrom et al., 1994b). PI 3-kinase is also involved in the prevention of cell death by apoptosis by the activation of AKT/PKB, which directly phosphorylates the pro-apoptotic Bcl-2 family member, Bad, and therefore couples this signalling pathway to the death machinery (Datta et al., 1997; Dudek et al., 1997; Franke et al., 1997; Kauffmann-Zeh et al., 1997; Yao and Cooper, 1995; Yao and Cooper, 1996). In addition PI 3 kinase has been demonstrated to have a role in endocytosis and vesicular trafficking (Joly et al., 1995; Joly et al., 1994; Li et al., 1995a; Martys et al., 1996; Shpetner et al., 1996), neurite outgrowth (Kimura et al., 1994) and insulin stimulated glucose transport (Cheatham et al., 1994; Hara et al., 1994; Okada et al., 1994). Mutations within PI 3-kinase have also been shown to increase lifespan in C.elegans (Morris et al., 1996). Furthermore, a PI 3 kinase has recently been shown to be a retrovirally-encoded oncogene in avian sarcoma virus 16 (Chang et al., 1997) and an activated form of PI 3-kinase has been cloned from an induced murine lymphoma (Jimenez et al., 1998).

The PI 3-kinase family is divided into classes; the most studied, and the one discussed here is Class IA (Fruman et al., 1998). Class IA PI 3-kinases phosphorylate phosphoinositide phospholipids on the free 3-position, and can phosphorylate, PtdIns, PtdIns 4-P, PtdIns 4,5-P^2 and PtdIns 5-P in vitro. Activation of PI 3-kinases in vivo however appears to mainly produce PtdIns 3,4-P^2 and PtdIns 3,4,5-P^3 (Fruman et al., 1998; Stephens et al., 1991). These proteins comprise of a catalytic subunit of 110-120 kDa and a regulatory subunit. The three mammalian catalytic subunits in this class are referred to as p110^α, p110^β and p110^δ. They interact with their regulatory subunits through their amino-terminal domains and have a carboxy-terminal catalytic domain. The regulatory subunits are called p85^α, p85^β, p55^α, p55^γ and p50^α (the final three proteins are splice variants of p85^α) and are generally termed the p85 subunit. p85^α and p85^β contain an SH3 domain in their amino terminus, then a proline rich region and a region of homology to Rho-GAPs. The carboxy terminus contains the p110 binding region (referred to as the inter-SH2 domain) flanked by two SH2 domains. The splice variants still contain the carboxy-terminal domain but are lacking the amino-terminal domains including the Rho-GAP-like domain (Fruman et al., 1998).
1.2.10.1 Activation of PI 3-kinase

PI 3-kinase activity is regulated by several mechanisms. Direct phosphorylation of the catalytic p110 subunit is necessary for its kinase activity and serine phosphorylation of p85 causes inhibition of PI 3-kinase activity, and tyrosine phosphorylation of p85 also occurs (Carpenter et al., 1993; Dhand et al., 1994; Kavanaugh et al., 1994). Activation is also mediated through interaction with a variety of signalling proteins. For example, many growth factor receptors activate PI 3-kinase directly. Growth factors stimulate autophosphorylation of tyrosine residues within the intracellular domain of their receptors which create a specific binding site recognised by the SH2 domains of the p85 subunit. Binding of PI 3-kinase to the receptor causes an increase in PI 3-kinase activity probably because it is brought into the vicinity of its lipid substrates. This can be blocked by mutation of these phosphotyrosines (Vanhaesebroeck et al., 1996). PI 3-kinase is also regulated by and associated with non-receptor tyrosine kinases, v-src, v-abl, lck, fyn and lyn in response to cellular activators that stimulate these enzymes. They are thought to bind the proline rich region of p85 through their SH3 domains (Kapeller and Cantley, 1994). PI 3-kinase activity is also activated by cell attachment to extracellular matrix (ECM) which may be an important survival signal. Adhesion-mediated activation of PI 3-kinase is thought to be via integrin activation of focal adhesion kinase (FAK). Autophosphorylated FAK has been shown to bind the p85 subunit of PI 3-kinase and activate PI 3-kinase activity (Chen et al., 1996a). p85α also binds to Cdc42 and Rac1 in vitro and exogenous Rac and Cdc42 can increase PI 3-kinase activity in vitro (Zheng et al., 1994). However, in contrast, another group has demonstrated that Rac and Cdc42 are unable to stimulate PI 3-kinase activity, while Ras is able (Rodriguez-Viciana et al., 1994). In addition, PI 3-kinase has been shown to be activated by direct interaction of the p110 subunit with GTP bound Ras which also locates it to the plasma membrane (Rodriguez-Viciana et al., 1994).

1.2.10.2 PI 3-kinase, a Ras effector

Several lines of evidence support the case for PI 3-kinase as an effector of Ras. Firstly, the p110 subunit of PI 3-kinase interacts directly with the effector domain of Ras-GTP in vitro and in vivo. (Rodriguez-Viciana et al., 1994). Secondly, transfection of Ras, but not activated Raf, upregulates the cellular levels of 3' phosphorylated phosphatidylinositols (the products of PI 3-kinase phosphorylation) and co-transfection of the p110 and p85 subunits of PI 3-kinase with Ras increases 3' phosphorylated phosphatidylinositol production above that with Ras alone. Moreover, a dominant negative Ras mutant is able to attenuate the production of 3' phosphorylated phosphatidylinositol in response to growth factors (Rodriguez-Viciana et al., 1994). This implies that Ras is important for growth factor stimulation of PI 3-kinase activity.
The remaining ability of growth factors to stimulate PI 3-kinase activity in the presence of RasN17 indicates that there are alternative pathways for activation. Thirdly, prenylated Ras has been shown to directly induce PI 3-kinase activity in an in vitro liposome reconstitution system (Rodriguez-Viciana et al., 1996). This implies that the role of Ras in PI 3-kinase activation may be to localise PI 3-kinase to the plasma membrane and bring it in contact with its lipid substrate.

Finally, several experiments have shown that PI 3-kinase is an important component of Ras transformation. Firstly, a mutant of the p85 subunit that acts in a dominant negative manner, suppresses the ability of RasV12, but not v-Src, to form foci and colonies in soft agar (Rodriguez-Viciana et al., 1997). Secondly, PI 3-kinase has been shown to cooperate with activated Raf to cause focus formation. Thirdly, co-expression of H-RasV12C40, which is able to bind PI 3-kinase, but not Raf-1, and H-RasV12S35, which has the reciprocal binding properties, results in synergistic focus formation. The same is true when one Ras effector mutant is co-expressed with the reciprocal effector (Rodriguez-Viciana et al., 1997).

1.2.10.3 PI 3-kinase regulation of the cell cycle

A requirement for PI 3-kinase activity for stimulation of DNA synthesis by serum and specific mitogens has been suggested by a variety of experiments. Firstly, platelet derived growth factor (PDGF) stimulated DNA synthesis is reported to be dependent on PI 3-kinase because PDGF receptor mutants that no longer bind PI 3-kinase are unable to respond mitogenically to PDGF in human hepatoma and canine kidney epithelial cells (Valius and Kazlauskas, 1993). However it should be borne in mind that these sites may be used by other signalling molecules. Secondly, microinjected antibodies specific for p110α and inhibitory to PI 3-kinase function, prevent PDGF and epidermal growth factor (EGF) stimulation of DNA synthesis in 3T3 fibroblasts. However this is not true for all growth factors, since colony stimulating factor-1, bombesin and lysophosphatidic acid are able to stimulate DNA synthesis in a PI 3-kinase independent manner (Roche et al., 1994). Thirdly, insulin stimulated DNA synthesis is inhibited by microinjection of the SH2 domain of the p85 subunit of PI 3-kinase, which acts in a dominant negative fashion (Jhun et al., 1994). Finally, a specific inhibitor of PI 3-kinase, LY 294002 inhibits serum stimulation of DNA synthesis in 3T3-L1 adipocytes (Cheatham et al., 1994).

PI 3-kinase activity is also able to stimulate DNA synthesis. For example constitutively activated PI 3-kinase in 3T3-L1 adipocytes stimulates mitogen independent DNA synthesis (Frevert and Kahn, 1997). Also microinjection of a PI 3-kinase activating antibody stimulates DNA synthesis in quiescent Chinese hamster ovary cells, however this activation appears to be dependent on Ras since it is partly inhibited by anti-Ras
antibodies (McIlroy et al., 1997). Thus PI 3-kinase is a strong candidate as a Ras effector and appears to have an important role in cell cycle progression.

1.2.10.4 Downstream targets of PI 3-kinase

1.2.10.4.1 Akt/PKB and its role in cell survival

PI 3-kinase has been shown to be necessary and sufficient for activation of Akt/PKB (also occasionally referred to as RAC, related to Δ and Ζ kinases) by growth factors. Akt/PKB has been demonstrated to have an important role in cell survival, through direct interaction with and subsequent inhibition of Bad, a protein that inhibits activation of the survival factor Bcl-2 (Datta et al., 1997; del Peso et al., 1997; Downward, 1998). Akt/PKB is thought to be activated by PDK1 which has been shown to phosphorylate its activation loop. PDK-1 is activated by a lipid product of PI 3-kinase activity, PI(3,4,5)P₃ (Alessi and Cohen, 1998). Thus PI 3-kinase activation can directly influence and promote cell survival. The significance of AKT in the regulation of cell growth and survival is emphasised by the amplification of Akt2/PKBβ in 12% of ovarian carcinomas, 3% of breast carcinomas and 10% of pancreatic carcinomas (Downward, 1998). Akt/PKB can also be activated in responses to cellular stresses of the type that activate the JNK and p38 MAP kinase cascades by a wortmannin insensitive pathway which implies that activation is independent of PI 3-kinase. The mechanism by which this occurs is unknown.

Other functions of Akt/PKB include the induction of protein synthesis through activation of p70S6 kinase; the activation of glycogen synthesis through inactivation of glycogen synthase kinase-3 (GSK-3) which is also implicated in a variety of other intracellular signalling pathways including control of API and Creb transcription factors and regulation of APC; the induction of glycolysis through 6-phosphofructo-2-kinase (PFK-2) and finally transducing the metabolic effects of insulin such as stimulation of glucose uptake and GLUT4 translocation.

1.2.10.4.2 Other downstream targets

PI 3-kinase activity is also required upstream of the Rho family small GTPase, Rac to promote Rac-mediated membrane ruffling, by growth factors or activated Ras, since it can be inhibited by specific inhibitors or dominant negative mutants of PI 3-kinase (Wennstrom et al., 1994a; Rodriguez-Viciana et al., 1997). However, the role of PI 3-kinase in Rac activation is not clear since a constitutively activated PI 3-kinase mutant was only able to reproduce the effects on the cytoskeleton, but not Rac and Rho-regulated gene transcription from the serum response element (SRE) or the transcription factors Elk1 and AP-1 (Reif et al., 1996). This may mean that PI 3-kinase is required to mediate downstream effects of Rac, but is not involved in direct activation of Rac itself.
In addition to being implicated as a Ras effector, PI 3-kinase may also have a function upstream of Ras. A constitutively active p110 subunit was able to stimulate transcription from the c-fos promoter which was sensitive to inhibition by a dominant negative Ras mutant and furthermore increased the levels of Ras-GTP and Raf activation when expressed in Xenopus oocytes, as well as stimulating oocyte maturation (Hu et al., 1995b). Furthermore, antibodies that specifically activate PI 3-kinase activity and stimulate DNA synthesis can be blocked by dominant negative Ras mutants (McIlroy et al., 1997).

PI 3-kinase also activates p70S6 kinase. The ability of the PI 3-kinase antibodies described above to induce DNA synthesis is also blocked by anti-p70S6 kinase antibodies or rapamycin (McIlroy et al., 1997). Both Akt/PKB and Rac have demonstrated ability to activate p70S6 kinase downstream of PI 3-kinase (Welch et al., 1998). p70S6 kinase is thought to have an important role in progression through the G1 phase of the cell cycle, because inhibition of the protein with rapamycin, causes cells to accumulate in G1 (Grammer et al., 1996). p70S6 kinase activation can also be mediated by PKCs independently of PI 3-kinase (Chung et al., 1994).

PI 3-kinase also appears to mediate growth factor activation of PKC\(\lambda\) and PKCe, possibly through 3 phosphoinositol dependent kinase (PDK) 1, since their activation by EGF or platelet-derived growth factor (PDGF) is inhibited by wortmannin and a dominant negative PI 3-kinase mutant (Akimoto et al., 1996; Moriya et al., 1996).

1.2.11 Ral-GEFs and their roles as Ras effectors

The Ral-GEF family currently consists of five members, Ral-GDP dissociation stimulator (Ral-GDS), Ral-GDS like factor (Rlf), Ral-GDS-like (RGL), RGL2 and Ral-GDS related (Rgr) (Albright et al., 1993; D'Adam et al., 1997; Kikuchi et al., 1994; Peterson et al., 1996; Wolthuis et al., 1996). These proteins act as nucleotide exchange factors for the Ras related, Ral family of small GTPases. Ral proteins, like Ras, are found exclusively in the membrane fraction of cells and are found mostly in endocytic and exocytic vesicles (Bielinski et al., 1993; Volknad et al., 1993).

Ral-GDS, Rlf and RGL2 have been shown to interact with Ras in a GTP-dependent fashion both in vitro and in vivo (Hofer et al., 1994; Kikuchi et al., 1994; Peterson et al., 1996; Spaargaren and Bischoff, 1994; Wolthuis et al., 1996). Ral-GDS, Rlf and RGL increase the amount of Ral-GTP in cells by stimulating Ral GDP/GTP exchange activity. This exchange activity is upregulated by co-transfection of activated Ras implying that Ras has a role in Ral-GEF activation (Murai et al., 1997; Urano et al., 1996). This is further evident because dominant negative Ras mutants inhibit Ral activation by growth factors (Wolthuis et al., 1997). Rlf availability appears to be rate limiting for Ras activation.
activation of the c-fos promoter upon transient transfection since Rif is unable to activate it alone, but increases c-fos promoter activity induced by Ras\textsuperscript{V12}. A role for both Ral-GEFs and Raf downstream of Ras in c-fos promoter activation is supported by two observations. Firstly, a dominant negative mutant of Ral (which is believed to compete out Ral-GEFs) or the MEK inhibitor PD 98059, partially inhibit Ras activation of the c-fos promoter. (Wolthuis et al., 1997). Secondly, in co-transfection experiments RGL and RafCAAX co-operate to activate the c-fos promoter to a similar degree to Ras\textsuperscript{V12} while they have a meagre effect when transfected individually (Murai et al., 1997). It also appears that Ral-GEFs have a role in Ras transforming potential. A dominant negative mutant of Ral inhibits the focus forming ability of Ras\textsuperscript{V12} (Urano et al., 1996). Furthermore expression of RalGDS, activated Ral or a Ras effector mutant (Ras\textsuperscript{V12G37}) that specifically interacts with Ral-GDS, is able to enhance the focus forming ability of v-Raf and Ras\textsuperscript{V12} (Urano et al., 1996; White et al., 1996). Thus there is a convincing case for Ral-GEFs as necessary Ras effectors.

Rif has also been implicated in mitogenesis because an activated form of Rif, Rif CAAX, is able to stimulate colony formation in low mitogen conditions in NIH 3T3 cells (Wolthuis et al., 1997). It is thought that Ral may be the mediator of Ral-GEF signalling, however this is not yet thoroughly established. The function of Ral proteins is largely unknown. Putative downstream targets of Ral are Phospholipase D which been shown to bind Ral via its amino-terminus (Jiang et al., 1995b) and Ral interacting protein 1 (RLIP76) (also known as Ral interacting protein 1 (RIP) and Ral binding protein 1(RalBP1)) which is believed to act as a GAP to the Rho family GTPases, Cdc42 and Rac (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). This interaction could potentially link the Ras and Rho family mediated pathways.

1.2.11.1 \textit{P120\textsuperscript{GAP} as a Ras effector}

The RasGAP, p120\textsuperscript{GAP} was the first candidate Ras effector identified. It was shown to bind to the Ras effector domain in a GTP-dependent manner of both wild type and oncogenic Ras. Mutations in the effector domain also inhibited binding (Adari et al., 1988; Cales, 1988). p120\textsuperscript{GAP} became implicated as a Ras effector because it was able to reproduce the effects of Ras in inhibiting muscarinic potassium channel currents in an in vitro system. This was dependent on its amino-terminal SH2 and SH3 domains which were sufficient for these effects, in the absence of the Ras binding domain (Martin et al., 1992). Furthermore, an amino-terminal fragment of p120\textsuperscript{GAP} was also shown to stimulate transcription from the c-fos promoter, however, this effect was dependent on Ras function, suggesting that it co-operated with other Ras effectors to stimulate this effect, however whether Ras activation, or an alternative activator of p120\textsuperscript{GAP} is required for activation of c-fos promoter transcription is not clear (Medema et al., 1992). In contrast,
overexpression of an amino terminal fragment of p120\textsuperscript{GAP}, has also been shown to interfere with Ras effects in mammalian cells. For example, it inhibited the transforming activity of oncogenic Ras, but not Raf (Clark et al., 1993); it blocked Ras activation of JNK, but not ERKs (Clark et al., 1997c), and it also interfered with cytoskeletal organisation (McGlade et al., 1993). Thus possibly the amino terminus of p120\textsuperscript{GAP} mediates multiple functions which are differentially affected by overexpression of this fragment. Furthermore, certain Ras-mediated effects in Xenopus have been reported to be dependent on p120\textsuperscript{GAP}. The use of blocking peptides has demonstrated that the SH3 domain of p120\textsuperscript{GAP} was essential for Ras-mediated germinal vesicle breakdown (GVBD) (Duchesne et al., 1993). Moreover, inhibition of the SH3 domain of p120\textsuperscript{GAP} with a monoclonal antibody prevented Ras activation of Cdc2 and expression of c-mos, but not ERK (Pomerance et al., 1996). However, certain mutations in the effector domain of Ras were found that impaired p120\textsuperscript{GAP} binding but did not reduce Ras focus forming ability. This indicated that p120\textsuperscript{GAP} signalling was not necessary for Ras transforming activity (Marshall and Hettich, 1993). Thus p120\textsuperscript{GAP} may be required for some Ras-mediated effects. However the mechanism by which it exerts these effects is not yet clear. This may involve p190\textsuperscript{RhoGAP} or another phosphoprotein that interacts with the amino terminus, p62. Furthermore, the function of p120\textsuperscript{GAP} downstream signalling in mammalian cells is not known.

1.2.11.2 Mediation of Ras effects by Ras effector pathways

Activation of Ras affects many cellular processes, many of which have been discussed above. Some of these processes can be induced by one Ras effector-mediated pathway and others require collaboration between two or more. The need for multiple Ras effector pathways to induce cellular transformation has been discussed above (Joneson et al., 1996b; White et al., 1995). Effector pathways from Ras have contrasting roles in their response to c-myc induced apoptosis in Rat1 fibroblasts. Ras\textsuperscript{V12} enhances c-myc induced apoptosis. Ras effector domain mutants, and activated Raf mutants demonstrated that this is caused by the Raf activated effector pathway. In contrast, activation of a constitutively activated p110 subunit of PI 3-kinase, or the Ras\textsuperscript{V12C40} effector domain mutant that activates PI 3-kinase activity provides protection from c-myc induced apoptosis (Kauffmann-Zeh et al., 1997). Thus cellular responses to Ras effector pathways can be very different and in order to see the protective effect of Ras in these cells it was necessary to prevent activation of Raf. This raises the question, can the cell preferentially activate one Ras effector pathway over another? One group has demonstrated a mechanism by which this may occur. Endothelin-1 (ET-1) induces Ras activation in rat glomerular mesangial cells through activation of a G-protein coupled receptor and subsequent coupling of Shc and Grb2 to the RasGEF, Sos. Addition of ET-1 to these
cells induces two separate peaks of Ras activation. The first peak begins 2 min after ET-1 addition and drops to background levels after 15 min because of a negative feedback mechanism in which ERK phosphorylates Sos. The first Ras peak directly correlates with stimulation of ERK activity and relatively weak PI 3-kinase activity, both of which drop to background levels. The second peak occurs at around 30 min, when Sos has reverted to a non-phosphorylated form. This induces a high level of PI 3-kinase activity, but no ERK activation. The reason for the inactivity of ERK is thought to be due to the upregulation of the ERK phosphatase, MKP1, which begins 30 min after ET-1 stimulation (Foschi et al., 1997). Thus differential activation of Ras effector pathways can be achieved and one mechanism by which this occurs is by the induction of regulatory proteins that are specific to a single pathway.

Another mechanism that may be used to differentiate between Ras effectors is through one effector having preferential binding affinity for Ras over another. This has been shown to occur between Raf and Ral-GDS. PKA phosphorylates both proteins, however, PKA phosphorylation of Raf, reduces its affinity for Ras, but does not affect the affinity of Ral-GDS. Furthermore, the addition of forskolin to cells (which upregulates PKA activity and downregulates Raf activity), increases ectopic Ral-GDS association with endogenous Ras in response to Ras activation by EGF, suggesting that Ral-GDS is preferentially binding Ras over Raf (Kikuchi and Williams, 1996).

Therefore it appears that another level of control of Ras-mediated effects derives from factors which do not directly influence Ras activation, but affect the availability of its downstream targets thus influencing the specificity of Ras signalling.
1.3 Rac and Rho-family proteins

1.3.1 Background

Rac is a member of the Ras superfamily of small GTPases. Specifically it is a member of the Rho (Ras homologous) subfamily which have around 55% amino acid identity to each other. Three Rac proteins have so far been identified, Racl, 2 and 3 (see Table 1). This overview will concentrate on Racl, however other Rho family GTPases, especially RhoA and Cdc42, are also relevant to Rac function and so will also be discussed.

After identification as homologues of Ras, the Rho family of GTPases were found to be involved in cytoskeletal reorganisation, through control of actin. Activation of Rho family members can cause profound changes to the actin cytoskeleton in certain cell-types. The cytoskeletal changes are different for Racl, RhoA and Cdc42 and have contributed to the growing understanding of the upstream activation and downstream targets of the Rho family. Activation of Rac causes formation of lamellipodia. Lamellipodia are actin sheets that protrude from the edges of cultured fibroblasts and many motile cells. When lamellipodia fold back on themselves they form membrane ruffles. Inhibition of growth factor signalling using dominant negative Racl mutants prevents lamellipodia formation (Ridley et al., 1992). Activation of Cdc42 forms filopodia. These are protruding spikes formed by the extension of a tight bundle of actin filaments in the direction of the protrusion. Activation of RhoA causes actin stress fibre formation. Stress fibres are bundles of actin filaments that traverse the cell and terminate in focal adhesions that are complexes which adhere to the extracellular matrix (ECM). Racl and Cdc42 also stimulate production of distinct integrin-containing complexes, however these are morphologically distinct from focal adhesions formed by RhoA and are termed focal complexes (Hotchin and Hall, 1996).

Rho GTPases also have roles in transcriptional regulation, cell growth control, membrane trafficking and development (Van Aelst, 1997).

1.3.2 Activation of Rho GTPases

Like Ras, Rho family proteins act as molecular switches. They are active when in a GTP bound conformation, and inactivated after GTP hydrolysis. This reaction is regulated by RhoGEFs, RhoGAPs and RhoGDI (GDP dissociation inhibitors).

1.3.2.1 RhoGEFs

Multiple proteins have been found to act as exchange factors that activate Rho GTPases by exchanging GDP for GTP. Table 2 lists the known RhoGEFs. Most RhoGEFs are...
oncogenic and they share two features. Firstly they have a Dbl homology (DH) domain. Deletion analysis has shown that this region is necessary and sufficient for exchange activity in vitro (Hart et al., 1991a; Ron et al., 1991). It is also necessary for the oncogenicity of RhoGEFs in vivo (Hart et al., 1994). Secondly, in each RhoGEF, the DH domain is followed by a pleckstrin homology (PH) domain. This region has been shown to be necessary for subcellular localisation of GEFs in vivo (Zheng et al., 1996b).

The guanosine exchange activity of Vav is activated by tyrosine phosphorylation, probably by the Src family tyrosine kinase, Lck. It is unclear however, whether this is a general mechanism of activation for RhoGEFs (Crespo et al., 1997; Han et al., 1997).

In addition to these shared domains, RhoGEFs also contain other regulatory domains, such as catalytic, protein-protein interaction, and protein-lipid interaction domains, these may mediate upstream or downstream regulatory mechanisms. Of note is that Sos, which was originally identified as a RasGEF, has a DH domain distinct from its RasGEF domain, which specifically displays GEF activity towards Rac1. This may provide a link between Ras and Rac signalling pathways (Nimnual et al., 1998). The substrate specificity of RhoGEFs varies between family members. For example Vav has exchange activity towards RhoA, Rac1 and Cdc42, whereas Tiam1 is specific for Rac1 (Crespo et al., 1997; Habets et al., 1994; Han et al., 1997). The specificity of cellular responses may be regulated by differential activation of RhoGEFs leading to distinct patterns of activation of Rho family members.

Table 2 RhoGEFs

<table>
<thead>
<tr>
<th>RhoGEF</th>
<th>Substrate specificity</th>
<th>Biological properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbl</td>
<td>Cdc42, RhoA</td>
<td>oncogenic, implicated in Ewings sarcoma</td>
<td>(Hart et al., 1991b)</td>
</tr>
<tr>
<td>Lbc</td>
<td>RhoA</td>
<td>oncogenic</td>
<td>(Zheng et al., 1995)</td>
</tr>
<tr>
<td>Lfc</td>
<td>RhoA</td>
<td>oncogenic</td>
<td>(Glaven et al., 1996)</td>
</tr>
<tr>
<td>GEF-H1</td>
<td>Rac1, RhoA</td>
<td>co-localises with microtubules</td>
<td>(Ren et al., 1998)</td>
</tr>
<tr>
<td>Lsc</td>
<td>RhoA</td>
<td>oncogenic</td>
<td>(Glaven et al., 1996)</td>
</tr>
<tr>
<td>Tiam1</td>
<td>Rac1</td>
<td>metastatic and oncogenic</td>
<td>(Habets et al., 1994)</td>
</tr>
<tr>
<td>Vav</td>
<td>Rac1, RhoA, Cdc42</td>
<td>oncogenic, implicated in lymphocytic proliferation and lymphopenia</td>
<td>(Crespo et al., 1997; Han et al., 1997)</td>
</tr>
<tr>
<td>FGD1</td>
<td>Cdc42</td>
<td>implicated in faciogenital dysplasia</td>
<td>(Olson et al., 1996; Zheng et al., 1996a)</td>
</tr>
<tr>
<td>Trio</td>
<td>Rac1, RhoA</td>
<td>cell migration ?</td>
<td>(Debant et al., 1996)</td>
</tr>
<tr>
<td>Ost</td>
<td>RhoA, Cdc42 (binds to GTP-)</td>
<td>oncogenic</td>
<td>(Horii et al., 1994)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>Rac1)</th>
<th>Substrate specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcr</td>
<td>Rac1, RhoA, Cdc42 (GAP for Rac1)</td>
<td>implicated in leukaemia</td>
<td>(Chuang et al., 1995)</td>
</tr>
<tr>
<td>Abr</td>
<td>Rac1, RhoA, Cdc42 (GAP for Rac1 and Cdc42)</td>
<td>?</td>
<td>(Chuang et al., 1995)</td>
</tr>
<tr>
<td>Sos</td>
<td>Rac1</td>
<td>oncogenic, RasGEF</td>
<td>(Nimnual et al., 1998)</td>
</tr>
<tr>
<td>p115RhoGEF</td>
<td>RhoA</td>
<td>oncogenic, also GAP for small G proteins: G_{12} and G_{13}</td>
<td>(Hart et al., 1998; Hart et al., 1996; Kozasa et al., 1998)</td>
</tr>
<tr>
<td>PIX</td>
<td>Rac1?</td>
<td>binds to PAK</td>
<td>(Manser et al., 1998)</td>
</tr>
<tr>
<td>Dbs</td>
<td>Cdc42, RhoA</td>
<td></td>
<td>(Whitehead et al., 1997)</td>
</tr>
<tr>
<td>mNET1</td>
<td>RhoA</td>
<td>oncogenic</td>
<td>(Alberts and Treisman, 1998)</td>
</tr>
</tbody>
</table>

1.3.2.2 RhoGAPs

The Rho family is negatively regulated by GTPase activating proteins (GAPs). The RhoGAP domain bears no similarity to the RasGAP domain. Proteins that contain a RhoGAP domain are listed in Table 1.3. Substrate specificity of RhoGAPs is also variable; the GAP domain of Bcr is able to increase GTPase activity of Rac1, but not RhoA in vitro, and if microinjected into cells can prevent Rac1-mediated membrane ruffling, but not RhoA-mediated stress fibre formation. Alternatively, p190GAP preferentially stimulates GTP hydrolysis on RhoA and RhoA-mediated stress fibre formation (Ridley et al., 1993). Interestingly, p190GAP is found complexed with the RasGAP, p120GAP, in Src transformed or growth factor stimulated cells (Settleman et al., 1992). This may formulate another link between Ras and Rho pathways since the interaction makes the SH3 domain of p120GAP more accessible to interacting proteins (Hu and Settleman, 1997). In addition, the Ral target, RalBP1, which acts as a GAP towards Cdc42 and Rac, may be regulated by Ras, through RalGAP activity (Wolthuis et al., 1998).

It is also likely that some RacGAPs act as Rac effector proteins. For example, N- and β-chimerin act as GAPs specific for Rac and microinjection of their GAP domains inhibits Rac1-mediated lamellipodia formation (Diekmann et al., 1991; Leung et al., 1993). However, microinjection of the full length N-chimerin induces lamellipodia and filopodia formation in fibroblasts and neuroblastoma cells. This is dependent on N-chimerin's ability to bind Rac1 and Cdc42, but not on GAP activity. Such actin reorganisation is inhibited by dominant negative mutants of Rac1 and Cdc42 (Kozma et al., 1996), which implies that N-chimerin is involved in mediating Rac and Cdc42 downstream signals.
### Table 1.3 RhoGAPs

<table>
<thead>
<tr>
<th>RhoGAPs</th>
<th>Target Specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50RhoGAP</td>
<td>preferentially Cdc42, but also RhoA and Rac1</td>
<td>(Barfod et al., 1993; Lancaster et al., 1994)</td>
</tr>
<tr>
<td>Bcr</td>
<td>preferentially Rac1, but also Cdc42</td>
<td>(Diekmann et al., 1991)</td>
</tr>
<tr>
<td>Abr</td>
<td>Rac1, Cdc42</td>
<td>(Tan et al., 1993)</td>
</tr>
<tr>
<td>N-Chimerin</td>
<td>Rac1</td>
<td>(Diekmann et al., 1991)</td>
</tr>
<tr>
<td>β-Chimerin</td>
<td>Rac1</td>
<td>(Leung et al., 1993)</td>
</tr>
<tr>
<td>p190GAP</td>
<td>preferentially RhoA, but also Rac1, Cdc42</td>
<td>(Settleman et al., 1992)</td>
</tr>
<tr>
<td>p122</td>
<td>RhoA</td>
<td>(Homma and Emori, 1995)</td>
</tr>
<tr>
<td>Myr5</td>
<td>preferentially RhoA, but also Cdc42</td>
<td>(Reinhard et al., 1995)</td>
</tr>
<tr>
<td>RalBP1/RLIP76/RIP1</td>
<td>preferentially Cdc42, but also Rac1</td>
<td>(Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995)</td>
</tr>
<tr>
<td>Graf</td>
<td>RhoA, Cdc42</td>
<td>(Hildebrand et al., 1996)</td>
</tr>
<tr>
<td>3BP-1</td>
<td>Rac1, Cdc42</td>
<td>(Cicchetti et al., 1995)</td>
</tr>
</tbody>
</table>

### 1.3.2.3 RhoGDIs

The Rho family has an additional family of negative regulators, that Ras proteins do not have. These are termed Rho GDP dissociation inhibitors (RhoGDIs) and comprise RhoGDI, D4-GDI (LY-GDI) and RhoGDIγ. In vivo, microinjection of RhoGDI is able to inhibit Rac1 and RhoA-mediated, insulin- and hepatocyte growth factor (HGF)-induced membrane ruffling (Nishiyama et al., 1994).

These proteins preferentially bind to the GDP-bound form of the Rho protein and prevent dissociation with the GDP, thus preventing reactivation of the protein (Abo et al., 1991; Adra et al., 1997; Fukumoto et al., 1990; Lelias et al., 1993; Leonard et al., 1992; Scherle et al., 1993; Ueda et al., 1990). RhoGDIs are located in the cytoplasm and are able to relocate Rho proteins from the plasma membrane into the cytoplasm, possibly by binding their carboxy terminal isoprene (Gosser et al., 1997). RhoGDIs also bind GTP-bound Rho proteins and inhibit GAP-mediated and intrinsic GTP hydrolysis keeping them inactive, probably by preventing them from forming their active transition state. This is especially important in the case of Rac1 which has an intrinsic GTPase activity fifty times that of RhoA or Ras (Chuang et al., 1993b; Hancock and Hall, 1993; Hart et al., 1992).

A region of Rho family proteins is not found in other Ras-like proteins and is termed the Rho insert region. In Cdc42, deletion analysis has shown that this region is dispensable
for RhoGEF and RhoGAP activity and for RhoGDI binding and relocalisation, however it is necessary for the GDI to inhibit GTP hydrolysis and GDP dissociation (Wu et al., 1997).

1.3.2.4 Indirect regulators of Rho GTPases

Another family of proteins which may regulate Rho family activation, is the ERM (ezrin, radixin, moesin) family. Although RhoGEFs can activate Rho GTPases, they are unable to disrupt Rho-RhoGDI complexes. This implies that another factor is involved in Rho activation. Radixin (and other members of the ERM family) has been shown to interact separately with both the RhoGEF, Dbl and RhoGDI. It has been shown to reduce the inhibitory activity of RhoGDI (Takahashi et al., 1998; Takahashi et al., 1997). Thus, the ERM family may regulate Rho proteins by recruiting other regulatory proteins to Rho. Furthermore, ERM proteins were identified as the cytosolic activity that permitted activated RhoA to form stress fibres in permeabilised and extracted Swiss 3T3 cells (Mackay et al., 1997). In addition, the proposed role of ERM proteins in Rho regulation is substantiated by the observation that ERM proteins also bind actin (Martin et al., 1995b).

Biologically active lipids, such as arachidonic acid, phosphatidic acid and phosphatidylinositolts, have also been shown to dissociate Rac-RhoGDI complexes, with GTP-Rac-RhoGDI being more sensitive to disruption (Chuang et al., 1993a). This suggests a further level of Rac regulation since the production of lipid second messengers can be mediated by many factors (Carpenter et al., 1991).

1.3.3 Upstream activation of Rac and the Rho family

Rho family proteins can be activated by many upstream elements, although how these signals are relayed to the Rho proteins remains unclear at present. Signals activating Rho family GTPases can be transmitted through seven transmembrane receptors, RTKs and integrin receptors. Figure 1.8 gives a summary of upstream activators and downstream effects. Insulin, PDGF, bombesin and phorbol ester (phorbol 12-myristate 13-acetate (PMA)) are able to induce lamellipodia formation. This is inhibited by a dominant negative Rac1 mutant, indicating that these compounds signal to the actin cytoskeleton through Rac (Ridley et al., 1992). The same is true of activated Ras which is also dependent on Rac to trigger lamellipodia formation (Ridley et al., 1992).
Figure 1.8 Upstream signalling to Rho family members and their effect on the cytoskeleton
The signalling pathways are activated by ligand-stimulated G-protein coupled receptors (SR), receptor tyrosine kinases (RTK) and integrin receptors (IR). GEFs are shown in green, kinases are shown in red and GTPases in yellow. Direct interactions are shown as unbroken arrows and indirect activation as dashed arrows. Shown as a blue arrow is phosphorylation of phosphatidylinositol 4,5 biphosphate (PIP$_2$) by PI 3-kinase to PI (3,4,5)P$_3$ (PIP$_3$). Interaction of G$_{13}$ and p115$^{RhoGEF}$ has recently been shown to stimulate its GEF activity towards RhoA. Furthermore, activation of stress fibres by LPA is sensitive to inhibitors of tyrosine phosphorylation, eg tyrophostin. Other interactions are described in the text.
The ability of mitogens and Ras to activate Rac appears to be partly mediated by PI 3-kinase. PDGF activates Rac1 by Wortmannin sensitive and insensitive mechanisms. The PDGF-induced increase in the amount of Rac-GTP is blocked by the PI 3-kinase inhibitor Wortmannin, whereas the decrease in GTPase activity is independent of PI 3-kinase inhibition (Hawkins et al., 1995). Nevertheless, the effects on the actin cytoskeleton by PDGF, IGF-1 and insulin, (but not bombesin, EGF and PMA), are inhibited by Wortmannin (Kotani et al., 1994; Nobes et al., 1995; Wennstrom et al., 1994a). Furthermore, dominant negative forms of PI 3-kinase completely inhibit RasV12, PDGF, IGF-1 and insulin stimulated membrane ruffling (Hawkins et al., 1995; Kotani et al., 1994; Rodriguez-Viciana et al., 1997). These data indicate that PI 3-kinase has a vital role in mediating activation of Rac1 and Rac-induced lamellipodia formation by RTKs and Ras, but that Rac activation can also occur by mechanisms independent of PI 3-kinase.

Regulation of Rac by PI 3-kinase appears to be mediated by its lipid targets. A PI 3-kinase substrate, PI(4,5)P2, has been shown to inhibit the activity of the RhoGEF, Vav, by inhibiting its activation by the Src family tyrosine kinase, Lck. Conversely, a lipid product of PI 3-kinase activity, PI(3,4,5)P3, upregulates Vav phosphorylation and activation by Lck (Han et al., 1998). Moreover the RasGEF, Sos, has RacGEF activity which is enhanced by the presence of RasV12 and RasV12C40 (the effector domain mutant that binds PI 3-kinase, but not RasGEFs or Raf), but not RasV12S35 (which does not bind PI 3-kinase). This suggests that Ras may regulate Sos activation of Rac1 and that this may involve PI 3-kinase (Nimnuai et al., 1998). However, Ras has been demonstrated to stimulate Rac-mediated transcriptional activation independently of the PI 3-kinase inhibitor LY 294002 in T lymphocytes, indicating that activation of Rac by Ras can occur independently of PI 3-kinase activity (Genot et al., 1996; Genot et al., 1998). Wortmannin does not prevent filopodia formation by Cdc42, nor stress fibre formation by RhoA (Nobes and Hall, 1995; Nobes et al., 1995) indicating that the involvement of PI 3-kinase may be distinct to Rac activation.

In addition to external stimuli, Cdc42 is able to activate Rac1, and Rac1 is able to activate RhoA. Stimulation of Cdc42 signalling, using Bradykinin or Cdc42V12, leads firstly to filopodia formation, and subsequently to lamellipodia formation. The formation of lamellipodia is prevented by the expression of a dominant negative Rac mutant (Nobes and Hall, 1995). Activation of Rho by Rac, may involve Rac-mediated induction of arachidonic acid and its metabolites, leukotrienes, since these are required for Rac-mediated EGF induction of Rho dependent stress fibres (Peppelenbosch et al., 1995). This cascade of activation of the Rho GTPases does not activate all Rho GTPase effects. For example, transcriptional activation from the SRE by Rac and Cdc42 is independent of Rho (Hill et al., 1995).
Thus Rho GTPases appear to be a point of convergence for many distinct upstream signals.

1.3.4 Rho GTPases in integrin signalling

Racl, RhoA and Cdc42 also regulate the assembly of integrin-containing complexes at the plasma membrane, which link the actin cytoskeleton to the ECM. In addition to containing proteins with structural functions, the adhesion complexes also contain putative signalling proteins such as Src and focal adhesion kinase (FAK). Integrin ligation to ECM proteins is essential for cell cycle progression (Giancotti, 1997). Integrins are known to signal to the Raf-MEK1-ERK pathway, and Rho GTPases appear to be involved in mediating this link. For example, ERK becomes activated when NIH 3T3 cells are plated on fibronectin, an integrin ligand. This is mediated by Rho since activation of ERK is sensitive to a specific inhibitor of Rho, C3 transferase, or dominant negative Rho mutants, and can be partially stimulated by activated mutants of Rho or the RhoGEF, lbc (Renshaw et al., 1996). Fibronectin-mediated activation of Raf-1, MEK1 and ERK is also blocked by a dominant negative p85 subunit and the chemical inhibitors, wortmannin and LY294002, demonstrating a dependence on PI 3-kinase function which may implicate Rac-mediated activation of PAK (see Figure 1.9) (King et al., 1997). Furthermore, two RhoGEFs, lbc and dbl, have been demonstrated to relieve the anchorage-dependence but not the requirement for mitogens of murine 3T3 fibroblasts, further implicating Rho as a mediator of integrin signalling (Schwartz et al., 1996). Rac and Cdc42 have also been shown to be necessary for integrin-mediated invasion by mammary epithelial cells and collagenase expression in synovial fibroblasts (Keely et al., 1997; Kheradmand et al., 1998).

1.3.5 Cellular responses to Rac activation

1.3.5.1 Activation of MAP kinase cascades

JNK and p38 appear to be major targets for Rho proteins. However, the properties of specific Rho family members vary between cell types. Rac1, Rac2 and Cdc42, but not Rho, are able to regulate the activation of JNK and p38, but not ERK in Hela, NIH 3T3 and Cos cells. In addition, the oncogenic RhoGEFs, Dbl and Ost, are also able to stimulate JNK activation (Coso et al., 1995; Minden et al., 1995; Olson et al., 1995; Zhang et al., 1995a). Furthermore, dominant interfering mutants of Rac1 are able to interfere with JNK activation in response to v-Src and EGF in Hela and NIH 3T3 cells respectively (Minden et al., 1995). In Cos cells, interleukin-1 activation of JNK is inhibited by a dominant interfering Cdc42 allele (Bagrodia et al., 1995b). However in human kidney 293T cells, Ras and Rac are unable to activate JNK, but RhoA, B and C and Cdc42 can (Teramoto et al., 1996b).
1.3.5.2 Rac involvement in ROS-mediated signalling

GTP bound Rac1, Rac 2 and Ras have all been demonstrated to increase the cellular levels of reactive oxygen species (ROS) (Finkel, 1998). Rac1 or Rac2 is required as a component of NADPH oxidase which regulates the oxidative killing mechanisms in human phagocytic neutrophils. Rac1, in combination with p47^{PHOX}, p67^{PHOX} and cytochrome b_{558} is able to generate super oxide in \textit{in vitro} recombination assays (Bokoch, 1994).

Furthermore, stimulation of mitogen independent DNA synthesis by activated Ras but not activated Raf, was partially inhibited by a scavenger of oxygen free radicals, N-acetyl-l-cysteine (NAC), indicating ROS are required for DNA synthesis and that generation of ROS by Ras is independent of Raf (Irani \textit{et al.}, 1997). Rac may be the downstream mediator from Ras involved in this response because superoxide production correlates with the ability of Rac to induce DNA synthesis in cell-lines expressing Rac effector domain mutants, whereas it is independent of JNK activation and actin reorganisation. Moreover, inhibition of superoxide with chemical inhibitors inhibits the mitogenic effects of Rac1 (Joneson and Bar-Sagi, 1998).

1.3.5.3 Transcriptional activation

Rho, Rac and Cdc42 have also been implicated in transcriptional regulation. Rho is required for transcriptional activation by the serum response factor, SRF, in response to LPA, serum and AIF. Rac and Cdc42 are also able to potentiate SRF-induced transcription in the presence of the Rho inhibitor transferase (Hill and Treisman, 1995). This indicates that, different from regulation of the actin cytoskeleton, Rac and Cdc42 induce SRF-mediated transcription independently of Rho.

In cardiac muscle cells, Rho activity, in addition to ERK and JNK signals, is required for regulation of MEKK1 or phenylephrine stimulated transcription from an atrial natriuretic factor (ANF) promoter reporter construct (Thorburn \textit{et al.}, 1997).

Rac1, RhoA and Cdc42 are all able to induce NFkB transcriptional activity. NFkB is a nuclear transcription factor which is regulated by being retained in a complex with an inhibitor, I\kappa B, in the cytoplasm. It is released from this complex by phosphorylation of I\kappa B. Activation of NFkB transcriptional activity by TNF\alpha in Cos cells, is dependent on Cdc42 and RhoA. However, it is not inhibited by dominant negative mutants of Rac (Perona \textit{et al.}, 1997). In contrast, in Hela cells, activated Rac and IL-1\beta can induce NFkB activity and Rac^{N17} can inhibit the latter. Furthermore, induction of ROS by Rac is necessary for NFkB mediated transcription since it is inhibited by treatment of the cells with antioxidants (Sulciner \textit{et al.}, 1996). In contrast, activation of NFkB-mediated
transcription by UV light is independent of Rho GTPases (Perona et al., 1997).

Rac mediates changes in cell shape in rabbit synovial fibroblasts. This is stimulated by integrin α5 and occurs through induction of collagenase-1 gene expression. Stimulation of collagenase-1 transcription by Rac1 is dependent on NFκB-mediated transcription of IL-1, which is also sensitive to inhibition by antioxidants (Kheradmand et al., 1998).

1.3.5.4 Rac involvement in invasion

The RacGEF, Tiam1, was initially identified as an oncogenic factor that stimulated T lymphoma cells to become invasive in an insertional mutagenesis screen. Transfection of Tiam1 mutants induced an invasive phenotype in non-invasive T-lymphomas (Habets et al., 1994). Tiam1 also induces membrane ruffling which is inhibited by dominant negative Rac1^{N17} whereas ruffling is not inhibited by the Rho specific inhibitor, C3 transferase. Rac1^{V12}, but not RhoA^{V14}, is also able to confer an invasive phenotype on non-invasive T lymphoma cells indicating that these functions of Tiam1 are Rac-mediated (Michiels et al., 1995). NIH 3T3 cells expressing a deletion mutant of Tiam1 encoding the DH/PH domain can stimulate tumours in nude mice, however this region is not sufficient to induce membrane ruffling. Moreover, full length Tiam1 is able to induce membrane ruffling, but is unable to stimulate tumour formation (Leeuwen et al., 1997). This indicates that these two properties of Tiam1 are independent of each other, but may still function through Rac. In contrast to the observations above, expression of Rac^{V12} or Tiam1 in MDCK epithelial cells suppresses the invasive phenotype stimulated by Ras^{V12}, by restoring E-cadherin-mediated cell-cell adhesion (Hordijk et al., 1997). Although this may seem contradictory to the effect of Rac^{V12} and Tiam1 in T lymphoma cells, in each case, Rac^{V12} and Tiam1 alter the adhesive properties of the cells, in the former case, by increasing cell-ECM binding and in the latter, by increasing cell-cell interaction.

Ectopic Rac1 and Cdc42 have also been shown to transform differentiated mammary epithelial cells which demonstrated integrin-dependent polarisation in a three dimensional collagen matrix, into motile and invasive cells (Keely et al., 1997). The use of Rac effector domain mutants, dominant negative SEK/MEK4 mutants and specific chemical inhibitors, indicated that this Rac-mediated increase in motility and invasion is not dependent on the proposed Rac targets, PAK, JNK, PORI, and leukotrienes. However, activated PI 3-kinase was able to reproduce the effects of Rac and Rac-induced invasion is inhibited by the PI 3-kinase inhibitors, wortmannin and LY 294002 (Keely et al., 1997).

1.3.5.5 Rac involvement in transformation and growth control

The expression of some Rho family members have been shown to be upregulated in response to mitogenic signals. For example, the expression a Rac2 isoform specific to
haemopoietic cells is upregulated in response to T cell activation by phytohaemagglutinin (Reibel et al., 1991). Furthermore, RhoB was identified as an immediate early gene induced by the PDGF and EGF receptors, and the non-RTKs, v-Src and Fps (Jahner and Hunter, 1991). More recently, specific growth enhancing properties have been directly attributed to Rho family members. Rho function was shown to be necessary for serum induced G1 to S-phase transition in Swiss 3T3 cells by the use of the Rho specific inhibitor, C3 transferase (Yamamoto et al., 1993). Similarly, Rac1 and Cdc42 function were shown to be necessary for serum-induced cell cycle progression from quiescence in Swiss 3T3 cells (Lamarche et al., 1996; Olson et al., 1995). Furthermore, Rac1, Cdc42 and RhoA and B have been shown to be necessary for transformation by activated Ras. Dominant negative mutants of the Rho family are able to block morphological transformation, focus forming ability and soft agar colony formation by oncogenic mutants of Ras in fibroblast cell-lines. In addition, co-transfection of activated RhoA or B or Rac1 can synergistically increase focus formation induced by activated Raf, when Raf is expressed at such levels that its transforming ability is weak (Khosravi-Far et al., 1995; Prendergast et al., 1995; Qiu et al., 1997; Qiu et al., 1995a; Qiu et al., 1995b). This implies that Rac and Rho are activating a pathway parallel to Raf downstream of Ras that is essential for cell proliferation and transformation. The effects on growth and transformation of activated Rho family members alone on cells is not yet clear. Full malignant transformation by activated Rac and Rho has been reported (Perona et al., 1993; Qiu et al., 1995a), however, other groups observe much lesser effects. This will be discussed later in the context of this thesis.

1.3.5.6 Membrane Trafficking

Rac and Rho are thought to be involved in cellular processes such as secretion, endocytosis, phagocytosis and antigen presentation. These processes may involve the transport of vesicles that may accompany actin remodelling. For example, dominant negative mutants of Rac can inhibit the formation of clathrin coated pits, which are important in endocytosis (Lamaze et al., 1996). Furthermore, Rac is able to stimulate pinocytosis in Swiss 3T3 cells (Ridley et al., 1992). However, this does not occur in baby hamster kidney cells. In this case, dominant negative Rac mutants are unable to inhibit Ras^V12 stimulation of pinocytosis. An alternative GTPase, Rab5, appears to be the mediator (Li et al., 1997). Thus pinocytosis appears to occur through a variety of regulators in different cell types. In addition, activated Rac and Rho also stimulate exocytosis of secretory vesicles in mast cells. GTP-γ-S induced exocytosis can be inhibited by dominant negative mutants of Rac and Rho. This may be linked to the ability of Rac and Rho to stimulate actin reorganisation in these cells (Norman et al., 1996).
1.3.6 Cellular Responses specific to effectors of Rac

In a similar manner to Ras, Rac1 and other Rho family members have many putative effectors. These are listed in Table 1.4. Rac1 and Cdc42 have many effectors in common, but RhoA effectors are mostly distinct. A sixteen amino acid sequence that is common to Rac and Cdc42 effector proteins, but not Rho effectors, has been designated Cdc42/Rac interactive binding, CRIB, and has aided identification of some putative Rac and Rho effectors (Burbelo et al., 1995). Although some Rac and Cdc42 effectors do not contain this motif, e.g. p70S6kinase, p85PI 3-kinase and IQGAPs. Some cellular responses to Rac have been attributed to specific effectors through the use of activated and dominant negative mutants (Van Aelst, 1997). In addition, the participation of certain candidate effectors in Rac-mediated responses has been dismissed since Rac effector domain mutants have been identified that induce the response but no longer bind the effector (Joneson et al., 1996a; Lamarche et al., 1996; Westwick et al., 1997).

Table 1.4 Rho family effector proteins

<table>
<thead>
<tr>
<th>Effector</th>
<th>Interacting GTPase</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPAK-1* (rat αPAK), hPAK-2*</td>
<td>Rac1, Cdc42</td>
<td>serine/threonine kinase, JNK activation, actin depolymerisation and focal adhesion disassembly</td>
</tr>
<tr>
<td>(p62αPAK), mouse mPAK3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rat p65αPAK)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLK2*, MLK3*</td>
<td>Rac1, Cdc42</td>
<td>MAPK kinase kinase, JNK activation</td>
</tr>
<tr>
<td>MEKK1, MEKK4* (MTK1)</td>
<td>Rac1, Cdc42</td>
<td>MAPK kinase kinase, JNK and p38 activation</td>
</tr>
<tr>
<td>p70S6kinase</td>
<td>Rac1, Cdc42</td>
<td>kinase for ribosomal subunit, required in growth factor stimulated DNA synthesis</td>
</tr>
<tr>
<td>IQGAP1^D, IQGAP2^D</td>
<td>Rac1, Cdc42</td>
<td>putative RasGAP, possibly involved in cytoskeletal organisation</td>
</tr>
<tr>
<td>p85 subunit of PI 3-kinase^D</td>
<td>Rac1, Cdc42</td>
<td>membrane ruffling</td>
</tr>
<tr>
<td>MSE55*</td>
<td>Cdc42, Rac</td>
<td>?</td>
</tr>
<tr>
<td>PI 5-kinase</td>
<td>Rac1, RhoA</td>
<td>possibly generating PI(4,5)P_2 which enhances actin polymerisation</td>
</tr>
<tr>
<td>POR1, POR2</td>
<td>Rac1</td>
<td>membrane ruffling</td>
</tr>
</tbody>
</table>

References:

Bagrodia et al., 1995b; Manser et al., 1995; Manser et al., 1994; Martin et al., 1995a; Teo et al., 1995;
Teramoto et al., 1996a;
Fanger et al., 1997; Gerwins et al., 1997;
Chou and Blenis, 1996;
Brill et al., 1996; Kuroda et al., 1996a;
Zheng et al., 1994;
Burbelo et al., 1995;
Ren et al., 1996;
Van Aelst et al., 1996.
| p67<sup>PHox</sup> | Rac1 | NADPH oxidase complex | (Diekmann et al., 1994) |
| POSH | Rac1 | JNK activation, NFκB activation, scaffold protein | (Tapon et al., 1998) |
| N-Chimerin ? | Rac1 | membrane ruffling | (Kozma et al., 1998) |
| α and β tubulin | Rac1 | possibly cellular translocation of Rac1 via microtubule network | (Best et al., 1996) |
| p35/cdk5 | Rac1 | regulation of neurite outgrowth, down regulation of PAK1 | (Nikolic et al., 1998) |
| MRCK (myotonic dystrophy kinase-related Cdc42 binding kinase) | Cdc42 | Filopodia formation, myosin light chain phosphorylation | (Leung et al., 1998) |
| WASP*, N-WASP* | Cdc42 | Actin organisation, implicated in Wiskott-Aldrich syndrome. N-WASP required for filopodia formation and actin depolymerisation | (Miki et al., 1998; Symons et al., 1996) |
| p120<sup>ACK</sup>*, ACK-2* | Cdc42 | non receptor tyrosine kinase | (Manser et al., 1993; Yang and Cerione, 1997) |
| CIP4 | Cdc42 | actin organisation | (Aspenstrom, 1997) |
| p160<sup>ROCK</sup>□/Rho-kinase/ ROKα*/ ROKβ*/ ROCKII | RhoA, (Rac1*) | Stress fibre formation, focal adhesions | (Fujisawa et al., 1996; Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996) |
| PKN/PRK2 | RhoA | Endocytosis? | (Quilliam et al., 1996; Watanabe et al., 1996) |
| citron*, CRIK, CRIK-SK | RhoA, Rac1* | cytokinesis | (Di Cunto et al., 1998; Madaule et al., 1995) |
| MBS (myosin-binding subunit of MLC phosphatase) | RhoA | muscle contraction, actin and myosin binding | (Kimura et al., 1996) |
| p140<sup>mDia2</sup>/ mDia2/ DFNA | RhoA | actin polymerisation, cytokinesis? Implicated in deafness | (Lynch et al., 1997; Watanabe and Madaule, 1997) |
| Rhophilin | RhoA | related to PRK1 | (Watanabe et al., 1996) |
| Rhothekin | RhoA | ? | (Reid et al., 1996) |
kinectin | RhoA | Kinesin binding, vesicle transport
---|---|---
(Alberts et al., 1998; Hotta et al., 1996)

* -indicates CRIB motif; □ -indicates does not contain CRIB motif; • -indicates also binds Rac1 in a GTP dependent manner

1.3.6.1 PAKs

The Rac and Cdc42 effector proteins, p21GTPase activated protein kinases, PAKs, are serine/threonine kinases that were initially found as proteins in brain cytosol that bound GTP-Rac and Cdc42, but not Rho. They antagonise intrinsic GTPase and GAP activity on Rac and Cdc42. Upon incubation with GTP-Rac or GTP-Cdc42 they become activated and exhibit autophosphorylation (Manser et al., 1994; Martin et al., 1995a).

PAKs have striking similarity to the S.cerevisiae gene Ste20. This is implicated in G protein signalling to a MAP kinase cascade. Mammalian PAKs have also been implicated in Rac signalling to the JNK and p38 MAP kinase pathways. Activated PAKs have been demonstrated to induce JNK activity (Bagrodia et al., 1995a; Teramoto et al., 1996b). Furthermore, activation of p38 by Rac and Cdc42 in Cos and Hela cells is inhibited by a kinase inactive mutant of PAK1 (Zhang et al., 1995a). JNK activation by Ras or Rac can also be inhibited by a kinase inactive mutant, PAK1R299 in Rat1 cells (Tang et al., 1997).

An amino terminal fragment of PAK that binds Rac-GTP is also able to block Rac and Cdc42-mediated activation of JNK (Minden et al., 1995; Teramoto et al., 1996b). However it is not clear whether these PAK mutants work by are blocking interaction of Rac and Cdc42 with other effectors in addition to PAK especially since inhibition by PAK1R299 is prevented by mutations which interfere with Rac and Cdc42 binding (Tang et al., 1997).

There is accumulating evidence that Rho GTPases may also have a role in regulating Raf-MEK1-ERK activation. Co-expression of activated Rho GTPases and wild type (wt) Raf-1, are able to synergistically activate MEK1, while neither is able to activate MEK1 alone. This ability of the Rho GTPases may be mediated by PAK1 since a kinase inactive mutant of PAK1 or a dominant negative mutant of Rac can inhibit RasV12 activation of MEK1. Furthermore, PAK1 has recently been shown to phosphorylate a serine residue (S298) of MEK1 which is important for Raf-1-MEK1 interaction (Frost et al., 1997; Frost et al., 1996). Moreover, PAK3 was recently identified as a kinase activity that phosphorylated a serine residue (S338) on Raf-1. This site regulates...
Raf-1 activation by Ras, Src and EGF. This group also showed that signal transduction through Raf-1 was dependent on activation of both the Ras and PAK pathways (King et al., 1998). Furthermore, the kinase inactive PAK1(mut) is able to inhibit transformation of Rat1 cells by Ras, but not by v-Raf, presumably because v-Raf does not require PAK1 for activation (Tang et al., 1997). In addition a PAK mutant that lacks the kinase domain but retains the motif, can also block transformation by Rac(mut) and Ras(mut) in rat 3Y1 cells (Osada et al., 1997). Interestingly, PAK1(mut) does not prevent Ras transformation in NIH 3T3 cells indicating a cell type specificity in terms of PAK function (Tang et al., 1997). Since PAK1 and PAK3 have been shown to be necessary for Ras activation of MEK1 and for transformation, this suggests that Raf is directly activated by Ras, but also requires PAK for activation which is indirectly activated by Ras, possibly through PI 3-kinase and Rac, see Figure 1.9.

There does not appear to be a role for PAK in Rac and Cdc42-mediated membrane ruffling since effector domain mutants that are unable to bind PAK are still able to induce lamellipodia. However, there is still a possibility that PAK does have an alternative role in actin organisation because Drosophila Pak1 is required for dorsal closure in Drosophila embryos which involves actin reorganisation (Harden et al., 1996). Moreover, expression of an activated PAK1/Cdc42 chimera or an activated PAK1 can mediate actin reorganisation and disassembly at the plasma membrane (Manser et al., 1997; Sells et al., 1997). Experiments with Rac effector domain mutants have shown that PAK binding to Rac may be required for induction of Cyclin D1 transcription of by Rac, but that it is not required for Rac stimulation of: transformation; G1 cell cycle progression; membrane ruffling; transcription from the SRE; or activation of JNK or p38 (Joneson et al., 1996a; Lamarche et al., 1996; Westwick et al., 1997). However, data using PAK mutants has suggested a requirement for PAK in Ras and Rac-mediated transformation. In one set of experiments, a PAK1 mutant lacking the kinase domain, but retaining the CRIB motif, blocked transformation and transcriptional activation of the TPA responsive element, by both Rac1(mut) and Ras(mut) in rat 3Y1 cells (Osada et al., 1997). Alternatively, kinase inactive PAK(mut) is able to block Ras, but not v-Raf mediated transformation in Rat1 fibroblasts, but not NIH 3T3 cells. Further mutations that prevented interaction of the mutant PAK(mut) with Rac and Cdc42 did not inhibit its ability to block transformation of Rat1 cells, or to block activation of ERK by Ras, indicating that for these effects it was not just blocking alternative Rac and Cdc42 effectors. However, Rac binding was necessary for inhibition of JNK activity, which indicates that JNK activity does not relieve the block on transformation (Tang et al., 1997).
1.3.6.2 PORI

The Rac effector, PORI, has been shown to induce membrane ruffling. The binding of PORI to Rac effector domain mutants also correlates well with their ability to stimulate membrane ruffling (Van Aelst et al., 1996). PORI is also able to co-operate with activated Ras to induce membrane ruffles, and a truncated PORI mutant is able to inhibit Rac¹¹² induced membrane ruffles. Furthermore, ARF6, the Ras effector, also interacts with PORI and induces alterations to the cytoskeleton which are sensitive to inhibition by a dominant negative PORI, but not Rac¹¹⁷ (D'Souza-Schorey et al., 1997). Thus Ras may influence cytoskeleton structuring through activation of different effector proteins using the same mediator.

1.3.6.3 p70S6kinase

Inhibition of p70S6kinase with rapamycin or inhibitory antibodies inhibits G1 cell cycle progression indicating the importance of this protein to cell proliferation (Grammer et al., 1996). p70S6kinase has been shown to interact with Rac and Cdc42 in vitro in a GTP dependent manner, and activated versions stimulate p70S6kinase activity in vivo. Cdc42 is unable to activate p70S6kinase in vitro; a similar situation to Ras-mediated activation of Raf. Moreover, dominant negative mutants of Rac and Cdc42 are able to inhibit EGF and PDGF induction of p70S6kinase activation (Chou and Blenis, 1996). Thus it appears that p70S6kinase is an effector of Rac and Cdc42. However, it is also regulated by PKC, AKT/PKB, and TOR, which may all play necessary roles in p70S6kinase regulation.

1.3.6.4 Rock and ROK

p160ROCK and ROKα interact with Rac1 and Cdc42 as well as with RhoA (Ishizaki et al., 1996; Joneson et al., 1996a; Lamarche et al., 1996). A Rac effector domain mutant that is deficient in its binding of p160ROCK was unable to induce G1 progression or lamellipodia formation, thus it may have a role in these processes (Lamarche et al., 1996).

1.3.6.5 MEKK4

Rac and Cdc42 activation of JNK in Cos cells may also be mediated by a putative Rac and Cdc42 effector, MEKK4 (a MAP kinase kinase kinase). One group reports that MEKK4 is able to activate JNK, and not p38 or ERK, and that a kinase inactive mutant of MEKK4 is able to block JNK activation by Rac and Cdc42 (Gerwins et al., 1997). However, another group identified MEKK4 as a potent activator of p38 and as a secondary activator of JNK in Cos and Hela cells. This group showed that activation of p38 by environmental stresses (UV light, anisomysin and osmotic shock) was sensitive to inhibition by a kinase inactive version of MEKK4, whereas TNFα activation of p38 was not inhibited (Takekawa et al., 1997). Importantly, it has also been demonstrated that activation of JNK activity by anisomycin, is not inhibited by dominant negative mutants.
of Rac1. Indicating that this is a Rac independent mechanism for JNK activation. Furthermore JNK activation by EGF is more sensitive to dominant negative mutant of Ras than Rac (Cosó et al., 1995; Minden et al., 1995).

1.3.6.6 MLK

Mixed-lineage kinase-3 (MLK-3), is able to bind Rac and Cdc42 in a GTP dependent manner. This protein has also been proposed as the main activator of JNK by Rac and Cdc42 in Cos cells since it was demonstrated that this kinase (and not PAK1) can activate JNK (Teramoto et al., 1996a).

1.3.7 Rho Family proteins and tumorigenesis

Unlike Ras, members of the Rho subfamily have not yet been found mutated in human tumours (Moscow et al., 1994). However, many proteins that have since been found to be exchange factors for the Rho family were first identified in NIH 3T3 cell transformation assays as oncogenes, for example, Dbl and Vav (see Table 2). In addition, Dbl is found expressed in the childhood tumour, Ewings sarcoma (Vecchio et al., 1989), and appears to affect DNA damage repair. Vav, is located on a region of chromosome 19 that is involved in karyotypic abnormalities in a variety of malignancies including melanomas and leukaemias (Martinerie et al., 1990). In addition, Bcr acts both as a RhoGEF and a RacGAP. Over 90% of all chronic myeloid leukaemia (CML) and 10-20% of acute lymphoblastic leukaemia involve the formation of the Philadelphia chromosome, which results in the generation of a BCR/ABL chimeric gene. Antisense oligonucleotides directed to the BCR/ABL junction are able to suppress the hyperproliferative growth of leukaemia cells from CML patients (Szczylik et al., 1991). This difference between the occurrence of Rho GTPases and their activators could derive from the ability of RhoGEFs to activate multiple Rho family members and that activation of more than one Rho family member is required for tumour promotion.
2. The Effect of Ras and Raf activation on the Cell Cycle
Chapter Two - Ras and Raf and the Cell Cycle

2.1 Introduction

Ras is found activated in 10-15% of all human tumours and this figure is much higher in specific types of tumour (Barbacid, 1987). This implies that it is an important growth regulatory gene and this has been borne out in studies investigating its ability to control cell cycle progression. Raf is a direct effector of 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) and can also be oncogenic, although it has only been found activated and/or upregulated in a very small number of human tumours (Chenevix-Trench et al., 1987; Graziano et al., 1987; Hajj et al., 1990; Riva et al., 1995; Sithanandam et al., 1989).

The role of Ras and Raf in regulating cell cycle progression has been shown to be complex and their modes of action are not fully understood. Early experiments showed that introduction of activated Ras and Raf could fully transform established cell-lines. They allowed cells to grow in reduced mitogen conditions, overcome contact inhibition, grow in the absence of anchorage and form tumours when injected into nude mice (Feramisco et al., 1984; Pritchard et al., 1995; Rapp et al., 1983; Smith et al., 1990; Stacey and Kung, 1984). A requirement for Ras and Raf in growth factor stimulated DNA synthesis has also been demonstrated in NIH 3T3 cells (Feig and Cooper, 1988; Howe et al., 1992; Kolch et al., 1991; Mulcahy et al., 1985; Schaap et al., 1993; Stacey et al., 1991). However Ras and Raf have been shown to control multiple aspects of cell growth and development in addition to mitogen-stimulated proliferation. Aberrant activation of Ras or Raf often causes cell cycle arrest, especially in primary cells. For example, oncogenic Ras and Raf cause a p53-dependent cell cycle arrest in Schwann cells which requires induction of the cyclin-dependent kinase inhibitor, p21\(^{CIP1}\) (Lloyd et al., 1997; Ridley et al., 1988). However the specificity of the Ras or Raf signal can be changed by the presence of an additional oncogenic lesion which can prevent p21\(^{CIP1}\) upregulation and allow mitogenic stimulation. For example, SV40 Large T antigen, which sequesters p53 or the loss of p53 is able to revert the growth inhibitory phenotype of activated Ras or Raf in Schwann cells (Lloyd et al., 1997; Ridley et al., 1988).

In addition to promoting cell cycle inhibition, Ras has also been shown to change cell fate. Expression of activated Ras in human or murine fibroblasts causes a phenotype that resembles premature senescence (Serrano et al., 1997). This may be a protective mechanism utilised by the cell to prevent constitutive activation of a mitogenic signal transduction pathway by oncogenes. Furthermore, Ras and Raf have also been demonstrated to promote differentiation when overexpressed in cells in tissue culture (Noda et al., 1985; Wood et al., 1993). More importantly, overexpression of activated Ras and Raf in transgenic animals has also been shown to promote differentiation.
(Bailleul et al., 1990; O'Shea et al., 1996). Thus aberrant activation of Ras or Raf has physiological consequences which match those observed in vitro.

Although many consequences of Ras and Raf activation have been observed, the molecular mechanisms by which Ras and Raf specify these different responses remains unresolved. Studies of oncogene co-operation in primary cells have shown that viral oncogenes can alter the response of cells to Ras or Raf by differential regulation of cell cycle inhibitor proteins (Lloyd et al., 1997; Serrano et al., 1997). Interestingly, Raf activation has been reported to both initiate and inhibit DNA synthesis in NIH 3T3 fibroblasts (Pritchard et al., 1995; Samuels and McMahon, 1994). This indicates that these cells have the potential to reveal how cells are able to respond to the same signal in different ways. Thus we have used NIH 3T3 cells to investigate how Ras and Raf signal specificity is regulated.

The work in this chapter was performed in collaboration with Andreas Sewing and his work is acknowledged in the figure legends.

2.2 Results

2.2.1 Regulation of Raf

To investigate the effects of Raf on the cell cycle we generated a cell-line in which Raf activation could be induced when required. Catalytic proteins have previously been made regulatable by the addition of the hormone binding domain (HBD) of a steroid hormone receptor (Godowski et al., 1988; Samuels et al., 1993). The presence of the HBD inactivates these proteins, but addition of the relevant hormone releases the inhibition and causes rapid activation. This principle was used to generate an androgen receptor-Raf fusion protein. The carboxy terminus of RafCAAX (wild type Raf-1 fused to the membrane localisation signal of K-Ras (Leevers et al., 1994)) was amplified by PCR and digested with HindIII and EcoRI and cloned into pBluescript. This fragment was ligated to the HBD of the androgen receptor (AR) which had also been amplified by PCR and digested with BamHI and HindIII. The Raf-AR fragment was sub-cloned in frame into the replication defective, neomycin resistant retroviral vector, pLXSN3 with a Myc tag that is specifically recognised by the 9E10 monoclonal antibody (Evan et al., 1985). This retroviral vector is depicted in Figure 2.2a and is referred to as pLXSN RafAR (Sewing et al., 1997).

The retroviral producer cell-line GP+E was stably transfected with pLXSN RafAR and selected for resistance to G418 in media containing 10% charcoal and dextran stripped FBS and without phenol red. Phenol red and hormones in serum are able to activate steroid hormone receptors. A G418-resistant population of cells was obtained from which supernatant containing replication-defective retroviruses was collected. GP+E cells
that produced the empty virus, LXSN, were also made. NIH 3T3 cells were infected with either virus and selected for G418 resistance. These cells were consistently cultured in media without phenol red and stripped NCS. Polyclonal populations were obtained and are referred to as RafAR and LXSN cells.

To confirm that RafAR was expressed, cell lysates from RafAR or LXSN cells were separated by SDS-PAGE and analysed for RafAR expression by Western blotting using the 9E10 antibody. A protein of the expected size was expressed in RafAR cells and not in the empty vector cells (Figure 2.2b). To confirm that RafAR can be activated by the addition of androgens we looked at the ability of RafAR to activate the ERK MAP kinase pathway. The testosterone derivative, R1881 or the equivalent volume of ethanol (the solvent used to dissolve R1881) was added to quiescent RafAR or LXSN cells for 30 minutes. p42\(^{ERK2}\) was immunoprecipitated from cell lysates and its ability to phosphorylate myelin basic protein (MBP) was measured. R1881 stimulated a large increase in the level of ERK kinase activity indicating that RafAR had been activated (Figure 2.2c). This increase in ERK kinase activity did not occur upon addition of ethanol to either cell-line or upon addition of R1881 to LXSN cells.

2.2.2 Inhibition of cell cycle progression by RafAR

Raf has been implicated in both stimulation and inhibition of DNA synthesis in NIH 3T3 cells. We wished to determine whether the growth of NIH 3T3 cells could be inhibited by activation of RafAR. Tritiated thymidine incorporation analysis, which measures the DNA synthesis index of the cells, was performed on asynchronously growing RafAR cells after stimulation with R1881 or ethanol. DNA synthesis of RafAR cells dramatically decreased 18 h after treatment with R1881 to 20% of the thymidine incorporation of RafAR cells that had been treated with ethanol. This inhibition of growth is specific to RafAR since the growth of LXSN cells was unaffected by the addition of R1881 (Figure 2.3a).

To analyse the nature of the arrest, the DNA profile of the RafAR cell populations were examined by staining their DNA with propidium iodide (PI) and quantifying the total DNA content of each cell using fluorescent activated cytometry (FACS). RafAR cells treated with R1881 accumulated in the G1 phase of the cell cycle and 88% of the cells had a 2N DNA content. Only 68% of RafAR cells treated with ethanol were in G1 which is normal for an asynchronously growing population of cells (Figure 2.3b). To examine the percentage of cells transiting from G1 to S-phase, RafAR cells were pulse labelled with a DNA analogue, Bromodeoxyuridine (BrdU) for 2 h, after treatment with R1881 or ethanol for 16 h. The degree of BrdU incorporation compared with the total DNA content for each population was analysed by FACS. Activation of RafAR with R1881 caused a dramatic reduction in the number of cells entering S-phase. Only 1% of cells in the presence of R1881 entered S-phase compared to 25% of cells treated with ethanol (Figure
2.3b). Thus activation of RafAR in NIH 3T3 cells caused a cell cycle arrest in G1. This was very similar to the effect of ΔRaf:ER that had been observed in primary rat Schwann cells where Raf-1 activation caused a p53-dependent induction of p21\(^{Cip1}\) which resulted in a G1 arrest. In Schwann cells expression of Cyclins D1, A and to a small extent, E became upregulated and yet were inactive due to the concomitant upregulation of the cell cycle inhibitor, p21\(^{Cip1}\) (Lloyd et al., 1997).

To determine whether this growth inhibition by RafAR in NIH 3T3 cells was similar to that observed in Schwann cells, we examined the effect of activation of RafAR on a panel of cell cycle regulatory proteins. The expression levels of G1 cyclins, CDKs and CKIs were compared from subconfluent RafAR cells in the presence of 10% NCS after stimulation with R1881 or ethanol. Immunoblotting analysis following SDS-PAGE revealed that Cyclin D1, Cyclin E and p21\(^{Cip1}\) expression were upregulated, while the levels of CDK4, CDK6 and p27\(^{Kip1}\) remained unchanged (Figure 2.3c). This profile of protein expression was similar to that observed upon Raf activation in Schwann cells, implying that growth arrest in NIH 3T3 cells may be through a similar mechanism.

pRb has an important role in G1 to S-phase progression and is a substrate for active D-type cyclin and Cyclin E complexes. Phosphorylation and subsequent inactivation of pRb is a necessary requirement for cells to transit from G1 to S-phase upon stimulation with mitogens (Mittnacht, 1998). The phosphorylation state of pRb was examined by looking at its mobility shift upon SDS-PAGE. In the presence of R1881, pRb was hypophosphorylated compared with unstimulated cells, indicating that pRb was active (Figure 2.3c).

We have shown that activation of RafAR in asynchronously growing cells induces expression of G1 cyclins which are usually growth promoting and yet RafAR activation causes in cell cycle arrest. This indicates that they may not be active and that concurrent upregulation of p21\(^{Cip1}\) may be responsible for their inactivation. The kinase activities of the G1 cyclin complexes were assessed. Asynchronously growing RafAR cells were stimulated with R1881 or ethanol. Cyclins D1, E and H were immunoprecipitated and the ability of the cyclin associated complex to phosphorylate a specific substrate was determined. The associated kinase activities of Cyclin D1 and Cyclin E decreased by 3-5 fold upon activation of RafAR (Figure 2.3d). The kinase activity of Cyclin H, which is unable to bind p21\(^{Cip1}\) (Harper et al., 1995), remained unchanged.

Stimulation of RafAR must therefore induce an activity inhibitory to the G1 cyclins. Since p21\(^{Cip1}\) was upregulated by RafAR activation and had already been implicated as the responsible factor in the Raf-1-induced Schwann cell growth arrest (Lloyd et al., 1997) it was an obvious candidate. p21\(^{Cip1}\) binds cyclin-CDK complexes and inhibits their activation (el-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994; Xiong et al., 1994).
Therefore we assessed whether the increased expression of p21\textsuperscript{Cip-1} may be responsible for downregulation of Cyclin D\textsubscript{1} and E-associated kinase activity. To determine whether the level of p21\textsuperscript{Cip-1} present in the G1 cyclin/CDK complexes increased upon RafAR activation, CDK2 and CDK4 complexes were immunoprecipitated from R1881 treated and untreated cells. Immunoprecipitates were separated by SDS-PAGE and examined by Western blot for p21\textsuperscript{Cip-1} and CDK protein levels. Stimulation of RafAR activity increased the amount of p21\textsuperscript{Cip-1} complexing with both CDK4 and CDK2, although total CDK levels remained constant (Figure 2.4a).

The presence of extra p21\textsuperscript{Cip-1} in CDKs complexes upon RafAR activation suggested that the increased level of p21\textsuperscript{Cip-1} stimulated by RafAR may be sufficient to inhibit the G1 cyclin complexes. To determine whether there was an excess of an inhibitory activity in cells which contained activated RafAR, we tested the ability of R1881-treated RafAR cell lysates to inhibit a target of exogenous active Cyclin E complex. In addition, to determine whether p21\textsuperscript{Cip-1} was responsible for the inhibitory activity, lysates of cells that had been treated with R1881 for 16 h were immunodepleted with an anti-p21\textsuperscript{Cip-1} antibody (or immunoglobulins as a control) to remove p21\textsuperscript{Cip-1} protein (Figure 2.4c). Exogenous active cyclin E target complexes were provided by a Rat-1 cell line that expressed human Cyclin E (E6 cells) (Perez-Roger \textit{et al.}, 1997)) that could be distinguished from murine Cyclin E by a monoclonal antibody. Target extracts from E6 cells were mixed either with lysates (immunodepleted or normal) from RafAR cells that had been treated with R1881 or ethanol, or with a mock extract (lysis buffer). Human Cyclin E complexes were then immunoprecipitated from the lysate mixtures and their ability to phosphorylate histone H1 was determined. RafAR cell lysates treated with R1881 and mock-immunodepleted with immunoglobulins, were able to inhibit the Cyclin E-associated kinase activity of the E6 cells. However, p21\textsuperscript{Cip-1} immunodepleted lysates from R1881 treated cells could not (Figure 2.4b). Lysates from RafAR cells treated with ethanol were unable to inhibit Cyclin E-associated kinase activity. This indicates that cells stimulated with R1881 contain active p21\textsuperscript{Cip-1} which is responsible for the inhibition.

G1 cyclin-associated kinase activity is necessary for mitogen stimulation of DNA synthesis. We have shown that in NIH 3T3 cells activation of RafAR downregulates Cyclin D\textsubscript{1} and Cyclin E-associated kinase activities by inducing p21\textsuperscript{Cip-1} which binds to CDK2 and CDK4 complexes and that p21\textsuperscript{Cip-1} is necessary for inhibition of cyclin-associated kinase activity. Therefore p21\textsuperscript{Cip-1} is necessary for the G1 specific arrest we observe in R1881 stimulated RafAR cells.

To determine if Raf-1 was able to transcriptionally upregulate p21\textsuperscript{Cip-1} we examined the ability of RafAR to induce expression from a luciferase reporter construct under the control of the full length human p21\textsuperscript{Cip-1} promoter (pCip-1-Luc) (a kind gift from X.Lu). RafAR cells were transiently transfected with pCip-1-Luc and a control lacZ reporter.
construct. Addition of R1881 for 24 h induced p21^{Cip-1} promoter activity to 3 fold above background levels Figure 2.5. This indicates that RafAR activity was able to stimulate p21^{Cip-1} transcription.

To confirm that p21^{Cip-1} was responsible for the Raf-induced arrest we decided to obtain direct genetic evidence using mouse embryo fibroblasts (MEFs) from transgenic mice. We determined whether activation of RafAR would inhibit the growth of wild type (wt) MEFs. To this end RafAR was subcloned into pLXP3, a derivative of pLXSN3 that contains a puromycin resistance gene in place of the neomycin resistance gene. This was necessary because MEFs from transgenic mice are already neomycin resistant. This plasmid was transiently transfected into a viral producer cell-line, Bosc 23 cells and viral supernatant was collected 48 h later. wtMEFs were infected with LXP3 RafAR viral supernatant and selected for resistance to puromycin, a polyclonal population was obtained. Asynchronously growing RafAR wtMEFs were stimulated with R1881 or ethanol and a tritiated thymidine incorporation assay was performed. Activation of RafAR for 16 h inhibited cellular DNA synthesis by over 50% (Figure 2.6a). This indicated that wtMEFs responded to RafAR activation in a similar manner to NIH 3T3 cells. We primarily wished to determine whether p21^{Cip-1} was necessary for RafAR-induced growth inhibition. Therefore, RafAR expressing MEFs from p21^{Cip-1} null mice were generated in the same manner as the wt MEFs and their DNA synthesis in response to RafAR activation was measured. Activation of RafAR for 16 h in these cells did not cause any growth inhibition (Figure 2.6a). This proved that p21^{Cip-1} was necessary for RafAR-induced growth inhibition in MEFs.

p53 is a direct regulator of p21^{Cip-1} (el-Deiry et al., 1994; el-Deiry et al., 1993) and has been shown to be necessary for Ras and Raf inhibition of primary cells (Lloyd et al., 1997; Serrano et al., 1997). To determine if the p21^{Cip-1}-mediated arrest we observe is dependent on p53, p53 null MEFs were analysed for their response to RafAR activation. p53 null MEFs were infected with LXP3 RafAR virus in the manner described above. Activation of RafAR in these cells caused inhibition of growth and an upregulation of p21^{Cip-1} (Figure 2.6). This indicated that growth inhibition and p21^{Cip-1} upregulation by Raf activity was not dependent on p53.

As shown earlier, Cyclin D1 is upregulated by RafAR activation. Cyclin D1 overexpression is able to stimulate proliferation (Lovec et al., 1994; Resnitzky, 1997; Zhu et al., 1996b) but has also been implicated in cell cycle inhibition (Del Sal et al., 1996) and senescence (Dulic et al., 1993; Lucibello et al., 1993). We wished to determine whether it had a role in RafAR-mediated growth inhibition. Thus Cyclin D1 null MEFs were infected with LXP3 RafAR retrovirus. Upon RafAR activation in asynchronously growing cells, their rate of DNA synthesis was reduced (Figure 2.6a), indicating that Cyclin D1 was not necessary for growth inhibition by RafAR. In addition p21^{Cip-1} was
also induced suggesting that Cyclin D1 also had no role in p21<sup>Cip-1</sup> induction (Figure 2.6b).

Thus we have shown that stimulation of RafAR activity is able to inhibit cell cycle progression from G1 to S-phase in asynchronously growing murine fibroblasts. Furthermore, it has been shown biochemically and genetically that this arrest is dependent on upregulation the cell cycle inhibitor, p21<sup>Cip-1</sup> which occurs transcriptionally and is independent of p53 and Cyclin D1 function.

### 2.2.3 Stimulation of DNA synthesis by RafAR

We have established that activation of RafAR is able to induce a G1 specific growth arrest in fibroblasts. However activated Raf has also been shown to act as a mitogen in NIH 3T3 cells (Fukui <i>et al.</i>, 1985; Pritchard <i>et al.</i>, 1995; Rapp <i>et al.</i>, 1983; Smith <i>et al.</i>, 1990). We were interested to find an explanation for these apparently contrasting effects. The different mitogenic properties of Raf family members provided clues as to how this apparent contradiction could be rationalised. An oncogenic form of A-Raf was shown to stimulate DNA synthesis in NIH 3T3 cells, whereas similarly activated forms of Raf-1 and B-Raf could not (Pritchard <i>et al.</i>, 1995). These Raf mutants also activated the ERK MAP kinase pathway to different extents. A-Raf stimulated the least ERK activity while B-Raf induced the most. Therefore the ability of A-Raf to stimulate DNA synthesis may have been related to its lesser ability to activate ERK activity implying that the strength of Raf signal generated may be a factor in determining their mitogenic ability.

To determine if the degree of Raf-1 activation was able to determine proliferative responses of cells, we tested whether it was possible to regulate the strength of the RafAR signal by analysing its ability to stimulate ERK activity. We treated quiescent, confluent, serum-deprived cultures of RafAR cells with the indicated concentrations of testosterone for 30 minutes and measured p42<sup>EzK2</sup> kinase activity. The degree of ERK kinase activity increased proportionally with greater concentrations of testosterone (Figure 2.7a). This demonstrated that RafAR could be activated to different signal strengths depending on the concentration of hormone.

Thus we investigated whether the induction of different levels of Raf-1 activity could stimulate G1 to S-phase progression with different efficiencies in quiescent cells. Confluent, serum-deprived cultures of RafAR cells were treated with the indicated concentrations of testosterone for 16 h. Their DNA synthesis was then analysed by tritiated thymidine incorporation. Activation of RafAR with gradually increasing concentrations of testosterone increased cellular DNA synthesis to a maximum of 4 fold above basal rates at 10 nM testosterone. Higher concentrations of testosterone failed to induce this mitogenic response and at the highest concentrations thymidine incorporation rates were reduced to below background levels (Figure 2.7b). In parallel to the thymidine incorporation assay, cell lysates were also taken for analysis by Western blot and
assessed for Cyclin D1 and p21<sup>Cip-1</sup> expression. Cyclin D1 expression levels became elevated in response to 10 nM testosterone and remained high in the presence of 100 nM testosterone. In contrast p21<sup>Cip-1</sup> was induced only by 100 nM testosterone. Thus different strengths of RafAR activation were able to differentially upregulate Cyclin D1 and p21<sup>Cip-1</sup>. Induction of Cyclin D1 correlated with induction of DNA synthesis, while induction of p21<sup>Cip-1</sup> correlated with a G1 arrest and we demonstrated earlier that p21<sup>Cip-1</sup> is responsible for RafAR-mediated inhibition of DNA synthesis. Thus RafAR is able to both stimulate and inhibit DNA synthesis by differentially regulating expression of cell cycle regulatory proteins depending on its strength of activation.

### 2.2.4 Investigating the generality of the Raf response

To confirm that this dual regulation by RafAR is a general response to Raf-1 activation we investigated the ability of other activated Raf-1 mutants to inhibit cell cycle progression. An alternative Raf-steroid receptor fusion protein which had been caused Raf-1-induced cell cycle arrest in Schwann cells was tested. To confirm that the steroid hormone binding domains had no role in the Raf-1 mediated arrest we also tested the ability of RafCAAX to cause growth inhibition by inducing its expression using a tetracycline repressible promoter. RafCAAX is wild type Raf-1 fused to a membrane localisation sequence which makes it constitutively active (Leevers et al., 1994; Stokoe et al., 1994). In addition we wished to determine whether Ras, which directly activates Raf in vivo, could elicit similar cellular responses.

### 2.2.5 The effect of a different Raf-hormone receptor protein on cell cycle progression

ΔRaf:ER has the CR3 domain of human Raf-1 at its amino terminus which is fused to the hormone binding domain of the human oestrogen receptor at its carboxy terminus (Samuels et al., 1993) (Figure 2.8a). This protein is activated by oestrogens and its antagonists, for example, β-estradiol and 4-OH tamoxifen (4-OHT) respectively. ΔRaf:ER induces p21<sup>Cip-1</sup>-dependent growth arrest in Schwann cells (Lloyd et al., 1997) and can prevent growth factor stimulated DNA synthesis in NIH 3T3 cells (Pritchard et al., 1995; Samuels and McMahon, 1994). NIH 3T3 cells infected with pLXSN ΔRaf:ER were a kind gift from A.Lloyd and are referred to as ΔER cells. The ability of ΔRaf:ER to stimulate dose-dependent stimulation and inhibition of DNA synthesis was tested. To determine whether the activity of ΔRaf:ER could be induced to different strengths, confluent serum-deprived cultures of ΔER cells were stimulated with the indicated concentrations of β-estradiol, ethanol or 10% NCS for 30 min. The degree of ΔRaf:ER activity was gauged by its ability to induce ERK phosphorylation which can be observed as a mobility shift when proteins are separated on a MAP kinase shift gel and
immunoblotted for p42\textsuperscript{Erk2} expression. The phosphorylated form of p42\textsuperscript{Erk2} increased proportionally with the concentration of β-oestradiol in the media (Figure 2.8b). Ethanol-treated ΔRER cells demonstrated no ERK phosphorylation shift indicating that ΔRaf:ER is inactive in unstimulated cells. In addition, the blot was stripped and reprobed with antibodies specific for tyrosine 204 and threonine 202 dual phosphorylated p42/44 ERK1/2. Phosphorylation of these residues is essential for ERK activity (Boulton \textit{et al.}, 1991; Payne \textit{et al.}, 1991). The shifted band of the MAP kinase shift blot correlated precisely in position and intensity with that recognised by anti-phospho-ERK antibody (Figure 2.8b). Thus ΔRaf:ER signal strength could be regulated.

The ability of ΔRaf:ER to stimulate DNA synthesis in quiescent NIH 3T3 cells was then investigated. Confluent, serum-deprived, quiescent cultures of ΔRER cells were stimulated with the indicated concentrations of β-oestradiol or 10% NCS for 25 h and a tritiated thymidine incorporation assay was performed. DNA synthesis gradually increased with increasing hormone concentration to a maximum of two fold above basal levels in the presence of 1.5 nM β-oestradiol. As we had observed with RafAR, higher concentrations of β-oestradiol were less able to initiate DNA synthesis in the cells and no induction of DNA synthesis was observed at the maximal concentration of hormone (Figure 2.8c). The addition of serum to the cells was much more mitogenic than ΔRaf:ER activation, it induced thymidine incorporation 5 fold above background levels. Thus similarly to RafAR, ΔRaf:ER has a narrow window in which it is able to stimulate DNA synthesis.

The ability of ΔRaf:ER activation to inhibit mitogen-stimulated growth in a dose dependent manner was tested. ΔER cells, exponentially growing in 10% NCS, were stimulated with 4-OH tamoxifen at the indicated concentrations for 18 h. DNA synthesis was measured by tritiated thymidine incorporation. DNA synthesis was reduced in proportion to the concentration of hormone added to the cells (Figure 2.8d). This indicates that like RafAR, ΔRaf:ER is able to inhibit DNA synthesis in the presence of mitogens. Furthermore, we have shown that Raf-1-mediated inhibition is sensitive to the strength of the Raf-1 signal.

We can conclude that dose-dependent regulation of the cell cycle is a common property of Raf-steroid hormone receptor fusion proteins.

\subsection*{2.2.6 The effects of \textit{RafCAAX} expression on cell cycle control}

It was necessary to establish that RafAR and ΔRaf:ER-mediated growth arrest was a genuine property of Raf-1 and that the different cellular responses to Raf-1 signals of varying strengths were a general phenomenon and not specific to Raf-steroid hormone receptor fusion proteins.

Therefore we utilised a tetracycline regulatable promoter to regulate expression of RafCAAX (Leevers \textit{et al.}, 1994). Transcription of RafCAAX is driven by a
Cytomegalovirus (CMV) promoter. This is inhibited in the presence of tetracycline due to tet O sequences within the promoter that bind tetracycline and repress transcription. Removal of tetracycline from the growth media allows transcription to occur (Gossen and Bujard, 1992). This method of regulation was developed as a two plasmid system by Gossen and Bujard (1992) and was then assimilated into a retroviral vector containing a puromycin resistance gene, based on pBabePuro (Morgenstem and Land, 1990a) and called pBPSTR1 (Paulus et al., 1996). This is depicted in Figure 2.9. Doxycycline, a derivative of tetracycline, has a higher binding affinity for tet O sequences than tetracycline, is soluble in dH₂O and is commonly used in place of tetracycline (M. Gossen personal communication).

RafCAAX was subcloned as a blunt fragment into the Pmel site of pBPSTR1 to form pBPSTR1 RafCAAX. The amino terminus of RafCAAX had been tagged with a peptide derived from human c-myc which is specifically recognised by the 9E10 antibody (Evan et al., 1985) to distinguish this mutant from endogenous Raf-1.

Cell-lines were generated in which RafCAAX expression could be regulated. pBPSTR1 and pBPSTR1 RafCAAX were transiently transfected into Bosc 23 viral producer cells in the presence of doxycycline, supernatant was collected and used to infect NIH 3T3 cells. Infected NIH 3T3 cells were selected for puromycin resistance in the presence of 100 ng/ml of doxycycline and clonal populations were derived by ring cloning and are referred to as ST and STRCX cells.

STRCX clones were then tested for their ability to induce RafCAAX expression. Two dishes of each clone were grown in DMEM with 10% NCS in the presence or absence of doxycycline for 48 h. Cell lysates were then separated by SDS-PAGE and analysed for RafCAAX expression by Western blot. Out of 16 STRCX clones tested, 4 had a significant induction of RafCAAX expression upon removal of doxycycline. Most of the clones did not express RafCAAX and one did not respond to doxycycline (Figure 2.10).

The ability of RafCAAX to inhibit mitogen-induced DNA synthesis was then analysed. Subconfluent cultures of four regulatable STRCX clones and a polyclonal population of ST cells maintained in DMEM with 10% NCS in the presence or absence of doxycycline for 48 h. DNA synthesis was measured by tritiated thymidine incorporation analysis. Removal of doxycycline from each STRCX clone caused some inhibition of DNA synthesis. Clones STRCX6 and 15 were inhibited to the greatest extent, to a half and a quarter of their normal growth rates respectively (Figure 2.11). Thus activation of Raf-1 signals by three different mechanisms can inhibit serum-stimulated DNA synthesis indicating that this is a genuine property of Raf-1.

The ability of RafCAAX to induce cell cycle progression was also investigated. Initially we tested whether RafCAAX expression levels could be differentially regulated by varying the concentration of doxycycline added to the growth media. Confluent, serum-
deprived, quiescent cultures of STRCX 6 cells were incubated in the indicated levels for 25 h. STRCX6 cells became gradually more morphologically transformed in response to higher levels of RafCAAX expression (Figure 2.12a). Cell lysates were separated by SDS-PAGE and analysed for RafCAAX expression by Western blot. Induction of RafCAAX expression was stimulated when doxycycline concentrations were lowered to 1 ng/ml and increased upon further reductions in doxycycline concentration. Maximal RafCAAX expression occurred on complete removal of doxycycline (Figure 2.12a). The levels of endogenous Raf-1 were analysed by stripping antibodies from the blot and re-probing with an anti-Raf-1 antibody. Raf-1 levels remained constant, indicating that expression of RafCAAX did not affect the expression of endogenous Raf-1 protein (Figure 2.12b).

We proceeded to test whether RafCAAX could initiate DNA synthesis and whether different expression levels of RafCAAX would affect this. Confluent, serum-deprived quiescent cultures of STRCX6 cells were incubated in the indicated levels of doxycycline for 25 h and DNA synthesis was measured by tritiated thymidine incorporation analysis. DNA synthesis increased to a maximum of two fold above basal levels in 0.5ng/ml doxycycline (Figure 2.12). However as doxycycline concentrations were reduced beyond 0.5 ng/ml DNA synthesis was reduced. At maximal RafCAAX expression, in the absence of doxycycline, DNA synthesis levels were higher than basal levels but lower than maximal rates (Figure 2.12). This indicated that the same effects on cell cycle control occurred with RafCAAX as with the Raf-hormone receptor fusions. I.E. that Raf-1 activation to a low level is stimulatory and yet higher levels of induction become inhibitory.

2.2.7 Regulation of the Cell Cycle by Ras\textsuperscript{V12}

Ras is able to directly activate Raf (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Zhang et al., 1993). Therefore we were interested to know whether activated Ras was also able to provoke similar cellular responses to activated Raf-1. Importantly, Ras, but not Raf, is frequently found activated in human tumours (Barbacid, 1987). This heightens the importance of understanding the molecular mechanisms behind its regulation of cell growth. As discussed earlier activated Ras acts mitogenically in cell-lines such as NIH 3T3 fibroblasts and yet has also been shown to inhibit the growth of primary cells (Ridley et al., 1988; Serrano et al., 1997). Thus we investigated the ability of Ras to regulate cell cycle progression in NIH 3T3 cells.

Induction of Ras\textsuperscript{V12} expression was achieved by using the tetracycline repressible pBPSTR1 vector described above (Figure 2.9). Ras\textsuperscript{V12} was subcloned as a BamHI fragment into pBPSTR1 to make pBPSTR1 Ras\textsuperscript{V12}. pBPSTR1 and pBPSTR1 Ras\textsuperscript{V12} were stably transfected into a viral producer cell-line, GP+E cells. Viral supernatants were collected from these pooled cells and used to infect NIH 3T3 fibroblasts. Infected
cells were selected for puromycin resistance in the presence of doxycycline to obtain clones which were tested for regulatability in the same manner as STRCX cells. Cells infected with virus derived from pBPSTR1 RasV12 were referred to as STRas cells. Lysates from STRas or ST cells cultured in the presence or absence of doxycycline were analysed for expression of RasV12 by SDS-PAGE and Western blotting. The antibodies used to detect RasV12 are also able to pick up endogenous Ras protein, however Ras expression was almost undetectable in ST cells indicating that the Ras expression we detected was probably RasV12. Sixteen STRas clones were tested and RasV12 was significantly induced in ten clones. Three clones did not express observable levels of RasV12 and three clones had a high background of RasV12 expression (Figure 2.13). Clone number 18 was chosen for further investigation and is referred to as STRas c18.

The effect of RasV12 expression on the DNA synthesis of asynchronously growing cells was investigated. Subconfluent STRas c18 cells in DMEM with 10% NCS were cultured in the indicated concentrations of doxycycline for 65 h. DNA synthesis was measured by a tritiated thymidine incorporation assay. In addition, lysates were taken and separated by SDS-PAGE and analysed by Western blot for RasV12 and p21Cip1 expression. Titration of doxycycline induced RasV12 and p21Cip1 expression in a dose-dependent manner (Figure 2.14b). Induction of RasV12 inhibited DNA synthesis in a dose dependent manner to a quarter of their DNA synthesis levels in the absence of RasV12 (Figure 2.14). The DNA profile of the cells was also analysed by propidium iodide staining using FACS. This revealed that upon RasV12 expression cells accumulated in the G1 phase of the cell cycle in a dose-dependent fashion from 64% in the presence of 100 ng/ml of doxycycline to 75% in its absence. Therefore RasV12 was able to inhibit cell cycle progression at high expression levels which correlated with p21Cip1 expression. This response is very similar to the effect of activated Raf-1, indicating that inhibition of proliferation is a general response to hyperactivation of the Ras-Raf signalling pathway.

Activated Ras has been shown multiple times to induce DNA synthesis in NIH 3T3 cells (Feramisco et al., 1984; Stacey and Kung, 1984). In addition, it has been reported that although RasV12 is able to morphologically transform cells in the complete absence of mitogens (Lloyd et al., 1989), RasV12 needs to synergise with other mitogenic factors, such as insulin to induce DNA synthesis (Morris et al., 1989). We analysed the RasV12-induced entry into S-phase and also whether RasV12 could initiate DNA synthesis in the complete absence of mitogens.

To determine when RasV12 expression occurs a time course of RasV12 expression was performed on synchronously growing STRas c18 cells which had had doxycycline removed at the indicated time points. RasV12 expression was induced 8-10 hours after doxycycline removal. Expression levels continued to rise until the final 24 h time-point of this experiment (Figure 2.15a). To determine when RasV12 expression reached a
consistent level the experiment was repeated with longer time points. The levels of Ras^{V12} induction reached a steady state between 24 and 36 h (Figure 2.15b). Ras^{V12} has a very long half-life, 42 h, while normal wild-type Ras, has a half-life of 20 h. This may account for the long period of protein accumulation (Ulsh and Shih, 1984).

To determine when Ras^{V12} induction elicited a cellular response a time course of ERK activation was performed. Confluent serum-deprived, quiescent cultures of STRas c18 cells were harvested at the indicated time points after doxycycline removal and assayed for p42^{ERK2} kinase activity. p42^{ERK2} became active 8 h after doxycycline removal (Figure 2.15c). This indicated that a cellular response to Ras^{V12} occurred at the same time or very soon after its expression.

Confluent cultures of STRas c18 cells were incubated in low mitogen conditions (either DMEM alone or DMEM with 0.25% serum) for 48 h and then doxycycline was washed out of the cells and the cells were cultured in the specific low mitogen conditions for the indicated time periods. Cells were then assayed for their ability to incorporate tritiated thymidine which had been added to the cells 2 h prior to harvest. The largest induction of DNA synthesis occurred between 20 and 24 h after doxycycline removal (Figure 2.15d). DNA synthesis reached higher levels in the presence of 0.25% NCS. Since Ras^{V12} induction took 8-10 h, this suggested that cells took approximately 14 h to progress cells from quiescence into S-phase upon Ras^{V12} induction. However it must also be noted that Ras^{V12} protein levels increased for at least 24 h and this may have influenced Ras^{V12} induced S-phase entry. Induction of Ras^{V12} in presence of 0.25% NCS induced the highest level of DNA synthesis, although Ras^{V12} also induced DNA synthesis in the absence of exogenous mitogens. Therefore serum may assist Ras^{V12} induction of DNA synthesis or alternatively a low concentration of serum in the growth media may affect cell survival. Autocrine factors induced by Ras^{V12} may also have contributed to the mitogenic ability of Ras^{V12}. To investigate this further one could block growth factor receptors on the cell surface by using a general growth factor receptor inhibitor, for example suramin and then test whether Ras^{V12} was still able to stimulate DNA synthesis in the absence of growth factor signalling.

2.2.8 Differential effects of Ras and Raf-1 activation

In comparison to Raf-1, Ras was a much more potent mitogen. In our experiments we have activated Raf-1 steroid hormone receptor fusion proteins with an extensive range of hormone concentrations, across its mitogenic window, and expressed a range of levels of RafCAAX and yet Raf-1 signals have only induced a maximum of four fold induction of DNA synthesis over background levels (Figure 2.7, Figure 2.8 and Figure 2.12). In comparison, Ras^{V12} is able to induce DNA synthesis 10-15 fold over background values (Figure 2.15). When activated strongly, Raf-1 induces p21^{Cip-1} which causes cell cycle arrest. Weak signals which do not induce p21^{Cip-1}, but induce Cyclin D1, initiate DNA
synthesis. Therefore we were interested in whether the reason for the superior ability of Ras\textsuperscript{V12} to induce DNA synthesis over Raf was caused by differences in p21\textsuperscript{Cip-1} regulation.

Thus we examined the profile of p21\textsuperscript{Cip-1} expression upon stimulation of DNA synthesis by Ras\textsuperscript{V12} in low mitogen conditions. Confluent, serum-deprived quiescent cultures of STRas c18 cells were incubated in the presence or absence of doxycycline and DNA synthesis at the indicated time points was measured by thymidine incorporation analysis. Cell lysates were taken for Western blot analysis. p21\textsuperscript{Cip-1} protein became upregulated only after the cell had already entered S-phase, 22 h after removal of doxycycline (Figure 2.16). In a similar experiment performed using STRCX 6 cell, p21\textsuperscript{Cip-1} was upregulated at least 14 h after doxycycline removal. This was prior to the induction of S-phase. In addition the fold induction of DNA synthesis over their respective background values by Ras\textsuperscript{V12} is six times higher than that of RafCAAX (Figure 2.16). Therefore Ras and Raf-1 activation differentially regulate p21\textsuperscript{Cip-1}, and this correlates with their relative abilities to induce DNA synthesis.

Since p21\textsuperscript{Cip-1} expression is dependent on the strength of activation of Raf-1, the delayed induction of p21\textsuperscript{Cip-1} by Ras\textsuperscript{V12} may occur because Ras\textsuperscript{V12} is unable to activate ERK signals to the same degree as RafCAAX. Alternatively, Ras\textsuperscript{V12} may actively downregulate p21\textsuperscript{Cip-1} expression through a Raf-independent effector pathway. One would expect that if the former hypothesis were true that Ras\textsuperscript{V12} and Raf-1 would have similar mitogenic abilities and yet the potency of Ras\textsuperscript{V12} to stimulate DNA synthesis is much stronger than activated Raf-1. A requirement for a Raf-independent pathway downstream of Ras for DNA synthesis induction is also indicated by experiments performed with Ras effector domain mutants. A Ras\textsuperscript{V12} mutant, which can activate Raf and the ERK MAP kinase pathway, is unable to stimulate DNA synthesis and yet DNA synthesis can be induced if this mutant is co-expressed with another Ras effector domain mutant that cannot activate ERK (Joneson et al., 1996b). Our results suggest that this pathway would be able to downregulate p21\textsuperscript{Cip-1} induction by Raf-1.

To investigate whether a Ras-mediated pathway could counteract Raf-1 mediated upregulation of p21, we generated a cell-line in which ΔRaf:ER could be activated in the presence of activated Ras\textsuperscript{V12}. ΔRER cells were infected with pBPSTR1 and pBPSTR1 Ras\textsuperscript{V12} retroviral supernatant and selected for resistance to G418 and puromycin in the absence of doxycycline. These cells were pooled into a polyclonal population and are referred to as ΔRER/ST and ΔRER/Ras\textsuperscript{V12} cells.

To test whether Ras was able to counteract induction of p21\textsuperscript{Cip-1} by ΔRaf:ER, sub-confluent serum-deprived, quiescent cultures of ΔRER/ST and ΔRER/Ras\textsuperscript{V12} cells were treated with the indicated concentrations of 4-OH tamoxifen. Western lysates were taken, separated by SDS-PAGE and Western blotted. Expression of Ras\textsuperscript{V12} in ΔRER/Ras\textsuperscript{V12}
cells was confirmed by immunoblotting with the pan-ras antibody. In addition these cells also had a higher basal level of ERK phosphorylation which is indicated by the mobility shift in Figure 2.17a. This indicated that Ras$^{V12}$ was active in these cells.

Even though ERK was activated in ΔRER/Ras$^{V12}$ cells, p21$^{Cip-1}$ expression was downregulated. This indicated that Ras$^{V12}$ could actively inhibit p21$^{Cip-1}$ expression. Furthermore, the presence of Ras$^{V12}$ also attenuated the ability of ΔRaf:ER to induce p21$^{Cip-1}$ when cells were stimulated with 4-OH tamoxifen. Thus in the presence of strong ERK activation, Ras$^{V12}$ was able to counteract induction of p21$^{Cip-1}$. Thus in addition to upregulating p21$^{Cip-1}$ through Raf, Ras can also suppress p21$^{Cip-1}$, probably through another effector pathway.

We also tested the ability of these cells to induce DNA synthesis by measuring their ability to incorporate BrdU. ΔRER/Ras$^{V12}$ cells either unstimulated or treated with 1 nM 4-OH tamoxifen had a higher level of DNA synthesis than the control cell-line, which correlated with the reduced level of p21$^{Cip-1}$. However, stronger activation of ΔRaf:ER inhibited DNA synthesis in ΔRER/Ras$^{V12}$ cells to a greater extent than control cells even though p21$^{Cip-1}$ levels were reduced. Therefore it appears that an additional p21$^{Cip-1}$-independent inhibitory mechanism is induced in response to the combination of Ras and Raf activation. NIH 3T3 cells are null for the cell cycle inhibitors p16$^{INK4}$ and p19$^{ARF}$, eliminating possible involvement of these proteins. However, these cells do contain the other cell cycle inhibitory proteins for example p15$^{INK4B}$ and p27$^{Kip1}$.

Attempts were also made to generate a cell line in which Ras and Raf could both be regulated by infecting either STRas cells with LXSN ΔRafAR retrovirus or RafAR cells with BPSTR1 Ras$^{V12}$ retrovirus. However none of the clones selected were able to regulate Ras$^{V12}$ expression. Therefore a system in which Ras and Raf could both be regulated seemed inviable.

2.3 Summary

p21$^{Cip-1}$, induced by activation of Raf-1 or Ras independently of p53 function, causes inhibition of DNA synthesis in the presence of serum by entering and inhibiting the activities of cyclin-CDK complexes. In addition, activated Ras and Raf-1 are able to stimulate mitogen-independent DNA synthesis. However the specificity of the cellular response to Raf-1 depends on the strength of the Raf-1 signal. In the absence of serum, weak to moderate activation of Raf-1 induces Cyclin D1 which correlates with mitogenic ability. In contrast strong Raf-1 signals induce p21$^{Cip-1}$ and a cell cycle arrest in G1.
Therefore we conclude that Raf-1 is able to control opposing cellular responses because different intensities of Raf-1 stimulate differential gene expression.

Ras$^{V_{12}}$ is also able to inhibit DNA synthesis in the presence of mitogens, however in the absence of serum, the potency of Ras$^{V_{12}}$ to stimulate DNA synthesis is much stronger than activated Raf-1. This correlates with an ability to repress and delay p21$^{Cip-1}$ expression in comparison to Raf-1 activation. Furthermore, Ras is able to attenuate Raf-1-mediated p21$^{Cip-1}$ induction. This indicates that Ras$^{V_{12}}$ induces an activity that is able to downregulate p21$^{Cip-1}$ through a Raf-independent signal transduction pathway which alters a potentially inhibitory signal into a mitogenic one. In the next chapter we explore this hypothesis by activating known Ras effector pathways and testing their abilities to attenuate Raf-1-mediated p21$^{Cip-1}$ induction and inhibition of DNA synthesis.
2.4 Figures - Chapter Two
Figure 2.2 LXSN RafAR

a) pLXSN RafAR PCR amplified fragments encoding the kinase domain of c-Raf-1 (CRIII aa 305 to 648) fused to the CAAX motif of Ki-Ras (Ki-Ras aa 169 to 188) and the hormone binding domain of the human androgen receptor (HBD huAR aa 646 to 917) were cloned in frame with an amino terminal 9E10 Myc epitope (Myc tag) into the replication deficient retroviral vector pLXSNS.

b) Expression of RafAR in RafAR cells. RafAR and LXSN cells were grown in DMEM with 10% stripped NCS. Cells were lysed and 50 $\mu$g of protein was subject to SDS-PAGE then analysed by Western blotting using the 9E10 antibody directed against the Myc tag.

c) RafAR is activated by R1881. Confluent cultures of RafAR and LXSN cells were incubated in DMEM with 0.1% stripped NCS for 48 h and stimulated with 0.01% ethanol, 500 nM R1881 or 10% stripped NCS for 30 min. p42$^{ERK2}$ was immunoprecipitated from 100 $\mu$g of protein. Immunocomplexes were collected on protein A sepharose beads, assayed for kinase activity with MBP as the substrate and subject to SDS-PAGE. The gel was then dried and exposed to X-OMAT film.

This experiment was performed by A.Sewing
Figure 2.2

a) 

Myc tag

HBD huAR (aa646-917)  CR3 Raf-1 (aa305-648)

RafAR

CAAX Motif

LTR LTRSV Neo

HBD huAR

CR3 Raf-1

RafAR

LXSN

b) 

LX Raf SN AR

RafAR

RafAR

c) 

R1881 (500 nM)  

RafAR  LXSNS

NCS

MBP
Figure 2.3 Raf causes cell cycle arrest

a) Activation of RafAR inhibits DNA synthesis. Subconfluent cultures of RafAR and LXSN cells growing in DMEM with 10% stripped NCS were treated with 0.01% ethanol or 500 nM R1881 for 16 h. DNA synthesis was measured by incubation with tritiated thymidine for 2 h prior to harvesting and assaying for thymidine incorporation.

b) RafAR activation inhibits cells in G1. Subconfluent cultures of RafAR and LXSN cells growing in DMEM with 10% stripped NCS were treated with 0.01% ethanol or 500 nM R1881 for 16 h. DNA synthesis was measured by incubation with BrdU for 2 h prior to harvesting. Cells were fixed in 70% ethanol and stained with propidium iodide (PI) for detection of total DNA and with anti-BrdU-FITC conjugated antibody for detection of DNA synthesis. Cells were then analysed by FACS.

c) Effect of RafAR on protein expression. Subconfluent cultures of RafAR and LXSN cells growing in DMEM with 10% stripped NCS were treated with 0.01% ethanol or 500 nM R1881 for 16 h prior to lysis. 30-50 µg of protein was separated by SDS-PAGE and analysed by Western blotting. The blots were probed with antibodies against Cyclin E (sc-481), CDK2 (sc163), CDK4 (sc260), p21^{Cip1} (sc-757), p27^{Kip1} (sc-528), Cyclin D1 (287.3) and pRb (14001A).

d) RafAR activation downregulates G1 cyclin-dependent kinase activity. Cyclin E and Cyclin H were immunoprecipitated from 100 µg of protein using sc-481 and sc-609 respectively, Cyclin D1 was immunoprecipitated from 300 µg of protein using DCS 11. Immunocomplexes were collected on protein A beads (protein G beads in the case of Cyclin D1) and their kinase activity towards specific substrates were determined. Substrates were Histone H1 for Cyclin E, GST-pRb for Cyclin D1 and GST-CDK2 for Cyclin H.

This experiment was performed by A.Sewing
Figure 2.3

a) 3H Thymidine Incorporation

- +
R1881
RalAR LXS

b) Cell Number
+EtOH  +R1881

+EtOH  +R1881

Propidium Iodide

- +
R1881

CDK2
CDK4
Cyclin D1
Cyclin E

- +
R1881

p21
p27
pRb

- +
R1881

αCyclin E
αCyclin D1
αCyclin H

H1
pRb
CDK2
Figure 2.4 \( p21^{Cip-1} \) is responsible for Raf-1-dependent inhibition of G1 Cyclin-associated kinase activity

a) RafAR activation induces \( p21^{Cip-1} \) to enter CDK complexes. Cell lysates were taken from RafAR cells growing in DMEM with 10% stripped NCS that had been treated with 0.01% ethanol or 500 nM R1881 for 16 h. 250 \( \mu \)g of protein was used to immunoprecipitate CDK2 and CDK4, which were collected on protein A sepharose beads, boiled in SDS-sample buffer and separated by SDS-PAGE with a sample of total protein lysate in one lane. Gels were transferred to PVDF membranes and probed for \( p21^{Cip-1} \), CDK2 and CDK4 with sc-757, sc-163 and sc-260 antibodies respectively.

b) \( p21^{Cip-1} \) causes inhibition of Cyclin E-associated kinase activity. The inhibitory activity of lysates before and after activation of RafAR was tested with human Cyclin E-CDK2 complexes expressed in Rat1 cells (Perez-Roger et al., 1997) as a target. Lysates were prepared from RafAR cells treated with 0.01% ethanol or 500 nM R1881 for 24 h. Samples of lysate from cells treated with R1881 were immunodepleted with either an antibody to \( p21^{Cip-1} \) (sc-757) or with 2 \( \mu \)g unrelated immunoglobulin G (IgG). Target extract (10 \( \mu \)g) containing human Cyclin E-CDK2 complexes was mixed with 25 \( \mu \)g of extract, either from uninduced cells or from cells treated with R1881 depleted with IgG or anti-p21. As a control target extract mixed with lysis buffer was used. The mixed extracts were incubated at 20°C for 30 min. Cyclin E-CDK2 complexes were immunoprecipitated with an antibody specific for human Cyclin E (sc-198) and kinase activity was measured using histone H1 as the substrate.

c) Analysis of \( p21^{Cip-1} \) levels in lysate of the experiment described in the legend to panel b. Lysates of the cells after activation with antibodies to \( p21^{Cip-1} \) and IgG and before depletion were analysed by Western blotting with an antibody against \( p21^{Cip-1} \) (sc-757).

This experiment was performed by A.Sewing

Figure 2.5 RafAR induces transcription from the \( p21^{Cip-1} \) promoter

70 to 80% confluent RafAR cells were transiently co-transfected with 3.5 \( \mu \)g of a full length human \( p21^{Cip-1} \) promoter-luciferase construct (pCip-1-Luc) and a 2.5 \( \mu \)g of \( \beta \)-galactosidase reporter construct (CH110-lacZ) using the standard calcium phosphate protocol. The precipitate was removed after 12 h and after another 12 h the cells were treated with 500 nM R1881 or 0.01% ethanol for 24 h. Cells lysates were harvested and assayed for luciferase and \( \beta \)-galactosidase activity. The luciferase values were normalised against \( \beta \)-galactosidase activity as a control for transfection efficiency.

This experiment was performed by A.Sewing
Figure 2.4

a)

\[\text{IP}\]
\[\alpha\text{CDK2} \quad \alpha\text{CDK4} \quad \text{lysatex}\]

\(\text{R1881 (500 nM)}\)

\[\quad - \quad + \quad - \quad + \quad + \]

\[\text{p21Cip1}\]

\[\text{CDK2}\]

\[\text{CDK4}\]

b)

\(\text{RafAR extract}\)

\(\text{R1881 (500 nM)}\)

\(\text{histone H1}\)

\[\text{IgG} \quad \alpha\text{p21Cip1}\]

\[\text{immunodepletion}\]

\(\text{p21Cip1}\)

c)

\[\text{IgG} \quad \alpha\text{p21Cip1}\]

\[\text{immunodepletion}\]

Figure 2.5

\[\text{Luciferase activity (arbitrary units)}\]

\[\text{R1881 (500 nM)}\]
Figure 2.6 p21$^{Cip-1}$ is responsible for Raf-dependent inhibition of growth in MEFs

Analysis of RafAR function in knockout MEFs. wt MEFs null for p53 (p53/-), p21$^{Cip-1}$ (p21$^{Cip-1}$/-), or Cyclin D1 (D1/-) were infected with pLXSN3 RafAR retrovirus. Subconfluent polyclonal populations of puromycin resistant cells growing in 10% foetal bovine serum (FBS) were treated with ethanol (0.01%) or 500 nM R1881 for 16 h:

a) Growth inhibition after activation of RafAR. DNA synthesis was measured by incubation with tritiated thymidine for 2 h prior to harvesting and assaying for incorporation.

b) Induction of p21$^{Cip-1}$ and Cyclin D1 after hormone treatment. 50 µg of protein from lysates of knockout MEFs were separated by SDS-PAGE and analysed by Western blotting for expression of p21$^{Cip-1}$ and Cyclin D1 with sc-757 and 287.3 antibodies respectively.

This experiment was performed by A.Sewing
Figure 2.6

Thymidine incorporation (cpm)

- WT
- p53-/-
- D1-/-
- p21-/-

RNAi (500 nM)
Figure 2.7 RafAR is able to stimulate DNA synthesis in a dose-dependent manner

a) RafAR is able to stimulate different levels of ERK activity. Confluent cultures of RafAR cells were incubated in DMEM with 0.1% stripped NCS for 48 h. Cells were then stimulated with the indicated concentrations of testosterone for 16 h. Cell lysates were taken and p42\(^{Erk2}\) was immunoprecipitated and its kinase activity was tested with myelin basic protein (MBP) as the substrate.

b) RafAR can induce DNA synthesis in a dose-dependent manner. Confluent cultures of RafAR cells were incubated in DMEM with 0.1% stripped NCS for 48 h. Cells were then stimulated with the indicated concentrations of testosterone for 18 h. DNA synthesis was measured by incubation with tritiated thymidine 2h prior to harvesting and assaying for incorporation.

c) RafAR differentially upregulates p21\(^{Cip1}\) and Cyclin D1 expression. Confluent cultures of RafAR cells were incubated in DMEM with 0.1% stripped NCS for 48 h. Cells were then stimulated with the indicated concentrations of testosterone for 16 h. Cell lysates were taken and 30 \(\mu\)g of protein were separated by SDS-PAGE and western blotted, the membrane was cut into two pieces and then analysed for expression of Cyclin D1 and p21\(^{Cip1}\) with 287.3 and sc-757 antibodies respectively.

This experiment was performed by A.Sewing.
Figure 2.7

a) MBP

b) 3H Thymidine Incorporation (cpm)

Testosterone (nM)

0 1 2 5 10 20 50 500

Testosterone (nM)

0 2 10 50 500

c) Cyclin D1

p21^{Cip1}

Testosterone (nM)
Figure 2.8 Activation of $\Delta$Raf:ER provokes a similar response to RafAR

a) $\Delta$Raf:ER consists of the CRIII domain of human c-Raf-1 (aa 305 to 648) fused to the amino terminus of an EcoRI/Clal fragment consisting of the hormone binding domain of the HE14 allele of the human oestrogen receptor (Samuels et al., 1993).

b) $\Delta$Raf:ER activates ERK phosphorylation in a dose-dependent manner. Confluent cultures of $\Delta$RER cells were incubated in DMEM containing 0.25% NCS for 36 h. Fresh media with 0.25% or 10% NCS with indicated concentrations of $\beta$-oestradiol was added for 30 min. Cells were then lysed and proteins were separated on a MAP kinase shift gel by SDS-PAGE and analysed sequentially for total p42$^{Erk}$ and phosphorylated p42$^{Erk}$ and p44$^{Erk}$ by Western blotting with 122.2 and phospho p42/44 MAP kinase antibodies respectively.

c) $\Delta$Raf:ER induces mitogen-independent DNA synthesis in a dose-dependent manner. Confluent cultures of $\Delta$RER cells were incubated in DMEM containing 0.25% NCS for 36 h. Cells were then incubated in fresh media containing 0.25% or 10% stripped NCS as indicated, the indicated concentrations of $\beta$-oestradiol or 0.01% ethanol and tritiated thymidine for 22 h. The cells were then assayed for tritiated thymidine incorporation.

d) $\Delta$Raf:ER causes inhibition of cell cycle progression. Subconfluent cultures of $\Delta$RER/ARlf cells (described in Chapter 3) were cultured in DMEM with 10% stripped NCS. Fresh media containing 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) was added to the cells for 16 h. DNA synthesis was measured by the addition of tritiated thymidine 3 h prior to harvest and analysis.
Figure 2.8

a) CR3 Raf-1 HBD huER

b) pp42ERK2 p42ERK2 ppERK2 ppERK1

\[
\begin{array}{c}
\text{p-oestrodiol (nM)} \\
0 & 0.1 & 1.0 & 5.0 & 50.0 & 500.0 & 10% NCS
\end{array}
\]

\[
\begin{array}{c}
\text{NCS} \\
0 & 0.5 & 1.0 & 1.25 & 2.5 & 5.0 & 500.0
\end{array}
\]

c) 3H Thymidine Incorporation (cpm)

\[
\begin{array}{c}
\text{3H Thymidine Incorporation (cpm)} \\
0 & 2 & 4 & 6 & 8 & 10 & 12 & 14 & 16
\end{array}
\]

\[
\begin{array}{c}
\text{4-OHT (nM)} \\
0 & 25 & 50 & 100
\end{array}
\]
Figure 2.9 Tetracycline repressible retrovirus, pBPSTR1

The pBPSTR1 retrovirus consists of a cytomegalovirus promoter (CMV) containing tet operator (tetO) sequences upstream of a cDNA of the gene to be induced (gene X). A simian virus 40 promoter (SV40) drives expression of a VP16-tetracycline repressor fusion protein (tTA) which is a transcriptional activator. In the absence of tetracycline or doxycycline tTA binds to the tetO sequences and activates transcription from the CMV promoter. Tetracycline and doxycycline bind tTA and prevent its interaction with tetO sequences. The puromycin resistance gene is regulated by the long terminal repeat (LTR) of the retrovirus.
Figure 2.9

Repression in the presence of Dox/Tet

Activation in the absence of Dox/Tet
**Figure 2.10 RafCAAX expression is inducible in STRCX clones**

Clones of STRCX were selected in the presence of doxycycline (100 ng/ml) and 2.5 μg/ml puromycin. Two dishes of each clone were cultured in the presence or absence of doxycycline (100 ng/ml) in DMEM with 10% NCS for 72 h. Cells were harvested and 50 μg of protein lysate was separated by SDS-PAGE and analysed by Western blot for expression of RafCAAX using the 9E10 antibody.

**Figure 2.11 RafCAAX expression inhibits DNA synthesis**

Subconfluent of STRCX clones 6, 11, 15 and 16 and ST cells were incubated in DMEM with 10% NCS in the presence or absence of doxycycline (100 ng/ml) for 46 h. DNA synthesis was measured by addition of tritiated thymidine for 3 h prior to harvest and assaying for incorporation.
Figure 2.10

![Western blot images with lanes labeled 1 to 18, showing varying intensities of the target protein RafCAAX, with Dox (100 ng/ml) treatment indicated by + and - signs.]

Figure 2.11

![Bar graph showing 3H Thymidine Incorporation (cpm) for samples RCX 6 to ST, comparing + Dox and - Dox conditions with error bars indicating variability.]*
Figure 2.12 RafCAAX induction of DNA synthesis is dose dependent

a) RafCAAX expression levels are regulatable. Confluent cultures of STRCX 6 cells were incubated in DMEM containing 0.25% serum and doxycycline (100 ng/ml) for 48 h. The doxycycline was washed out and fresh media with the indicated concentrations of doxycycline was applied to the cells for 28 h. Photographs of the cells were taken prior to harvesting. 50 μg of total protein was separated by SDS-PAGE and analysed by Western blot. Membranes were sequentially probed for RafCAAX and endogenous Raf-1 using 9E10 and sc-227 respectively.

b) RafCAAX induces DNA synthesis in a dose-dependent manner. Confluent cultures of STRCX 6 cells were incubated in DMEM containing 0.25% serum and doxycycline (100 ng/ml) for 48 h. The doxycycline was washed out and fresh media containing tritiated thymidine and the indicated concentrations of doxycycline was applied to the cells for 28 h. Cells were assayed for tritiated thymidine incorporation
Figure 2.12

a)  

```
Dox (ng/ml)  100  0  100  5  1  0.5  0.1  0
ST          0   0   0   0   0   0   0   0
STRCX 6     0   0   0   0   0   0   0   0
```

Raf CAAX
Raf-1

Dox (100 ng/ml)  No Dox

b)  

```
Dox (ng/ml)  100  5  1  0.5  0.1  0
3H Thymidine Incorporation (cpm)  2000  2000  4000  4000  4000  4000
```

No Dox
Figure 2.13 Ras\textsuperscript{V12} expression is inducible in STRas clones

Clones of STRas were selected in the presence of doxycycline (100 ng/ml) and 2.5 μg/ml puromycin. Two dishes of each clone were cultured in the presence or absence of doxycycline (100 ng/ml) in DMEM with 10% NCS for 72 h. Cells were harvested and 30 μg of protein lysate was separated by SDS-PAGE and analysed by Western blot for expression of Ras using the Y13-259 antibody.
Figure 2.13

Dox (100 ng/ml)  

+  -  +  -  +  -  +  -  +  

Ras
Figure 2.14 Ras$^{V12}$ expression inhibits DNA synthesis

Sparse cultures of STRas c18 and ST cells were incubated in DMEM with 10% NCS in the indicated concentrations of doxycycline for 65 h.

a) Ras$^{V12}$ expression inhibits serum-stimulated DNA synthesis. DNA synthesis was measured by the addition of tritiated thymidine to the cells 4 h prior to harvesting.

b) Ras$^{V12}$ expression levels are regulatable and induce p21$^{Cip-1}$. Lysates were taken and 30 μg of protein was separated by SDS-PAGE and analysed by Western blot. Membranes were sequentially probed for Ras and p21$^{Cip-1}$ using pan-ras and sc-397 antibodies respectively.

c) Ras$^{V12}$ expression retards cells in G1. Cells were trypsinised, fixed in 70% ethanol, permeabilised and stained with propidium iodide. The DNA content of the cells was then analysed by fluorescence activated cell scanning.
Figure 2.14

a) 3H Thymidine Incorporation (cpm)

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</tbody>
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b) Western Blot

- Ras
- p21^{Cip1}

<table>
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<th>ST</th>
</tr>
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C) Table

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Figure 2.15 Ras\textsuperscript{V12} expression induces mitogen-independent DNA synthesis

a & b) Ras\textsuperscript{V12} expression is induced at 8-10 h. Subconfluent cultures of STRas c18 cells in DMEM with 10% NCS and doxycycline (100 ng/ml) had doxycycline washed away and were incubated in its absence for the indicated time periods. Cells were then harvested and 30 \(\mu\)g of protein was separated by SDS-PAGE and analysed by Western blot for expression of Ras using the pan-ras antibody. For (b) the membrane was stripped and re-probed for p21\textsuperscript{CIP1} expression with sc-397 antibody.

c) Ras\textsuperscript{V12} induction stimulates p42\textsuperscript{ERK2} kinase activity. Confluent cultures of STRas c18 cells were incubated in DMEM with 0.25% NCS in the presence of doxycycline (100 ng/ml) for 48 h. Doxycycline was then gently washed out of the cells and the cells were harvested at the indicated time points. 100 \(\mu\)g of protein was immunoprecipitated with 122.2 antibody and immunocomplexes were analysed for p42\textsuperscript{ERK} kinase activity using MBP as a substrate.

d) Ras\textsuperscript{V12} expression induces DNA synthesis in the absence of mitogens. Confluent cultures of STRas c18 cells were incubated in the presence of doxycycline (100 ng/ml) in either DMEM alone or DMEM with 0.25% NCS for 48 h. Doxycycline was washed out of the cells and the cells were incubated for the indicated time periods in the absence of doxycycline. Tritiated thymidine was added for 2 h prior to harvesting for analysis of DNA synthesis.
Figure 2.15

a) Minus ras c18

b) p21Cip1

c) MBP

d) Thymidine Incorporation (cpm)

Minus Dox (h)
Figure 2.16 Ras\(^{\text{V12}}\) and RafCAAX differentially upregulate p21\(^{\text{Cip-1}}\)

Confluent cultures of STRas c18 cells or STRCX cells (as indicated) were incubated in the presence of doxycycline (100 ng/ml) in DMEM with 0.25% NCS for 48 h. Doxycycline was washed out of the cells and the cells were incubated for the indicated time periods in the absence of doxycycline.

a) Ras\(^{\text{V12}}\) induces a higher level of DNA synthesis than RafCAAX. Tritiated thymidine was added for 2 h prior to harvesting for analysis of DNA synthesis. The figure shows fold increase in DNA synthesis over background since the STRas and STRCX experiments were carried out at different times.

b) Western lysates were taken and 50 µg of protein were separated by SDS-PAGE and analysed by Western blot. Membranes were probed for Ras, p21\(^{\text{Cip-1}}\) and Cyclin D1 expression with pan ras, sc-397 and 287.3 antibodies respectively.
Figure 2.16

a) 

Fold increase in thymidine incorporation/cell

Time minus Dox (h)

b) 

Stras

Ras

p21Cip1

StrCX

p21Cip1

Cyclin D1

β-tubulin
Figure 2.17 Ras\textsuperscript{V12} downregulates p21\textsuperscript{Cip-1} induction by ΔRaf:ER

a) Expression of Ras\textsuperscript{V12} reduces p21\textsuperscript{Cip-1} expression and induction. Confluent cultures of ΔRER/Ras\textsuperscript{V12} and ΔRER/ST cells were incubated in 0.25% stripped NCS for 24 h. Cells were then trypsinised and seeded subconfluently on new dishes in DMEM with 0.25% DMEM for 24 h. Fresh media was then applied containing 10% stripped NCS and serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) for 24 h. Cell lysates were taken and 40 μg of protein was separated by SDS-PAGE and analysed by Western blot for expression of Ras, p21\textsuperscript{Cip-1}, Cyclin D1 and β-tubulin as a loading control with pan ras, sc-6246, 287.3 and T4026 antibodies. In addition, 10 μg of protein was separated on a MAP kinase shift gel and analysed for p42\textsuperscript{Erk2} phosphorylation with 122.2 antibody.

b) Induction of DNA synthesis by Ras\textsuperscript{V12} is inhibited by ΔRaf:ER. Confluent cultures of ΔRER/Ras\textsuperscript{V12} and ΔRER/ST cells were incubated in 0.25% stripped NCS for 24 h. Cells were then trypsinised and seeded subconfluently on new dishes in DMEM with 0.25% DMEM for 24 h. Fresh media was then applied containing 0.25% stripped NCS and serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) for 24 h. BrdU (10 μM) was applied to the cells 4 h prior to harvesting the cells for analysis of DNA synthesis by FACS. Cells were fixed in 70% ethanol and stained with propidium iodide (PI) for detection of total DNA and with anti-BrdU-FITC conjugated antibody for measurement of DNA synthesis by FACS analysis.
Figure 2.17

a) 

4-OHT nM

0 1 10 100

\[ \text{ARER/Ras}^{V12} \]

\[ \text{ARER/ST} \]

Ras

ppERK2

ERK2

p21^{Cip-1}

Cyclin D1

\[ \beta\text{-tubulin} \]

b) 

Percentage BrdU Incorporation

0 2 4 6 8 10

0 1 10 100

4-OHT nM

\[ \Delta\text{RER/ST} \]

\[ \Delta\text{RER/Ras} \]
3. The Effects of PI 3-kinase, Rlf and RhoA on Raf-1

Regulation of the Cell Cycle
3.1 Introduction

In the previous chapter we have observed that RasV12 has a greater ability to induce DNA synthesis than Raf-1. We have proposed that the differences may be caused by the ability of Ras to activate a Raf-independent pathway which downregulates p21Cip-1 expression induced by Raf-1 activation.

In addition to the Raf family, two other protein families have met the criteria required to be considered genuine Ras effectors. PI 3-kinases and Ral-GEFs are both able to bind Ras in a GTP-dependent manner, they are able to reproduce downstream effects of Ras, and interference with their function inhibits cellular responses to Ras activation (Campbell et al., 1998). This was discussed at length above. Furthermore, both have been shown to have a mitogenic influence on the cell. Intact PI 3-kinase binding sites are required on the PDGF receptor for PDGF stimulation of DNA synthesis (Valius and Kazlauskas, 1993). Moreover, dominant negative PI 3-kinase mutants, neutralising PI 3-kinase antibodies or specific chemical inhibitors to PI 3-kinase are able to inhibit growth factor and serum-stimulated DNA synthesis (Cheatham et al., 1994; Jhun et al., 1994; Roche et al., 1994). They are also able to inhibit Ras-mediated transformation (Rodriguez-Viciana et al., 1997). Moreover, a constitutively active mutant of PI 3-kinase has been shown to support mitogen-independent DNA synthesis (Frevert and Kahn, 1997). Constitutively active Ral-GDS like factor (Rlf), has been shown to promote colony formation in reduced mitogen conditions (Wolthuis et al., 1997). A dominant negative mutant of Ral was also able to inhibit Ras-mediated transformation (Urano et al., 1996). In addition, experiments with Ras effector domain mutants have demonstrated that Ras-mediated DNA synthesis was dependent on activation of at least two effector pathways (Joneson et al., 1996b). This supports our hypothesis that Raf-1 requires additional Ras signals to efficiently induce DNA synthesis.

Therefore the ability of PI 3-kinase and RalGEFs to alter the cellular response to Raf-1 activation was tested to determine whether they could counteract Raf-1 induction of p21Cip-1 and increase mitogenic stimulation by Raf-1.

3.2 Results

3.2.1 Cell Cycle Regulation by Phosphatidylinositol-3' Kinase and Raf-1

To investigate the effect of PI 3-kinase on cell growth, cell-lines with constitutive PI 3-kinase activity were generated from ΔRER cells. Mammalian expression vectors, pEFBos rCD2p110 and pEFBos rCD2p110R-P (a kind gift from D.Cantrell) were stably co-transfected with the selection marker pJ6Puro into ΔRER cells and pools of puromycin
resistant ΔRER/p110 and ΔRER/p110R-P cells were generated. rCD2p110 is a fusion protein comprising of the p110 subunit of PI 3-kinase and the transmembrane domain of the rat CD2 cell surface antigen (Reif et al., 1996). The rCD2 domain serves to localise the p110 domain to the intracellular membrane causing constitutive activation (Reif et al., 1996). A control cell-line was also generated that expressed a kinase-inactive mutant, rCD2p110R-P, which had a single point mutation (R1130P) in its kinase domain (Dhand et al., 1994).

We tested whether PI 3-kinase activity could increase the efficiency of Raf-mediated DNA synthesis initiation and downregulate p21^Cip-1. Serum-deprived, sub-confluent, quiescent cultures of ΔRER/p110 and ΔRER/p110R-P cells were stimulated with 4-OH tamoxifen or ethanol at the indicated concentrations for 30 h and their DNA synthesis was measured by a tritiated thymidine incorporation assay. In addition cell lysates were taken, separated by SDS-PAGE and analysed by Western blot for expression of rCD2p110 fusions, Cyclin D1, p21^Cip-1, p27^Cip-1 and for ERK phosphorylation.

Expression of rCD2p110 and rCD2p110R-P was detected in ΔRER/p110 and ΔRER/p110R-P cells (Figure 3.1a). However, expression of rCD2p110R-P was very low compared to rCD2p110. The low expression may be due to a growth inhibitory effect of the mutant p110 molecule, possibly through sequestration, however a similar number of ΔRER/p110R-P puromycin resistant colonies were obtained as compared to the ΔRER/p110 transfectants. The degree of ERK phosphorylation acts as an indicator of ΔRaf:ER activity. ERK proteins were not phosphorylated in the absence of 4-OH tamoxifen and ERK phosphorylation was not detected in 1 nM 4-OH tamoxifen. However, addition of 10 nM 4-OH tamoxifen stimulated ERK phosphorylation and 100nM 4-OH tamoxifen resulted in the highest phosphorylation. There was no difference in the degree of phosphorylation between ΔRER/p110 and ΔRER/p110R-P cells, which indicated that PI 3-kinase activation did not increase ΔRaf:ER activation of ERK.

ΔRER/p110 cells had a two fold higher basal level of DNA synthesis than control cells which was further activated by stimulation of ΔRaf:ER with 1 nM 4-OH tamoxifen. However the combined induction of DNA synthesis by ΔRaf:ER and PI 3-kinase activation was only equivalent to the sum of the effect of each individually. In addition, ΔRER/p110 cells demonstrated a degree of resistance to maximal ΔRaf:ER activation and maintained a higher level of DNA synthesis than control cells in the same concentration of hormone (Figure 3.1b).

Unexpectedly, PI 3-kinase co-operated with Raf-1 to induce p21^Cip-1 expression. 1 nM 4-OH tamoxifen was insufficient to induce p21^Cip-1 by activation of ΔRaf:ER alone, however it could induce p21^Cip-1 in the presence of PI 3-kinase activity (Figure 3.1a). This indicated that PI 3-kinase changed the threshold of Raf activity required for p21^Cip-1
expression. In addition, the presence of PI 3-kinase activity allowed strong ΔRaf:ER activation to induce much higher expression levels of p21^{\text{Gip}} than ΔRaf:ER alone.

In spite of the co-operative induction of p21^{\text{Gip}} by PI 3-kinase in response to ΔRaf:ER activation, this did not prevent induction of DNA synthesis. Indeed weak activation of ΔRaf:ER by 1 nM 4-OH tamoxifen in the presence of PI 3-kinase stimulated more DNA synthesis than Raf alone even though p21^{\text{Gip}} has been induced in the ΔRER/p110 cells and not the control cells (Figure 3.1a and b). Furthermore cells treated with 10 nM 4-OH tamoxifen prevented DNA synthesis in cells with ΔRaf:ER alone while cells in the presence of PI 3-kinase activity had higher DNA synthesis levels. However much more p21^{\text{Gip}} was expressed in the presence of PI 3-kinase. This shows that p21^{\text{Gip}} concentration does not necessarily correlate with cell cycle progression.

To understand how PI 3-kinase induced DNA synthesis we examined the relative levels of Cyclin D1 and p27^{\text{Kip}}. The presence of PI 3-kinase activity increased the expression levels of Cyclin D1 and reduced expression of the cell cycle inhibitor p27^{\text{Kip}}. This may explain the ability of cells in the presence of PI 3-kinase activity alone to induce DNA synthesis. However ΔRaf:ER activation also reduced p27^{\text{Kip}} levels, which went down as p21^{\text{Gip}} level were induced, therefore, the reduction in p27^{\text{Kip}} could not explain the superior mitogenic ability of PI 3-kinase in the presence of strong Raf activity. In addition, Cyclin D1 levels in response to strong Raf activation are equivalent in the presence or absence of PI 3-kinase, which eliminated the possibility that Cyclin D1 was sequestering the extra p21^{\text{Gip}}. However another factor may be able to sequester the excess p21^{\text{Gip}}; the relative kinase activities of the cyclin-CDK complexes would need to be determined to investigate this further.

We also assessed whether in addition to inducing DNA synthesis in the absence of mitogens, PI 3-kinase was able to attenuate Raf-mediated inhibition of DNA synthesis in the presence of serum, since we had specifically demonstrated that this was dependent on p21^{\text{Gip}}. ΔRER/p110 and ΔRER/p110R-P cells were cultured at subconfluence in 10% NCS with the indicated concentrations of 4-OH tamoxifen for 16 h. Their DNA synthesis was measured by tritiated thymidine incorporation analysis. Unstimulated ΔRER/p110 and ΔRER/p110R-P cells had a similar level of DNA synthesis in the presence of NCS (Figure 3.1c). The presence of PI 3-kinase activity could attenuate, but not prevent ΔRaf:ER-mediated inhibition of DNA synthesis.

In conclusion, PI 3-kinase was unable to mimic the downregulation of p21^{\text{Gip}} by Ras, which suggests Ras does not use this pathway to affect p21^{\text{Gip}} levels. Indeed PI 3-kinase and Raf-1 co-operatively induced p21^{\text{Gip}}. However PI 3-kinase demonstrated mitogenic potential which correlated with downregulation of p27^{\text{Kip}} and induction of Cyclin D1. Furthermore PI 3-kinase was able to increase the ability of weak Raf activity
to induce DNA synthesis and also provided some resistance to inhibition of DNA synthesis by strong Raf-1 activation.

3.2.2 The effect of Raf-1 activation in the presence of RalGEF activity

Another family of Ras effectors are the RalGEFs. The effect of constitutive activation of this pathway was investigated to test whether it could attenuate the cellular response to ΔRaf:ER activation. Due to a possibility of synergy between RalGEFs and PI 3-kinase these experiments were carried out in the presence or absence of PI 3-kinase activity.

We used a constitutively activated form of the RalGEF family member, Rif in which the activation domain of Rif had been fused to the CAAX motif of Ki-Ras to localise the protein to the membrane (Wolthuis et al., 1996). This protein is referred to as RifCAAX. An inactive version of RifCAAX that has had its transactivation domain deleted (Wolthuis et al., 1996) was used as a control and is referred to as ΔRlfCAAX. Both proteins were fused to HA epitope tags for detection purposes. ΔRER cells were stably co-transfected with mammalian expression vectors, pmt RifCAAX or pmt ΔRlfCAAX (Both plasmids were kind gifts from J. Bos) and pEFBos rCD2p110 or pEFBos rCD2p110R-P and the selection marker pJ6Puro. These cells were selected for resistance to puromycin and four new cell lines, ΔRER2, ΔRER2/Rlf, ΔRER2/p110 and ΔRER2/Rlf/p110 were generated. These cell-lines were tested for protein expression. Lysates from each cell-line was subject to immunoprecipitation with the HA antibody and immunocomplexes were separated by SDS-PAGE and analysed by Western blotting with the HA antibody. RifCAAX was detected in ΔRER2/Rlf and ΔRER/Rlf/p110 cells, however ΔRlfCAAX was not detected in ΔRER2 or ΔRER2/p110 cells (Figure 3.2a). The lack of detectable expression may be due to a defect in the expression plasmid (although this plasmid gives the expected restriction enzyme digest pattern). It could also indicate that the protein acted in a dominant negative manner that could not be tolerated in the cells. However a similar number of ΔRER2 or ΔRER2/p110 puromycin resistant colonies were obtained as compared to the ΔRER2/Rlf and ΔRER/Rlf/p110 transfectants. Total cell lysates were also analysed for rCD2p110 expression by Western blotting. rCD2p110 was expressed in ΔRER2/p110 and ΔRER2/Rlf/p110 cells. However as in ΔRER/p110R-P cells above, expression of rCD2p110R-P was very low (Figure 3.2b).

The ability of these molecules to promote mitogen-independent DNA synthesis was tested in response to ΔRaf:ER activation. Serum-deprived, confluent cultures of ΔRER2, ΔRER2/Rlf, ΔRER2/p110 and ΔRER2/Rlf/p110 cells were stimulated with the indicated concentrations of 4-OH tamoxifen or ethanol for 30 h and their DNA synthesis was assessed by a tritiated thymidine incorporation assay.
In the absence of ∆Raf:ER activation, RlfCAAX induced a degree of mitogen-independent DNA synthesis, however this was very low compared to unstimulated ∆RER2/p110 cells expressing activated PI 3-kinase (Figure 3.2c). Expression of RlfCAAX did not significantly assist induction of DNA synthesis by weak ∆Raf:ER activation. Furthermore, it provided no protection against inhibition of DNA synthesis by strong Raf-1 signals.

Interestingly, in the absence of ∆Raf:ER activation, Rif activity co-operated with PI 3-kinase activity to induce a higher level of mitogen-independent DNA synthesis than PI 3-kinase alone or in combination with activated ∆Raf:ER. Thus Rif did have mitogenic ability, but only in combination with PI 3-kinase, not Raf. This implied that activation of Raf-independent Ras effector pathways were sufficient for DNA synthesis and that activation of Rif was permissive for PI 3-kinase stimulation of DNA synthesis. However, since both ∆RER2/p110 and ∆RER2/Rlf/p110 cells had similar DNA synthesis levels when treated with 10 and 100 nM 4-OH tamoxifen, the presence of Rif in addition to PI 3-kinase activity provided no more protection against Raf-mediated inhibition of DNA synthesis than PI 3-kinase alone.

Therefore it appeared that the RalGEF effector pathway could not attenuate DNA synthesis inhibition by ∆Raf:ER in the presence or absence of PI 3-kinase activity and so was not investigated further.

3.2.3 Rho signals and Raf upregulation of p21^{Cip-1}

The small GTPase RhoA has recently been proposed to have a vital role in Ras^{V12} induction of DNA synthesis. Olson and colleagues (1998) have demonstrated that in contrast to NIH 3T3 cells, microinjection of Ras^{V12} did not induce DNA synthesis in quiescent Swiss 3T3 cells. Quiescent Swiss 3T3 cells are thought to have no basal level of Rho activity because they are devoid of stress fibres, whereas quiescent NIH 3T3 cell contain stress fibres. Activation of Rho in quiescent Swiss 3T3 fibroblasts, by co-microinjection of RhoA^{V14} or application of *E.coli* cytotoxic necrotising factor-1 (CNF-1) which selectively stimulates Rho activity (Flatau *et al.*, 1997; Schmidt *et al.*, 1997), was shown to downregulate Ras-induced p21^{Cip-1} expression and allow induction of DNA synthesis. Furthermore, Ras^{V12} induced cell cycle progression in NIH 3T3 cells or serum-induced DNA synthesis in Swiss 3T3 cells was sensitive to the RhoA specific inhibitor, C3 transferase, which permitted induction of p21^{Cip-1} (Olson *et al.*, 1998). Thus Rho activity was able to inhibit Ras^{V12}-mediated upregulation of p21^{Cip-1} which then permitted a mitogenic response to Ras^{V12}.

The differences we observe between the ability of Ras^{V12} and Raf-1 activation to regulate p21^{Cip-1} may therefore be related to their relative abilities to induce Rho activity. Raf and
Rho signalling are thought to activate separate pathways because their co-activation leads to synergistic focus formation. In addition many possible mechanisms have been proposed that would permit Ras regulation of Rho activity. For example, the RasGAP, p120^GAP binds directly to the RhoGAP, p190^GAP (Settleman et al., 1992). In addition, RhoGEFs such as Vav are regulated by events which can be mediated by Ras, such as PI 3-kinase activity (Han et al., 1998). Ras^V12 has also been demonstrated to increase the nucleotide exchange activity of the RasGEF, Sos on the Rho family member, Rac1 (Nimnual et al., 1998). A link between the Ras effector family, the RalGEFs and Rho family proteins has also been implied since Ral binding protein 1 (RalBP1/RLIP1/RIP1) has been shown to have GAP activity towards Cdc42 and Rac1, but not RhoA (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). Furthermore, a function for RhoA downstream of Ras is suggested because dominant negative mutants of Rho have been shown to inhibit Ras-mediated focus formation (Khosravi-Far et al., 1995; Prendergast et al., 1995; Qiu et al., 1995b). These data taken in relation to the differences in p21^Cip-1 induction between activated Ras and Raf-1 in our experiments, support a model in which Ras^V12 could stimulate Rho activity in NIH 3T3 cells through a Ras effector pathway distinct from Raf which would inhibit Raf-mediated induction of p21^Cip-1. However, since serum induces Rho activity and we have observed induction of p21^Cip-1 and inhibition of DNA synthesis by Ras and Raf-1 activation in the presence of serum Rho may have a more subtle role for example, delaying the onset of p21^Cip-1 expression.

To investigate the possibility of RhoA involvement, a new cell-line was generated. ARER cells were stably co-transfected with a mammalian expression vector encoding a constitutively activated RhoA mutant, RhoA^V14 with a myc tag, pLink RhoA^V14 or the empty vector and the selection marker pJ6Puro. Cells were selected for resistance to puromycin and polyclonal populations were obtained and are referred to as ARER/Rho or ARER/plink.

The effect of RhoA activity on Raf-1-mediated induction of p21^Cip-1 was then tested. In addition we assessed whether activated RhoA would affect the ability of ΔRaf:ER to stimulate DNA synthesis. Quiescent, subconfluent, serum-deprived ARER/Rho and ARER/plink cells were stimulated with the indicated concentrations of 4-OH tamoxifen for 30 h. Western lysates were separated by SDS-PAGE and immunoblotted. DNA synthesis was measured by both tritiated thymidine and BrdU incorporation.

Expression of RhoA was detected by Western blotting using the 9E10 antibody to detect the Myc tag (Figure 3.3). Cyclin D1 levels were induced in unstimulated ARER/Rho cells compared to ARER/plink cells indicating that RhoA was active. This was also evident from the exaggerated transformed morphology these cells demonstrated upon
ΔRaf:ER activation in comparison to ΔRaf:ER activation alone. A similar morphological change was observed in cells expressing both activated Ras and RhoA (Khosravi-Far et al., 1995). However the significance of this change is unclear since the contribution of morphological alterations to transformation has not yet been explained.

In contrast to our expectations RhoA co-operated with Raf to induce p21^{Cip1}. This effect was very similar to the response of these cells to constitutive activation of PI 3-kinase. However, the presence of RhoA activity did not induce DNA synthesis and did not assist DNA synthesis induction by ΔRaf:ER. Indeed, in response to strong ΔRaf:ER activation with 100 nM 4-OH tamoxifen the degree of DNA synthesis was actually reduced in ΔER/Rho cells compared to ΔER/plink cells (Figure 3.3b and c). This may have been a response to the substantially higher levels of p21^{Cip1} in these cells compared to controls. The inability of RhoA to support DNA synthesis is consistent with reports which show RhoGEFs only poorly stimulate mitogen-independent growth (Schwartz et al., 1996). However this contrasts with experiments in which microinjection of activated RhoA was able to induce DNA synthesis (Olson et al., 1995).

We had observed a delay in the induction of p21^{Cip1} by Ras^{V12}, therefore we tested the rate of p21^{Cip1} induction by Raf-1 in the presence of RhoA to test whether RhoA delayed induction of p21^{Cip1} rather than inhibiting its expression. Serum-deprived, confluent ΔER/Rho and ΔER/plink cells were stimulated for the indicated time periods with 4-OH tamoxifen and then harvested. Cell lysates were separated by SDS-PAGE and analysed by Western blot. The presence of RhoA^{V14} accelerated p21^{Cip1} induction by ΔRaf:ER. 8 h after 4-OH tamoxifen addition p21^{Cip1} was absent from ΔER/plink cells, but was induced to a substantial degree in ΔER/Rho cells. p21^{Cip1} began to be induced in ΔER/plink cells by 12 h, but at a lower level than in the ΔER/Rho cells at either 8 or 12 h. In addition, the presence of RhoA activity was unable to prevent the reported transient upregulation of p21^{Cip1} by serum (Figure 3.3d) (Michieli et al., 1994).

RhoA induced Cyclin D1 expression in both unstimulated and stimulated ΔER/Rho cells. However, this was insufficient to stimulate DNA synthesis. The inability of RhoA^{V14} to stimulate mitogen-independent DNA synthesis may have been due to the high levels of p27^{Kip1} in the cells. ΔER/p110 cells also expressed a higher level of Cyclin D1 compared to control cells and superinduce p21^{Cip1} in a similar manner to the ΔER/Rho cells. However, in contrast to the ΔER/Rho cells, constitutive PI 3-kinase activity downregulated p27^{Kip1} expression. p27^{Kip1} levels were also downregulated by ΔRaf:ER activation and addition of serum. Therefore, downregulation of p27^{Kip1}, correlated with mitogenic signalling, and thus the inability of Rho to reduce p27^{Kip1} levels may have prevented initiation of DNA synthesis.
In addition, ΔRER/Rho cells had a higher level of ERK phosphorylation in the absence and presence of 4-OH tamoxifen. Rho signalling has been demonstrated to feed into the ERK MAP kinase module (Renshaw et al., 1997). However, since this did not occur if ΔRER/Rho cells were subconfluent, this may not be significant to the p21\(^{Cip-1}\) induction.

To test whether RhoA would behave differently in the presence of serum the ability of constitutively activated RhoA to attenuate ΔRaf:ER mediated cell cycle arrest was assessed in serum-stimulated asynchronously growing ΔRER/Rho and ΔRER/plink cells. However, similar to the experiments shown in Figures b and c, Rho did not alter Raf inhibition of DNA synthesis (Figure 3.3e).

Therefore, in contrast to its reported role, RhoA activation was unable to prevent upregulation of p21\(^{Cip-1}\) by ΔRaf:ER or by serum. Furthermore, it was unable to induce DNA synthesis or protect against DNA synthesis inhibition by ΔRaf:ER and actually reduced the low mitogenic ability of strongly activated ΔRaf:ER. This may be related to the superinduction of p21\(^{Cip-1}\) that occurred upon co-activation of RhoA and Raf-1 signals. These properties of RhoA\(^{V14}\) contrast its reported abilities upon microinjection or activation using CNF-1. This is possibly due to the different exposure of the cells to RhoA, caused by the different techniques of inducing Rho activity. Microinjection or CNF-1 treatment causes acute activation of RhoA, whereas our cells were exposed to constitutive RhoA activation which may also result in cellular adaptation. As we have demonstrated above the strength and duration of activation of a signal transduction pathway can have contrasting effects.

### 3.3 Summary

Although we have observed that Ras\(^{V12}\) can attenuate induction of p21\(^{Cip-1}\) by Raf, we have found no evidence from our experiments that constitutive activation of the PI 3-kinase and RafGEF Ras effector pathways can mimic the effects of Ras. Moreover, in contrast to its reported ability, constitutive expression of RhoA\(^{V14}\) does not suppress p21\(^{Cip-1}\) or counteract the effects of Raf. However, we have found that PI 3-kinase has mitogenic ability which may be related to its ability to downregulate p27\(^{Kip1}\) and induce Cyclin D1. Furthermore PI 3-kinase can increase the ability of Raf to induce DNA synthesis and surprisingly this occurs in the presence of high levels of p21\(^{Cip-1}\). In addition, although Rif is ineffectual as a mitogen when expressed alone, DNA synthesis is synergistically activated by PI 3-kinase and Rif. In contrast Rif is unable to alter the response of cells to Raf. Finally both PI 3-kinase and RhoA are able to co-operate with Raf-1 to induce p21\(^{Cip-1}\) which is contrary to the effect of Ras on Raf-1-mediated p21\(^{Cip-1}\) induction.
3.4 Figures - Chapter Three
Chapter Three - PI 3-kinase, Raf and Rho on Regulation the Cell Cycle by Raf-1

Figure 3.1 Activated Raf:ER and PI 3-kinase induce mitogen independent DNA synthesis

a) PI 3-kinase induces DNA synthesis alone and in co-operation with ΔRaf:ER. Subconfluent cultures of ΔRER/p110 and ΔRER/p110R-P cells were incubated in 0.25% stripped NCS for 36 h. Fresh media was then applied containing 0.25% stripped NCS and serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) and tritiated thymidine for 25 h. Cells were harvested and analysed for tritiated thymidine incorporation. In addition cells were counted and DNA synthesis was normalised for cell number.

b) Effect of PI 3-kinase activity and ΔRaf:ER activation on protein expression. Subconfluent cultures of ΔRER/p110 and ΔRER/p110R-P cells were incubated in 0.25% stripped NCS for 36 h. Fresh media was then applied containing 0.25% stripped NCS and serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) for 25 h. Cell lysates were taken and 25 µg of protein was separated by SDS-PAGE and analysed by Western blot for expression of rCD2, p21^{Cip1}, p27^{Kip1}, Cyclin D1, phosphorylated p42^{Erk2}/p44^{Erk1}, total p42^{Erk2} and β-tubulin as a loading control with anti-rCD2 sc-6246, sc-1641, 287.3, phospho p42/44ERK, 122.2 and T4026 antibodies respectively.

c) PI 3-kinase activity hinders ΔRaf:ER inhibition of DNA synthesis. Subconfluent cultures of ΔRER/p110 and ΔRER/p110R-P cells were incubated in 10% stripped NCS for 20 h. Fresh media was then applied containing 10% stripped NCS and the indicated concentrations of 4-OH tamoxifen or ethanol (0.01%) for 18 h. Tritiated thymidine was added to the cells 2.5 h prior to harvesting and analysing for thymidine incorporation.
Figure 3.1
a) RlfCAAX is expressed. ΔRER2, ΔRER2/Rlf, ΔRER2/p110 and ΔRER2/Rlf/p110 in DMEM with 10% NCS were harvested in Triton X-100 buffer. 400 µg of protein was immunoprecipitated with HA antibody overnight at 4°C. Immunocomplexes were then separated by SDS-PAGE and analysed by Western blotting with anti-HA antibody. Lane 1) ΔRER2; Lane 2) ΔRER2/Rlf/p110; Lane 3) ΔRER2/p110 cells; Lane 4) ΔRER2/Rlf cells

b) rCD2p110 protein is expressed. Confluent cultures of ΔRER2, ΔRER2/Rlf, ΔRER2/p110 and ΔRER2/Rlf/p110 cells were incubated in DMEM with 0.25% stripped NCS for 72 h. Cell lysates were taken and proteins were separated by SDS-PAGE and sequentially immunoblotted with anti rCD2 and 287.3 antibodies against rCD2p110 and Cyclin D1 respectively. Two different exposures of the rCD2 blot are shown. Lane 1) ΔRER2; Lane 2) ΔRER2/Rlf/p110; Lane 3) ΔRER2/p110 cells; Lane 4) ΔRER2/Rlf cells

c) DNA synthesis analysis of cell-lines in response to ΔRaf:ER activation. Confluent cultures of ΔRER2, ΔRER2/Rlf, ΔRER2/p110 and ΔRER2/Rlf/p110 cells were incubated in DMEM with 0.25% stripped NCS for 48 h. Fresh media was then applied containing 0.25% stripped NCS and serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%), for 25 h. To measure DNA synthesis tritiated thymidine was added to the cells 20 h prior to harvesting. Cells were assayed for thymidine incorporation. In addition cells were counted and DNA synthesis was normalised for cell number.
Figure 3.2

a) RlfCAAX

b) rCD2

non-specific band

Cyclin D1

1 2 3 4

1 2 3 4

14000
12000
10000
8000
6000
4000

ARER2 ARER2/Rlf

ARER2/p i

ARER2/Rlf

3H Thymidine Incorporation per cell

(absolute units)

∆RER2  ∆RER2/Rlf  ∆RER2/p i  ∆RER2/Rlf

0 nM 4-OHT  1 nM 4-OHT  10 nM OHT  100 nM OHT
Figure 3.3 The effects of Rho activity on Raf-mediated effects

a) Subconfluent cultures of ΔRER/Rho and ΔRER/plink cells growing in DMEM with 10% stripped NCS were treated with 100 nM 4-OH tamoxifen or ethanol (0.01%) for 18 h. DNA synthesis was measured by adding tritiated thymidine 3 h prior to harvesting and analysing for thymidine incorporation.

b, c and d) Confluent cultures of ΔRER/Rho and ΔRER/plink cells were incubated in 0.25% stripped NCS for 24 h. Cells were then trypsinised and seeded subconfluently on new dishes in DMEM with 0.25% DMEM for 24 h. Fresh media was then applied containing serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) and 0.25% or 10% stripped NCS if indicated for 30 h.

b) DNA synthesis was measured by addition of tritiated thymidine 4 h prior to harvesting the cells for analysis of thymidine incorporation.

c) BrdU (10 μM) was applied to the cells 4 h prior to harvesting the cells for analysis of DNA synthesis by FACS. Cells were fixed in 70% ethanol and stained with propidium iodide (PI) for detection of total DNA and with anti-BrdU-FITC conjugated antibody for detection of DNA synthesis.

d) Cell lysates were taken and 50 μg of protein was separated by SDS-PAGE and sequentially analysed by Western blot for expression of RhoA <sup>V14</sup>, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, Cyclin D1, phosphorylated p42<sup>Erk2</sup>/p44<sup>Erk1</sup>, total p42<sup>Erk2</sup> and β-tubulin as a loading control with 9E10, sc-6246, sc-1641, 287.3, DPERK, 122.2 and T4026 antibodies. Two different exposures of the p21<sup>Cip1</sup> Western blot are shown.

e) Confluent cultures of ΔRER/Rho and ΔRER/plink cells were incubated in 0.25% stripped NCS for 48 h. Fresh media was then applied containing serial dilutions of 4-OH tamoxifen at the indicated concentrations or ethanol (0.01%) and 0.25% or 10% stripped NCS if indicated for the indicated time periods. Cell lysates were taken and 50 μg of protein was separated by SDS-PAGE and sequentially analysed by Western blot for expression of RhoA <sup>V14</sup>, p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, Cyclin D1, phosphorylated p42<sup>Erk2</sup>/p44<sup>Erk1</sup>, total p42<sup>Erk2</sup> and β-tubulin as a loading control with 9E10, sc-6246, sc-1641, 287.3, DPERK, 122.2 and T4026 antibodies.
Figure 3.3
4. The Effect of Rac1 activation on the Cell Cycle
4.1 Introduction

Rac is a member of the Rho family of Ras-like small GTPases. Racl has been shown to be involved in actin and cytoskeletal reorganisation, membrane trafficking, activation of stress activated MAP kinase pathways, the generation of reactive oxygen species and regulation of transcription via the serum response element (SRE) and NFκB. Furthermore it is reported to have a role in the regulation of DNA synthesis and cellular transformation (Van Aelst, 1997; Zohn et al., 1998). Ras is believed to activate Rac since RasV12 induction of membrane ruffling is dependent on Rac function (Nobes et al., 1995; Ridley et al., 1992). Many putative mechanisms through which Ras may regulate Rac have been identified. These are summarised in Figure 1.7 and Figure 1.8. They include activation of Rac through the Ras effector, PI 3-kinase. This may occur through activation of RhoGEFs such as Vav and perhaps Sos which have been shown to be activated through pathways controlled by the lipid products of PI 3-kinase activity. In addition, Sos has GDP-GTP exchange activity towards both Rac and Ras through different domains. Ras may also regulate Rac through its RalGEF effector pathway since RBPl acts as a GAP for Rac and Cdc42.

Rac is also required for Ras transformation and co-operates with activated Raf-1 in focus formation assays (Khosravi-Far et al., 1995; Qiu et al., 1995a). This implies that Ras may mediate transformation through Rac and Raf through separate effector pathways. Furthermore, Rac is also required for serum stimulated DNA synthesis (Olson et al., 1995; Qiu et al., 1995a) and when constitutively activated has been shown to stimulate proliferation in the absence of adhesion (Khosravi-Far et al., 1995; Qiu et al., 1995a) or mitogens (Lamarche et al., 1996; Olson et al., 1995). Taken together with the requirement for Rac in Ras signal transduction, this implies that Rac has an important role as a downstream target of Ras in RasV12 induced DNA synthesis.

However, the mechanisms through which Rac regulates the cell cycle have not yet been elucidated. Therefore we have generated a cell-line in which activated Rac1 can be regulated to investigate the effect of Rac1 on the cell cycle.

4.2 Results

4.2.1 Generating cells encoding inducible Rac1V12

To generate a cell-line with inducible activated Rac1 a cDNA of constitutively activated Rac1 with a glycine to valine mutation at codon 12, (equivalent to the V12 mutation in Ras) was sub-cloned as a BamH1/EcoRV fragment from pEXV3 Rac1V12 (a kind gift from A.
Ridley) into the tetracycline regulatable retroviral vector described earlier, pBPSTR1 (Paulus et al., 1996) (Figure 2.8). This cDNA was tagged with the myc epitope that is specifically recognised by the 9E10 antibody (Evan et al., 1985). This construct will be referred to as pBPSTR1 Raci\textsuperscript{V12}. Attempts to make a stable GP+E cell-line producing BPSTR1 Raci\textsuperscript{V12} retrovirus were unsuccessful, therefore retrovirus was prepared from transiently transfected Bosc 23 cells. pBPSTR1 Raci\textsuperscript{V12} and pBPSTR1 viral supernatants were used to infect NIH 3T3 cells. They were then selected in the presence of doxycycline for resistance to puromycin. These cells will be referred to as STRAC and ST cells respectively. STRAC cells were both ring cloned and pooled. The STRAC polyclonal population was tested for its ability to regulate Raci\textsuperscript{V12} expression. Sub-confluent cells were grown in the presence or absence of doxycycline for 48 h in DMEM with 10% NCS. Cell lysates were separated by SDS-PAGE and analysed for expression of Raci\textsuperscript{V12} by Western blotting using the 9E10 antibody. We found that the pool expressed a very low level of Raci\textsuperscript{V12} in the presence of doxycycline which increased significantly upon removal of doxycycline from the media (Figure 4.1a). This indicated that Raci\textsuperscript{V12} expression in these cells was regulatable. Clones were then tested for Raci\textsuperscript{V12} expression and regulatability in the same way as the pool. Out of 51 clones tested, from two separate infection events, three regulatable clones were obtained (Figure 4.1b). The majority of clones did not express Raci\textsuperscript{V12} and a few others expressed a very low level of Raci\textsuperscript{V12}, but with no regulation. The three clones, which will be referred to as STRAC.C, STRAC.7 and STRAC.10, were used to investigate the effect of Raci\textsuperscript{V12} in NIH 3T3 cells.

Although Raci\textsuperscript{V12} has been reported to be a transforming oncogene in NIH 3T3 cells (Qiu et al., 1995a), we did not observe any morphological changes to STRAC cells upon removal of doxycycline. In the previous chapter induction of Ras\textsuperscript{V12} and RafCAAX in STRas and STRCX cells respectively, caused the cells to become spindly and refractile, features of morphological transformation in comparison with the uninduced cells (Figure 2.12). Therefore we had no confirmation that induction of Raci\textsuperscript{V12} in STRAC cells induced a cellular response. Raci\textsuperscript{V12} has been reported to stimulate transcription from the serum response element (SRE) (Hill et al., 1995), to activate the JNK MAP kinase (Minden et al., 1995), to induce mitogen- and anchorage-independent growth (Khosravi-Far et al., 1995; Olson et al., 1995; Qiu et al., 1995a) and to induce pinocytosis and formation of membrane ruffles (Ridley et al., 1992). We therefore tested whether induction of Raci\textsuperscript{V12} in the STRAC cells would elicit some of the reported biological effects of activated Rac.
4.2.2 Activation of the SRE by Rac

The serum response element (SRE) is a regulatory sequence found in many growth factor-regulated promoters, including that of \textit{c-fos} (Treisman, 1990). It is activated in response to serum, but also by other signals such as lysophosphatidic acid which signals through RhoA and AlF\textsubscript{4} which globally activates heterotrimeric G-proteins (Treisman, 1992; Treisman, 1994). Recent work has shown that transient transfection of Rac\textsuperscript{V12} in NIH 3T3 cells induces SRE activation from a co-transfected SRE reporter construct (Hill \textit{et al.}, 1995). Therefore the ability of Rac\textsuperscript{V12} to stimulate transcriptional activity from the SRE was tested in STRAC cells. Transient transfection assays were performed using a reporter construct p4\times SRE TKCAT (a kind gift from R.Treisman). This had a series of four serum response elements (CCCATATATGGG) inserted in front of a basic thymidine kinase (TK) promoter which was upstream of a chloramphenicol acetyl transferase (CAT) cDNA. SRE activation was tested in the three STRAC clones and ST cells. In addition, to ensure the SRE was able to respond to activated Rac1 in these cells, a mammalian expression vector encoding Rac1\textsuperscript{V12}, pEXV3 Rac1\textsuperscript{V12} or the empty vector were co-transfected. pEXV3 Rac1\textsuperscript{V12} had previously been shown to activate transcription from the SRE (Hill and Treisman, 1995). 16 h after transfection the cells were incubated in DMEM with 1\% NCS in the presence or absence of doxycycline for 48 h. To determine whether the SRE responded to serum in these cells and to provide a comparison for the degree of activation stimulated by Rac1\textsuperscript{V12}, 20\% NCS was added to control dishes of each cell type 12 h prior to harvesting. The cell lysates were then assayed for the ability to acetylate chloramphenicol by assessing CAT activity. Unexpectedly, neither STRAC cells from which removal of doxycycline had induced Rac1\textsuperscript{V12} nor cells which had been co-transfected with pEXV3 Rac1\textsuperscript{V12} stimulated any SRE transcriptional activation (Figure 4.2). In contrast, serum was able to stimulate transcription from the SRE to around 14 fold above basal levels, which indicates that the SRE reporter was functional and one pathway responsible for SRE activation was intact in these cells. This suggested that the SRE was unable to respond to Rac1\textsuperscript{V12} in these cells or possibly that a functional Rac1\textsuperscript{V12} was not being expressed from either the STRAC cells or the transfected vector.

We repeated the experiments in the NIH 3T3 cell-line that had been used in the published work to see if differences between the NIH 3T3 cell-lines could explain the difference we observed. A similar transient transfection assay was performed on our parental NIH 3T3 cell-line (referred to as NIH 1) and on the specific NIH 3T3 cells in which the work showing activation of the SRE by Rac1\textsuperscript{V12} had been performed (Hill \textit{et al.}, 1995), which for the purposes of these experiments, we named NIH 2 (a kind gift from R.Treisman). To control for differences in TK promoter activity and cell number in response to serum or Rac1\textsuperscript{V12}, we also used another pBLCAT2 derivative that has 4 yeast pheromone...
response elements (PRE) (ATGAAAC), in place of the SRE elements upstream of the TK promoter, p4xPRE TKCAT (also a kind gift from R. Treisman). The PRE is an element that responds to the yeast transcription factor STE 12, but has no basal activity in mammalian cells and is unresponsive to a variety of stimuli, such as serum, PDGF and anisomycin (personal communication E. Sahai). CAT activity stimulated in p4xPRE transfected cells was used to normalise SRE activity.

pEXV3 Rac1<sup>V12</sup> or the empty vector were co-transfected into NIH 1 and NIH 2 cells with p4xSRE TKCAT or p4xPRE TKCAT. The cells were maintained in DMEM with 1% NCS for 48 h and then harvested and assayed for CAT activity. If indicated, cells were stimulated by the addition of 20% NCS 12 h prior to harvesting. The resulting CAT activities were then normalised to give the fold increase in SRE-mediated transcriptional activation in response to serum or Rac1<sup>V12</sup> expression. In NIH 2 cells, both serum and Rac1<sup>V12</sup> stimulated transcriptional activity from the SRE in accordance with the published work (Figure 4.3a). However, SRE-mediated transcriptional activity in NIH 1 cells was only stimulated by serum and not by Rac1<sup>V12</sup> (Figure 4.3a). Thus it appears that there is a fundamental difference between the cell-lines and the NIH 1 cells are lacking a pathway linking Rac1<sup>V12</sup> to the SRE that is intact in NIH 2 cells.

To assess whether activation of the SRE by Rac1<sup>V12</sup> is a common property of rodent fibroblasts, we tested the activation of the SRE by Rac1<sup>V12</sup> and serum in a rat fibroblast cell-line, Rat 1 cells and in primary mouse embryo fibroblasts (MEFs), concurrently with both strains of NIH 3T3 cells. Either p4xSRE or p4xPRE were co-transfected with pEXV3 Rac1<sup>V12</sup> or the empty vector. Their CAT activity was determined after serum deprivation and after re-stimulation with serum if indicated.

The SRE in both Rat 1 cells and MEFs responded to serum stimulation; in Rat 1 cells it was stimulated 11 fold and in MEFs, 3 fold over cells transfected with p4xPRE (Figure 4.3b). However in both cell-types the SRE did not respond well to Rac1<sup>V12</sup> expression: transcription mediated by the SRE in Rat 1 cells increased by only 2 fold whereas it remained at background levels in MEFs.

Thus activation of the SRE by Rac1<sup>V12</sup> is not a general feature in fibroblasts. The inability of Rac1<sup>V12</sup> to reproduce the effects of serum indicates that Rac1 does not participate in a linear pathway between serum and the SRE. Furthermore it appears that stimulation of transcription from the SRE is not a vital aspect of Rac1 function in fibroblasts since NIH 1, Rat-1 and MEFs proliferate without this ability.

Our reason for this investigation into the SRE was to test whether induction of Rac1<sup>V12</sup> from the STRAC clones could induce cellular responses. However, this was not a suitable assay in which to test this since the SRE did not respond to activated Rac1 in these cells.
4.2.3 Activation of JNK/SAPK by Rac

Activated Rac1 and other Rho family members have been reported to activate the JNK MAP kinase pathway (Minden et al., 1995). This pathway is also activated in response to stress such as UV and γ-irradiation, osmotic stress, DNA damaging agents such as anisomycin or by RasV12 (Kyriakis and Avruch, 1996). JNK targets include transcription factors and other kinases which go on to regulate transcription (Figure 1.6). Therefore we tested the ability of Rac1V12 induction in STRAC.C cells to activate JNK kinase activity.

Confluent, quiescent cultures of STRAC.C and ST cells in DMEM with 0.5% NCS were incubated in the presence or absence of doxycycline for 16 h and then assayed for JNK activity. As a control, matched dishes of STRAC.C and ST cells in the presence of doxycycline were exposed to UV light, a stress stimulus. JNK was immunoprecipitated from cell lysates and its activity was determined by its ability to phosphorylate GST-c-jun. Induction of Rac1V12 was unable to activate JNK. In contrast, UV light stimulated a high level of JNK activity (Figure 4.4). Therefore since UV light but not Rac1V12 expression was able to activate JNK activity this suggests that the JNK pathway is unresponsive to Rac1V12 in these cells. However, again this did not inform us about the ability of RacV12 induction in STRAC cells to elicit a cellular response.

4.2.4 Induction of DNA synthesis by Rac1V12

It has been reported that Rac1 activity is both necessary and sufficient for DNA synthesis in Swiss 3T3 fibroblasts. Specifically, dominant negative Rac1 mutants were able to inhibit serum-stimulated DNA synthesis and microinjection of constitutively activated Rac1 into quiescent cells was shown to initiate DNA synthesis (Lamarche et al., 1996; Olson et al., 1995). Expression of activated Rac1 itself in Rat1 cells has been demonstrated to induce focus formation and tumours when injected into nude mice (Qiu et al., 1995a). Furthermore, a dominant negative mutant of Rac1 was able to inhibit Ras-mediated transformation of NIH 3T3 cells (Khosravi-Far et al., 1995; Qiu et al., 1995a). Many of the RacGEFs have also been shown to have oncogenic potential (Zohn et al., 1998). Thus it appears that Rac1 has an important role in mitogen stimulation of DNA synthesis which may involve its activation downstream of Ras. However the signals regulated by Rac1 that mediate these aspects of growth control have not yet been elucidated.

4.2.4.1 Rac1V12 does not support mitogen-independent growth

We tested whether Rac1V12 could stimulate colony formation in low mitogen conditions. 1x10^4 cells were cultured in the presence or absence of doxycycline for 3 weeks in 0.5%, 2% or 10% NCS. Rac1V12 was unable to support colony formation in 0.5% NCS.

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However, STRAC.C cells in the absence of doxycycline formed more numerous and dense colonies than cells in the presence of doxycycline in both 2% and 10% NCS (Figure 4.6a). This suggested that Rac\textsuperscript{V12} was able to assist, but not reproduce, the mitogenic abilities of serum.

We then tested whether Rac\textsuperscript{V12} induction could stimulate DNA synthesis in STRAC.C cells in reduced serum conditions. Confluent, serum-deprived, quiescent STRAC.C and ST cells were cultured in the presence or absence of doxycycline for 30 h. As a control, 10% NCS was added to equivalent dishes for the same time period in the presence or absence of doxycycline. DNA synthesis was measured by tritiated thymidine incorporation (Figure 4.6b). The induction of DNA synthesis by serum addition was assisted by the co-expression of Rac\textsuperscript{V12} which concurs with the colony formation assays. However, induction of Rac\textsuperscript{V12} did not stimulate DNA synthesis in serum-deprived conditions. Thus, in contrast to the published effect of Rac\textsuperscript{V12} in Swiss 3T3 cells, activated Rac1 was not able to induce mitogen-independent growth in NIH 3T3 cells. This may be due to cell type specific differences. We have already described differences in the abilities of two lines of NIH 3T3 cells to differentially regulate the SRE in response to Rac\textsuperscript{V12} (Figure 4.3). Alternatively this difference could be due to different expression levels of Rac\textsuperscript{V12} or the different methods of introduction of the activated oncogenes, microinjection induces a very high and rapid upregulation of the signal in a background of unstimulated cells, whereas we induced Rac\textsuperscript{V12} expression more slowly by relieving transcriptional repression.

4.2.4.2 Rac\textsuperscript{V12} does support anchorage-independent growth

In addition to requiring mitogens for cell cycle progression, non-transformed fibroblasts also require adhesion to a substratum. Expression of an activated oncogene such as Ras\textsuperscript{V12} enables cell cycle progression in the absence of anchorage, presumably by mimicking adhesion signals, such as those from specific integrin receptors. Activated Rac1 has also been shown to induce colony formation in soft agar assays (Khosravi-Far \textit{et al.}, 1995; Qiu \textit{et al.}, 1995a). Rac1 may have a role in integrin-mediated signalling. For example, dominant negative mutants of Rac1 and Ras, and the PI 3-kinase inhibitor wortmannin, are able to suppress integrin $\alpha 6\beta 4$ activation of JNK (Maniero \textit{et al.}, 1995).

We tested whether Rac\textsuperscript{V12} was able to induce anchorage-independent growth by assessing whether induction of Rac\textsuperscript{V12} caused STRAC.C cells to form colonies in soft agar. $5 \times 10^3$ and $2 \times 10^4$ cells of the three STRAC clones and polyclonal populations of ST, LXSN Rac\textsuperscript{V12}, LXSN Ras\textsuperscript{V12} and LXSN cells (NIH 3T3 cells which constitutively express Rac\textsuperscript{V12} and Ras\textsuperscript{V12} respectively and cells infected with the empty vector, kind gifts from A.Lloyd and P.Rodriguez-Viciana) were seeded in soft agar in the presence or
absence of doxycycline and cultured for 26 days. Colonies were then stained with 0.1% Neutral Red, photographed and counted (Table 4.1a & b and Figure 4.5)).

**Table 4.1a Formation of colonies in soft agar**

<table>
<thead>
<tr>
<th>Cell-line</th>
<th>Number of cells seeded</th>
<th>Colonies in presence of doxycycline</th>
<th>Percentage colony formation</th>
<th>Colonies in absence of doxycycline</th>
<th>Percentage colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST</td>
<td>5x10^3</td>
<td>10</td>
<td>0.2%</td>
<td>9</td>
<td>0.18%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>2</td>
<td>0.04%</td>
<td>4</td>
<td>0.08%</td>
</tr>
<tr>
<td>STRAC.C</td>
<td>5x10^3</td>
<td>53</td>
<td>1.06%</td>
<td>916</td>
<td>18.32%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>29</td>
<td>0.15%</td>
<td>2558</td>
<td>12.79%</td>
</tr>
<tr>
<td>STRAC.7</td>
<td>5x10^3</td>
<td>58</td>
<td>1.16%</td>
<td>386</td>
<td>7.72%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>59</td>
<td>0.3%</td>
<td>288</td>
<td>1.44%</td>
</tr>
<tr>
<td>STRAC.10</td>
<td>5x10^3</td>
<td>71</td>
<td>1.42%</td>
<td>767</td>
<td>15.34%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>45</td>
<td>0.23%</td>
<td>436</td>
<td>2.18%</td>
</tr>
</tbody>
</table>

**Table 4.1b Formation of colonies in soft agar**

<table>
<thead>
<tr>
<th>Cell-line</th>
<th>Number of cells seeded</th>
<th>Colonies</th>
<th>Percentage colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LXSN</td>
<td>5x10^3</td>
<td>147</td>
<td>2.94%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>122</td>
<td>0.61%</td>
</tr>
<tr>
<td>LXSN Rac¹¹²</td>
<td>5x10^3</td>
<td>908</td>
<td>18.16%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>2580</td>
<td>12.9%</td>
</tr>
<tr>
<td>LXSN Ras¹¹²</td>
<td>5x10^3</td>
<td>361</td>
<td>7.22%</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>2476</td>
<td>12.38%</td>
</tr>
</tbody>
</table>

Colonies in soft agar were formed by the three STRAC clones in the absence, but not in the presence, of doxycycline and no colony formation was observed with ST cells. Of the clones, STRAC.C had the highest colony formation with 18% of the seeded cells able to grow anchorage-independently in the absence of doxycycline compared with 1% in the presence of doxycycline. In the absence of doxycycline 7% and 12% of STRAC clones 7 and 10 respectively were able to grow in soft agar compared to 1.2% and 1.4% in the presence.

LXSN Rac¹¹² cells formed a similar number of colonies in soft agar as LXSN Ras¹¹² cells. However, LXSN Ras¹¹² cells formed colonies much sooner and they grew faster than LXSN Rac¹¹² cells.

The density at which the cells were seeded also appeared to be an important factor in the colony forming ability of the cells. For each cell-line, except LXSN Ras¹¹², whether in
the presence or absence of doxycycline, cells seeded at the higher density of $2 \times 10^4$ cells per 4 ml of agar had a lower percentage of colony formation. This was especially clear with STRAC.7 and STRAC.10 where the percentage colony formation in the absence of doxycycline dropped from 8% and 15% to 1.5% and 2% respectively. This may have been due to a limited supply of an essential factor in the growth media or alternatively that autocrine signals were excreted by the cells which inhibited growth. Both STRAC.C and LXSN Ras$^{V12}$ were less affected by culture at a higher density. Their percentage colony formation reduced from 18% to 13% in both cases. A higher percentage of LXSN Ras$^{V12}$ cells formed colonies at greater cell density, possibly due to mitogenic autocrine signals induced by Ras$^{V12}$.

We have demonstrated that Ras$^{V12}$ is able to replace an essential signal that is normally provided by cell contact with the substratum, but cannot replace signals provided by mitogens. However, it appears that Ras$^{V12}$ is able to co-operate with mitogenic signals to increase DNA synthesis levels. Importantly, stimulation of DNA synthesis by Ras$^{V12}$ is unlikely to involve JNK activity or transcriptional activation via the SRE since Ras$^{V12}$ does not stimulate these responses in STRAC cells.

4.2.4.3 Optimising conditions for Ras$^{V12}$-induced anchorage-independent growth

Colony formation in soft agar is a long term assay from which one is unable to retrieve cells for biochemical analysis. In order to perform biochemical analysis of Ras$^{V12}$-induced DNA synthesis we required a culture system in which short-term assays could be performed.

We tested three techniques: 1) A suspension matrix of methylcellulose (MC) with DMEM and 10% NCS referred to as MC matrix from which cells can be retrieved by diluting out the MC with PBSA (Assoian et al., 1989); 2) tissue culture dishes coated in agarose mixed with DMEM to which cells are unable to adhere (Guadagno and Assoian, 1991) and 3) tissue culture dishes coated with poly (2-hydroxyethylmethacrylate) (polyHEMA) which acts as a cell repellent.

Confluent, serum-deprived, quiescent STRAC.C cells were trypsinised and $1 \times 10^5$ cells were incubated in the presence of 10% NCS in either 2 ml of growth media on polyHEMA or agarose coated dishes. Alternatively, cells were mixed with 2 ml of MC matrix. All cells were cultured in the presence or absence of doxycycline in 10% NCS for 48 h and their DNA synthesis was assessed by tritiated thymidine incorporation analysis. In addition, the DNA content of cells incubated on polyHEMA coated dishes or in MC matrix was measured by propidium iodide staining and analysis by FACS.
Induction of Rac1\textsuperscript{V12} increased DNA synthesis in all three culture systems (Figure 4.7a). The largest fold increase in DNA synthesis occurred on polyHEMA coated dishes. The FACS analysis of PI content showed that polyHEMA cultures had a population of cells in the sub-G1 region with a DNA content less than 2N (Figure 4.7b). This suggested some cells were dying. However we chose this method of cultivation for the majority of our investigation since it gave the largest difference in DNA synthesis between cells in the presence and absence of doxycycline and we were specifically interested in performing biochemical analysis.

In addition, the ability of STRAC.C cells in suspension to induce Rac\textsuperscript{V12} was also investigated. Serum-deprived confluent STRAC.C cells were trypsinised and put into suspension on polyHEMA coated dishes in the presence or absence of doxycycline. Cells were harvested at the indicated time points and lysates were separated by SDS-PAGE and analysed for Rac\textsuperscript{V12} expression by Western blotting. Rac\textsuperscript{V12} expression reached maximum expression levels by 24 h and its regulation was unaffected by the lack of adhesion (Figure 4.7c).

The soft agar assay (Table 4.1) had indicated that the density at which the cells were cultured affected the colony forming potential of the cells. Therefore we assessed whether cell density affected the DNA synthesis of cells in suspension. Confluent, serum-deprived, quiescent, STRAC.C cells were resuspended in DMEM with 10% NCS and equal numbers were put into suspension at different densities on polyHEMA coated dishes in the presence or absence of doxycycline for 72 h. DNA synthesis was measured by thymidine incorporation analysis. The results showed that the DNA synthesis of the cells, both in the presence of doxycycline and with Rac\textsuperscript{V12} induction, was affected by the density at which the cells were cultured. Cells cultured at low density had a higher level of DNA synthesis than cells grown at high density. Nevertheless, Rac\textsuperscript{V12} was able to induce a higher level of DNA synthesis at each density indicating an ability to partially overcome the limiting or inhibitory factor involved (Figure 4.8). This concurs with the effect we observed in the soft agar assay.

**4.2.5 The effect of Rac1\textsuperscript{V12} on the G1 Cyclins**

Upon loss of anchorage, NIH 3T3 cells are reported to downregulate the expression and the associated kinase activities of Cyclins D1, E and A to varying extents even though serum is present in the medium (Bohmer et al., 1996; Guadagno et al., 1993; Schulze et al., 1996; Zhu et al., 1996b). In addition, in some cell-types, the expression levels of p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} have also been reported to be upregulated upon removal of anchorage. Ectopic expression of Cyclin D1 or Cyclin A in fibroblast cell-lines has also been reported to induce cell proliferation in non-adherent cells (Carstens et al., 1996; Guadagno et al.,
1993; Kang and Krauss, 1996; Resnitzky, 1997; Schulze et al., 1996). Furthermore, Cyclin A expression is specifically dependent on adhesion, independently of the kinase activity of Cyclin D and Cyclin E complexes. NRK cells have constitutive Cyclin D1- and E-associated kinase activities and pRb phosphorylation in the absence of anchorage but are dependent on adhesion for Cyclin A expression and subsequent cell cycle progression (Guadagno et al., 1993; Zhu et al., 1996b).

The reported effects of loss of anchorage on protein expression are not consistent and therefore we wanted to determine the effects on Cyclin D1, Cyclin A and p27^kip1 protein levels if anchorage was removed from STRAC.C cells in the presence of serum. Confluent, serum-deprived, quiescent STRAC.C cells were trypsinised and plated in 10% NCS either as a suspension culture on polyHEMA coated dishes or as an adherent culture on normal tissue culture plastic, for 30 h. Cell lysates were then separated by SDS-PAGE and analysed by Western blot for expression of Cyclin D1, Cyclin A and p27^kip1. As expected Cyclin D1 and A proteins were highly expressed in adherent, sub-confluent STRAC.C cells proliferating in 10% NCS. The levels of both Cyclin D1 and A were dramatically reduced in the absence of adhesion. p27^kip1 levelswere unaffected by the loss of adhesion (Figure 4.9).

It has been reported that Cyclin D1 mRNA levels become reduced upon removal of anchorage which implies that the downregulation of Cyclin D1 may be transcriptional (Zhu et al., 1996b). To determine whether the downregulation of Cyclin D1 protein we had observed was transcriptional, we examined the activity of the Cyclin D1 promoter upon removal of adhesion to substratum. A Cyclin D1 promoter reporter construct, pGL2 Cyclin D1 (a kind gift from J.Downward), containing 1.8 kb of the human Cyclin D1 promoter cloned upstream of a luciferase gene in the pGL2 vector (pGL2 Cyclin D1) was stably transfected into ST cells or a promoterless control were stably co-transfected into ST cells with pJ6Hygro, a plasmid encoding a hygromycin resistance gene. The cells were selected for hygromycin resistance and polyclonal populations were obtained. ST cell-lines containing pGL2 and pGL2 Cyclin D1 will be referred to as ST GL2 and ST D1 respectively.

To assess the regulation of the Cyclin D1 promoter upon removal of anchorage, confluent, serum-deprived, quiescent cultures of ST GL2 and ST D1 cells were trypsinised and resuspended in DMEM with 10% NCS. The samples corresponding to the zero hour time-point were harvested immediately and the others were incubated on polyHEMA coated dishes for the indicated times prior to harvesting. Cell lysates were analysed for luciferase activity with a luminometer by assaying the amount of light emitted upon cleavage of luciferin. The activity of the Cyclin D1 promoter decreased dramatically when the cells were suspended in 10% NCS (Figure 4.10). After 3 hours its activity was
reduced to 50% of the activity of cells which had been harvested immediately. After 9 h in suspension the activity of the Cyclin D1 promoter was reduced to 20%. The ST GL2 cells had a minimal level of luciferase activity that did not change in this experiment. Thus it appears that reduction of Cyclin D1 expression upon loss of anchorage occurs at least in part by transcriptional downregulation.

4.2.5.1 Rac1\textsuperscript{V12} regulation of cell cycle proteins

We have demonstrated that Cyclins D1 and A in STRAC.C cells become downregulated upon removal of anchorage. We tested whether Rac1\textsuperscript{V12} was able to induce DNA synthesis by inducing these proteins. Furthermore the pattern of induction of protein activation was determined in relation to the initiation of DNA synthesis.

Confluent, serum-deprived, quiescent STRAC.C cells were trypsinised and put into suspension on polyHEMA coated dishes in the presence and absence of doxycycline. Cells were labelled with tritiated thymidine for 2 h at the indicated time points to determine DNA synthesis levels. Cell lysates from parallel cultures were taken at the indicated times. In addition, cell lysates were taken from sub-confluent, adhered, asynchronously growing cells and confluent serum-deprived adherent cultures. Lysates were separated by SDS-PAGE and analysed for cyclin expression by Western blot. Alternatively they were assayed for Cyclin E- or Cyclin A-associated kinase activity.

DNA synthesis began 22 h after removal of doxycycline and maximal DNA synthesis occurred at 30 h (Figure 4.11a). Induction of Cyclin D1 and Cyclin E expression and Cyclin E-associated kinase activity occurred 22 h after removal of doxycycline which correlated with the initiation of DNA synthesis. However, Cyclin E expression levels increased by a meagre degree compared to the increase in Cyclin E-associated kinase activity. Cyclin A expression levels increased 30 h after removal of doxycycline and Cyclin A-associated kinase activity increased by two fold over its basal level 34 h after doxycycline removal and continued to increase thereafter (Figure 4.11b). Thus the earliest events following Rac1\textsuperscript{V12} induction are the expression of Cyclin D1 protein and upregulation of Cyclin E-associated kinase activity. After which Cyclin A protein and its associated kinase activity were induced.

Absolute comparisons between adhered and suspended STRAC.C cells are difficult since it was not technically possible to control for cell number and so the effect of cell proliferation and death were not accounted for. However, equal numbers of STRAC.C cells were adhered in 10% NCS or put into suspension in 10% NCS and assayed for DNA synthesis after 38 h. Adhered cells (in the presence of doxycycline) incorporated over 10 times more than Rac1\textsuperscript{V12} expressing cells in suspension (Data not shown). This indicates that adhesion gives a much greater proliferative signal than Rac\textsuperscript{V12}. This is
reflected in the difference of cyclin-associated kinase activities and Cyclin D1 and A protein levels, which are much higher in the adhered cultures than the suspension cultures even in the presence of Rac1V12. Interestingly, although the kinase activity of Cyclin E in the adhered culture is over seven times higher than the maximum activation by Rac1V12 in suspension, the expression level of Cyclin E protein is lower. This suggests that inhibitor proteins are important in regulating Cyclin E-associated kinase activity in suspension. Interestingly, in comparison to adhered STRAC.C cells arrested by serum deprivation, RacV12 expressing cells have a higher level of Cyclin E-associated kinase activity and Cyclins D1, E and A protein, which implies that these cells are progressing through the cell cycle. The low level of DNA synthesis may be due to cell death in the suspension cultures.

4.2.6 The role of PI 3-kinase in anchorage-independent DNA synthesis

We have demonstrated that Rac1V12 is able to propagate signals that are usually activated by adhesion. Thus it is probable that Rac1 is constitutively activating integrin-mediated pathways. Furthermore, activated Rac1 and Cdc42 (but not Rho) have been shown to activate PI 3-kinase in vitro (Bokoch et al., 1996). In contrast another group has reported that Rac1V12 is unable to activate PI 3-kinase in vitro (Rodriguez-Viciana et al., 1994). However, in mammary epithelial cells integrin-mediated invasion can be reproduced by the overexpression of Rac1V12, Cdc42V12 which require PI 3-kinase activity to elicit these effects (Keely et al., 1997). Therefore PI 3-kinase may be required downstream of Rac, particularly in transmitting signals from integrin receptors. However, there is also a lot of evidence which suggests PI 3-kinase also activates Rac. For example, activation of Rac1 by activation of the PDGF receptor is dependent on PI 3-kinase function and the production of PI (3,4,5)P3 lipids (Hawkins et al., 1995). Furthermore, activation of Rac1-mediated effects by PDGF, insulin and activated Ras can be inhibited by the specific PI 3-kinase inhibitors wortmannin and LY 294002 or dominant negative PI 3-kinase mutants (Kotani et al., 1995; Nobes et al., 1995; Rodriguez-Viciana et al., 1997). However, Rac1-mediated events can also be activated independently of PI 3-kinase (Genot et al., 1998; Hawkins et al., 1995). Thus it appears that the relationship between PI 3-kinase and Rac1 is complex. However, since Rac1V12 has been shown to require PI 3-kinase for integrin mediated signalling, we used the PI 3-kinase specific inhibitor, LY 294002 to test whether PI 3-kinase activity was necessary for Rac1V12-mediated anchorage-independent DNA synthesis. LY 294002 competes for the ATP-binding site of the catalytic subunit of PI 3-kinase (Vlahos et al., 1994). To assess the activation state of PI 3-kinase we analysed the phosphorylation of a downstream target of PI 3-kinase, Akt/PKB, using a
phospho-specific antibody made against the phosphorylated serine 473 on Akt/PKB. Phosphorylation of this serine is inhibited by wortmannin indicating a dependence on PI 3-kinase activity for phosphorylation (Alessi et al., 1996) and thus was a good indicator of PI 3-kinase activity.

4.2.6.1 Optimising the conditions for the use of LY 294002

To assess the optimal concentration of LY 294002 for inhibition of PI 3-kinase activity we tested the ability of different LY 294002 concentrations to prevent PI 3-kinase-mediated phosphorylation of Akt/PKB by EOF. Confluent, serum-deprived quiescent STRAC.C cells in DMEM with 5 μg/ml insulin were media changed into similar media containing the indicated concentrations of LY 294002 dissolved in DMSO (or DMSO alone as a control). Five hours later, EOF was added to the indicated dishes for 3 min prior to harvesting the cells. Cell lysates were separated by SDS-PAGE and the expression levels and phosphorylation status of Akt/PKB were analysed by Western blotting. Addition of EOF stimulated a high degree of Akt/PKB phosphorylation and this was partially inhibited by 2 and 5 μM LY 294002 and fully inhibited by the addition of 10 and 50 μM LY 294002 (Figure 4.12a). The ability of EOF to stimulate PI 3-kinase activity was not affected by DMSO. In addition, the phosphorylation status of p42"^"^ and p44"^"^ erk in response to LY294002 was assessed using a phospho-specific p44/42"^"^ antibody. The ERKs were still phosphorylated by EOF in the presence of LY 294002, however, the level of phosphorylation was reduced by about 4 fold in the presence of LY 294002. This indicated that either LY 294002 or an indirect effect was downregulating MEK activity. A similar reduction in MEK activity has been reported with another PI 3-kinase inhibitor, wortmannin in vivo, however when MEK activity in response to wortmannin was tested in vitro it was unaffected (Marra et al., 1995). This suggests that the inhibition of MEK is due to an effect of the reported cross talk between the Raf and PI 3-kinase pathways (Frost et al., 1997; King et al., 1998), rather than non-specific action of the inhibitor compound directly on MEK. In addition the inhibitor also slightly reduced the expression levels of Akt/PKB, which may indicate that a low level of PI 3-kinase activity (or an LY 294002 sensitive activity) is required to maintain Akt/PKB protein levels.

The duration of LY 294002 inhibition was also tested. Confluent, serum-deprived quiescent STRAC.C cells in DMEM with 5 μg/ml insulin and doxycycline were media changed into similar media containing 10 μM LY 294002 dissolved in DMSO or DMSO. EGF was added to the indicated dishes for 3 min at the indicated time points and the cells were then analysed for expression and phosphorylation of Akt/PKB. 10 μM LY 294002 was able to inhibit PI 3-kinase activity for up to 32 h (Figure 4.12b). This indicated that this inhibitor was suitable for analysing DNA synthesis induction.
4.2.6.2 Inhibition of PI 3-kinase activity in anchorage-independent DNA synthesis

The effect of LY 294002 on anchorage-independent DNA synthesis by Rac1V12 was tested. Confluent, serum-deprived quiescent STRAC.C cells in DMEM with 5 μg/ml insulin and doxycycline had the indicated concentration of LY 294002 or DMSO added to them for 1 h and then the cells were trypsinised and put into suspension on polyHEMA coated dishes in the presence or absence of doxycycline and DMEM with 10% NCS and the indicated concentration of LY 294002 or DMSO. Their DNA synthesis was determined by tritiated thymidine incorporation analysis after 30 h in suspension.

The basal DNA synthesis rates of cells in the presence of doxycycline and also the DNA synthesis rates of cells with induced Rac1V12 expression were inhibited by the addition of LY 294002 and this was a dose-dependent response. At 10 μM LY 294002 their DNA synthesis was 10% that of their uninhibited levels (Figure 4.12). However the induction of DNA synthesis by Rac1V12 remained constant at around 2-2.5 fold (see insert, Figure 4.13). Thus Rac1 induction of DNA synthesis appeared to be independent of PI 3-kinase activity. However, PI 3-kinase activity may be required for DNA synthesis but independently from the Rac1 signal. Alternatively, the inhibition of PI 3-kinase activity may be causing cell death which would reduce the DNA synthesis index. Akt/PKB is an important survival signal and has been shown to rescue a particular form of apoptosis, anoikis, in epithelial cells upon loss of adhesion. Therefore inhibition of Akt/PKB-mediated survival signals may be causing apoptosis in the absence of fibroblast adhesion. However confirmation of this would require further investigation.

4.2.7 The effect of Raf-1 activation upon the ability of Rac1V12 to control cell proliferation

The effect of activated Rac1 on cell cycle control was initially addressed as part of our larger question about the mechanisms used by Ras to govern cell proliferation. Rac1 had been shown to be an essential element downstream of Ras for the transformation and promotion of mitogen-independent DNA synthesis. Transformation assays have implicated Rac1 on a signalling pathway downstream from Ras and distinct from Raf due to the co-operation of Rac1 and Raf signals in focus formation assays. Thus we were interested in whether Rac1 could counteract the inhibitory properties of Raf and whether Rac1 and Raf signals could co-operate together to induce anchorage-independent DNA synthesis.

A cell-line in which both Raf and Rac1 activation could be induced separately was generated. STRAC.C cells were infected with GPE LXSN ΔRER viral supernatant and then selected for resistance to G418 and puromycin. These cells will be referred to as STRAC/RER cells. The ability of these cells to induce Rac1V12 and ΔRaf:ER activity and
the effect of these proteins on cell cycle regulatory proteins was tested. In addition the ability of these proteins to induce DNA synthesis was also tested. Confluent, serum-deprived, quiescent cultures of STRAC/RER cells were trypsinised and seeded onto polyHEMA coated dishes in the presence or absence of doxycycline (and BrdU for DNA synthesis analysis) and in the indicated concentrations of 4-OH tamoxifen or ethanol for 36 h. To assess DNA synthesis BrdU-treated cells were collected and a single cell suspension was made. Cells were then cytopspun onto glass slides, fixed and immunostained for BrdU incorporation and their total DNA was counterstained with Hoechst 33258. The percentage of BrdU positive cells was then assessed. Cell lysates were separated by SDS-PAGE and analysed by Western blotting. The ability of the STRAC/RER cells to induce DNA synthesis in the absence of anchorage after being placed in suspension from an asynchronously growing population was also investigated. STRAC/RER cells were incubated in MC matrix in the indicated conditions and their DNA synthesis was measured after 48 h by tritiated thymidine incorporation analysis.

Expression of Rac1\textsuperscript{V12} remained regulated by doxycycline in STRAC/RER cells, although strong Raf-1 activation slightly increased Rac1\textsuperscript{V12} levels. Dose-dependent phosphorylation of ERK2 occurred in response to activation of ΔRaf:ER by the indicated concentrations of 4-OH tamoxifen (Figure 4.14a). The effect of Rac1\textsuperscript{V12} expression and ΔRaf:ER activation was similar in both cases. Activation of ΔRaf:ER with 100 nM or 5 nM 4-OH-tamoxifen reduced the basal levels of anchorage-independent DNA synthesis by approximately 4 fold. Rac1\textsuperscript{V12} expression afforded a small degree of protection but DNA synthesis levels were still below basal levels. Weak activation of ΔRaf:ER had no effect on the ability of Rac1\textsuperscript{V12} to induce anchorage-independent DNA synthesis. Therefore it appeared that ΔRaf:ER activation could not act mitogenically in the absence of adhesion.

The expression levels of p21\textsuperscript{Cip-1} were analysed to determine if the ability of Rac1\textsuperscript{V12} to partially resist ΔRaf:ER mediated inhibition was due to an ability to inhibit p21\textsuperscript{Cip-1} upregulation. Induction of p21\textsuperscript{Cip-1} and Cyclin D1 by ΔRaf:ER were unaffected by Rac1\textsuperscript{V12}. However activation of ΔRaf:ER completely inhibited Cyclin A expression, thus this may be involved in ΔRaf:ER-mediated inhibition of DNA synthesis. In addition p27\textsuperscript{Kip1} levels were not downregulated by ΔRaf:ER activation as they had been in the absence of serum. This suggests that growth factors and adhesion may regulate p27\textsuperscript{Kip1} levels differently.

To test whether activation of Raf-1 could support anchorage-independent growth, STRCX 6 and STRCX 16 cells (Section 2.2.6), NIH 3T3 cells containing RafCAAX under the control of a doxycycline repressible promoter (Figure 2.9), were tested for their ability to form colonies in soft agar in the presence or absence of doxycycline. The
indicated number of STRCX 6 and 16 and STRAC.C cells were seeded in soft agar in the presence or absence of doxycycline and cultured for 20 days. Colonies were then stained with 0.1% Neutral Red, photographed and counted (Table 4.2).

<table>
<thead>
<tr>
<th>Cell-line</th>
<th>Number of cells seeded</th>
<th>Colonies in presence of doxycycline</th>
<th>Colonies in absence of doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAC.C</td>
<td>2x10^4</td>
<td>44</td>
<td>668</td>
</tr>
<tr>
<td>STRCX6</td>
<td>5x10^3</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>STRCX16</td>
<td>5x10^3</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2x10^4</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Induction of RafCAAX through removal of doxycycline did not induce formation of colonies in soft agar, in contrast to STRAC.C cells.

Therefore we have been unable to demonstrate any ability of Raf-1 activation, either through different levels of ARaf:ER activation or induction of RafCAAX to stimulate anchorage-independent growth. Furthermore, ARaf:ER demonstrated no ability to cooperate with Rac1^V12 to induce anchorage-independent growth. However, strong activation of ARaf:ER was able to inhibit the ability of Rac1^V12 to induce DNA synthesis in suspension. This demonstrated that ARaf:ER was able to inhibit anchorage-independent DNA synthesis. This implies that Raf-1 activation is not mitogenic in anchorage-independent conditions and is unable to replace adhesion-mediated signals.

4.2.8 RhoA^V14 can support anchorage independent growth

In the previous chapter we demonstrated that RhoA^V14 was unable to support mitogen-independent growth or co-operate with Raf to induce mitogen-independent growth. To determine whether RhoA^V14 had similar properties to Rac1^V12 we used the ARER/Rho cells to test whether RhoA^V14 was able to support anchorage-independent growth. ARER/Rho and ARER/plink cells were suspended in polyHEMA cultures from an asynchronously growing population. After 16 h 4-OH tamoxifen was added for a further 16 h and DNA synthesis was measured by tritiated thymidine incorporation analysis. ARER/Rho cells had 2-3 fold higher levels of DNA synthesis than control cells. In a similar manner to STRAC/RER cells, activation of ARaf:ER inhibited DNA synthesis to below basal levels in both cell-lines indicating that RhoA^V14 was unable to protect the cells from Raf-1-mediated cell cycle inhibition. Therefore we have shown that RhoA^V14 was able to support anchorage-, but not mitogen-independent DNA synthesis and thus has similar properties to Rac1^V12. Rac1 has been shown to activate Rho-mediated signal transduction (Nobes and Hall, 1995). However it is thought that Rac1 and RhoA act on
separate pathways in relation to cellular transformation because while activated Rac1 and PI 3-kinase are unable to co-operate with each other to induce focus formation, RhoA\textsuperscript{V14} can co-operate with either activated Rac1 or PI 3-kinase (Rodriguez-Viciana \textit{et al.}, 1997). This indicated that RhoA and Rac1 contributed separately to transformation.

\textbf{4.3 Summary}

Thus Rac1\textsuperscript{V12} is able to induce anchorage, but not mitogen-independent growth in NIH 3T3 cells. This may occur because Rac1\textsuperscript{V12} induction is sufficient to rescue the downregulation of G1 cyclins and their respective associated kinase activities that occurs upon loss of anchorage. These abilities are probably independent of serum response factor (SRF) and JNK activity since Rac1\textsuperscript{V12} does not activate these signals in STRAC cells. In addition, Rac1\textsuperscript{V12} is able to induce DNA synthesis independently of PI 3-kinase activity, however, PI 3-kinase activity appears necessary for either basal DNA synthesis or cell survival. Raf-1 activity is able to inhibit anchorage DNA synthesis induced by Rac1\textsuperscript{V12} probably through induction of p21\textsuperscript{Cip1}. Furthermore Raf-1 activity appears unable to induce or assist Rac1\textsuperscript{V12}-mediated anchorage-independent growth. Finally RhoA\textsuperscript{V14} demonstrates similar properties to Rac1\textsuperscript{V12} and is able to induce anchorage-, but not mitogen-independent DNA synthesis which is also sensitive to inhibition by Raf:ER activation.
4.4 Figures - Chapter Four
Figure 4.1 Rac1\textsuperscript{V12} expression is regulated in STRAC cells
After infection with BPSTR1 or BPSTR1 Rac1\textsuperscript{V12} NIH 3T3 cells were for resistance to 2.5mg/ml puromycin in the presence of doxycycline (100 ng/ml). Selected cells, referred to as ST and STRAC cells respectively, were then either ring cloned, or pooled to form a polyclonal population.

(a) Two dishes of the STRAC pool was cultured for 24 h in DMEM with 10% NCS in the presence of doxycycline. Doxycycline was then washed out of the cells and re-added to one dish and then incubated for a further for 48 h. Cell lysates were taken and 50 μg of protein was separated by SDS-PAGE gel and analysed by Western blotting using the 9E10 antibody.

(b) Pairs of clones of STRAC cells and the ST polyclonal population were cultured for 24 h in DMEM with 10% NCS in the presence of doxycycline. Doxycycline was then washed out of the cells and re-added to one dish and then incubated for a further for 72 h. Cell lysates were taken and 50 μg of protein was separated by SDS-PAGE gel and analysed by Western blotting using the 9E10 antibody.
Figure 4.1

a) [Image showing a gel with RacV12 labeled and Dox (100 ng/ml) indicated]

b) [Image showing a gel with RacV12 labeled and Dox (100 ng/ml) indicated]

2 13 16 C

6 7 10 11

35 36 37 38

39 40 41 ST
Figure 4.2 Rac1\textsuperscript{V12} expression does not induce SRE activity

The LipofectAmine reagent was used to transiently transfect ST, STRAC.C, STRAC.7 and STRAC.10 cells. Four dishes of each cell-line were transfected with 0.5 µg of p4xSRE, in addition, one dish of each cell-line was co-transfected with 0.5 µg of pEXV3 Rac1\textsuperscript{V12}, while the other three were co-transfected with 0.5 µg of pEXV3. Cells were continuously cultured in the presence of doxycycline unless otherwise indicated. The LipofectAmine-DNA mix was applied to the cells for 5 h after which cells were incubated in DMEM with 10% NCS for 16 h. Then the cells were washed in DMEM and cultured in DMEM with 1% serum. One dish that had been transfected with pEXV3 was cultured in the absence of doxycycline, while the rest were cultured in the presence of doxycycline. Another pEXV3 transfected dish was re-stimulated with 20% NCS 14 h prior to harvesting. Cells were harvested and analysed for CAT activity 48 h later. Each point was performed in triplicate.
Figure 4.2

![Graph showing CAT activity (cpm) with different conditions: Dox (100 ng/ml), pEXV3 RacV12, and NCS (20%). The graph has bars representing STR1, STRAC.C, STRAC.7, and STRAC.10 with various conditions indicated by + or - for each variable.]
Figure 4.3 The response of the SRE to Rac1\textsuperscript{VI12} differs in different rodent fibroblasts

a) The LipofectAmine reagent was used as described above to transiently transflect six dishes of NIH 1 and NIH 2 cells. Each cell-line was co-transfected with either 0.5 \( \mu \)g of p4xSRE or p4xPRE and 0.5 \( \mu \)g pEXV3 or p4xSRE and pEXV3 Rac1\textsuperscript{VI12}. In addition untransfected cells were used to provide a background measure of CAT activity. After transfection the cells were washed in DMEM and cultured in DMEM with 1\% NCS. Cells were harvested and analysed for CAT activity 48 h later. One dish of each cell-line transfected with p4xSRE or p4xPRE and pEXV3 was stimulated with 20\% NCS 14 h prior to harvesting. The CAT activity of the untransfected control was deducted from the CAT activities of the transfected cells. The factor of difference between CAT activity from unstimulated p4xPRE transfected cells and their serum-stimulated counterparts was used as a normalising factor for the CAT activities obtained from cells transfected with p4xSRE. Values are shown as fold activation over the unstimulated SRE which is taken as 1. Each point was performed in duplicate.

b) Transient transfections into NIH 1, NIH 2 and Rat1 cells were performed as above. Each cell-line was co-transfected with either p4xSRE or p4xPRE and pEXV3 or pEXV3 Rac1\textsuperscript{VI12}. The calcium phosphate method was used for MEFs with 2.5 \( \mu \)g of each vector and 5 \( \mu \)g of pUC19 carrier DNA. The precipitate was removed after 16 h and then MEFs were incubated in DMEM with 1\% NCS and treated as described above. The CAT activity of the untransfected control was deducted from the CAT activities of the transfected cells. The factor of difference between CAT activity from p4xPRE transfected cells unstimulated and those stimulated with serum or with Rac1\textsuperscript{VI12}, were used as normalising factors for the CAT activities obtained from p4xSRE transfected cells stimulated with serum or with Rac1\textsuperscript{VI12} respectively. Values are shown as fold activation over the unstimulated SRE which is taken as 1. Each point was performed in duplicate.
Figure 4.4 JNK is not activated by induction of Rac1V12

Confluent cultures of ST and STRAC.C cells were incubated in DMEM with 0.5% NCS and doxycycline. 36 h later, cells were washed with warm DMEM and one dish was incubated in the absence of doxycycline while the other two were cultured in the presence for a further 16 h. 30 min prior to harvesting, the media from one of the dishes in the presence of doxycycline was removed and reserved and 10 ml of PBSA was added to the dish, the cells were then subjected to 25 seconds of UV light. The PBSA was then removed and the media replaced. All cells were harvested 30 min later using JNK lysis buffer.

Figure 4.5 Induction of Rac1V12 allows colony formation in soft agar

STRAC.C, STRAC 10 and ST cells in DMEM with 10% (v/v) serum and doxycycline were washed and trypsinised. A soft agar assay was performed with 4x10^3 and 2x10^4 cells in the presence or absence of doxycycline for 26 days. Cells were then stained with 0.1% Neutral Red, photographed and colonies were counted. Dishes that were seeded with 2x10^4 cells are shown for ST, STRAC.C and STRAC.10 cells in the presence of doxycycline. The colony numbers are shown in Table 4.1.
Figure 4.4

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>STRAC.C</th>
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<tbody>
<tr>
<td>UV</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dox (100 ng/ml)</td>
<td>+</td>
<td>-</td>
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Figure 4.5

Dox (>100 ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>STRAC.C</th>
<th>STRAC.10</th>
<th>ST</th>
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<tr>
<td>+</td>
<td></td>
<td></td>
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<td>-</td>
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Figure 4.6 Rac1\textsuperscript{V12} is unable to induce mitogen-independent growth

(a) 1x10\textsuperscript{4} STRAC.C cells were seeded in full growth media with doxycycline (100 ng/ml). The next day they were washed and cultured in DMEM containing 10%, 2% or 0.5% serum in the presence or absence of doxycycline for 3 weeks. Then the dishes were stained with crystal violet and photographed.

(b) Confluent cultures of STRAC.C cells were serum deprived for 48 h in media containing 0.5% serum in the presence of doxycycline (100 ng/ml). Doxycycline was then washed out and the cells were cultured in the presence or absence of doxycycline for 30 h. Tritiated thymidine was added 6 h prior to harvesting and assaying for thymidine incorporation.
Figure 46

3H Thymidine Incorporation/cell (arbitrary units)

- NCS
- STRAC
- ST

Dox (100 ng/ml)
- 0.5%
- 2%
- 10%

- +
- -
Figure 4.7 Rac1^{V12} induces DNA synthesis in a variety of anchorage-independent culture methods

a) Confluent cultures of STRAC.C cells were incubated in DMEM with 1% NCS for 48 h. Cells were then trypsinised, resuspended in DMEM with 10% NCS and counted. 1x10^5 cells were plated in DMEM with 10% NCS and tritiated thymidine in the presence or absence of doxycycline on dishes coated with either polyHEMA or agarose or 1x10^5 cells were suspended in 2 ml of MC matrix. Cells were harvested after 48 h and assayed for tritiated thymidine incorporation.

b) Confluent cultures of STRAC.C cells were incubated in DMEM with 0.5% NCS for 48 h. Cells were then trypsinised, resuspended in DMEM with 10% NCS and counted. 3x10^6 cells were seeded in DMEM with 10% NCS in the presence of doxycycline either on dishes coated with polyHEMA or were suspended in 15 ml of MC matrix for 36 h prior to fixing and staining with propidium iodide and analysing with a FACScan machine.

c) STRAC.C cells were seeded confluent, then serum starved in DMEM with 0.25% NCS for 48 h in the presence of doxycycline (100 ng/ml). Cells were then placed into suspension on polyHEMA coated dishes in the presence or absence of doxycycline for stated time period. Cells were harvested, loaded onto an SDS-PAGE gel, western blotted and probed with 9E10 antibody. Lanes 11 and 12 are STRAC.C lysates from adhered cells cultured in DMEM with 0.25% (lane 11) or 10% (v/v) (lane 12) NCS in the presence of doxycycline. Lane 13 is a lysate from NIH 3T3 LXSN Ras^{V12} cells.

Figure 4.8 The basal rate of DNA synthesis is compromised in high density conditions

Confluent cultures of STRAC.C cells were incubated in DMEM with 0.5% NCS and doxycycline for 48 h. Cells were then trypsinised, resuspended in DMEM with 10% NCS and counted. 1x10^5 cells were incubated in 2.5 ml or 0.5 ml DMEM with 10% NCS in the presence or absence of doxycycline on 6 or 24 well dishes respectively coated with polyHEMA for 72 h. Tritiated thymidine was added for 9 h prior to harvesting and analysing the amount of thymidine incorporation.
Figure 4.7

(a) 

![Graph showing 3H Thymidine Incorporation (cpm) for different substrates: poly-HEMA, agarose coating, and methylcellulose. The graph compares + Dox and - Dox conditions.](image)

(b) 

![Histograms showing Propidium iodide counts for 6 and 24 well plates.](image)

(c) 

![Western blot showing RacV12 expression under different conditions.](image)

Figure 4.8

![Graph showing 3H Thymidine Incorporation (cpm) for different substrates: 6 and 24 well plates. The graph compares + Dox and - Dox conditions.](image)
Chapter Four - Rac and the Cell Cycle

Figure 4.9 Removal of adhesion causes downregulation of G1 Cyclins

Confluent cultures of STRAC.C cells were incubated in DMEM with 0.5% NCS for 48 h. Cells were then trypsinised, resuspended in DMEM with 10% NCS. Cells were seeded in DMEM with 10% NCS with doxycycline either on dishes coated with polyHEMA or on normal tissue culture plastic. Cells were incubated thus for 30 h prior to harvesting. Cell lysates were separated by SDS-PAGE and analysed by Western blotting. The blot was sequentially probed with 287-3, sc-596 and sc-528 antibodies against Cyclin D1, Cyclin A and p27Kip1 respectively.

Figure 4.10 Removal of adhesion rapidly downregulates Cyclin D1 transcription

ST GL2 and ST D1 cells were seeded confluenly and incubated in DMEM with 5 µg/ml insulin and doxycycline for 48 h. Cells were then trypsinised and resuspended on ice in DMEM with 10% NCS to inactivate the trypsin and counted. 2x10^5 cells of each cell-line was immediately harvested and frozen on dry ice. Other samples of equal cell numbers were seeded onto polyHEMA coated dishes in DMEM with 10% NCS and harvested 3 or 9 h later, then frozen. All samples were stored at -80°C overnight, prior to assaying for luciferase activity the following day. Each point was performed in duplicate.
Figure 4.9

Suspension
Adhered

- Cyclin D1
- Cyclin A
- p27Kip1

Figure 4.10

![Graph showing luciferase activity over time in suspension](image)

- ST GL2
- ST D1

Time in Suspension (h)

Luciferase activity (arbitrary units)
Figure 4.11 Induction of Rac1\textsuperscript{V12} induces S-phase and upregulates G1 cyclin expression and activity

Confluent cultures of ST and STRAC.C cells were incubated in DMEM with 0.5% NCS and doxycycline for 36 h. Cells were then trypsinised and resuspended on ice in DMEM with 10% NCS to inactivate the trypsin and counted.

a) For the thymidine incorporation assay, 1x10\(^5\) cells were seeded on polyHEMA coated dishes for the indicated times. Tritiated thymidine was added 2 h prior to harvesting for analysis of tritiated thymidine incorporation.

b) For biochemical analysis 6x10\(^5\) cells were seeded on polyHEMA coated dishes in DMEM with 10% NCS in the presence or absence of doxycycline and harvested at the time indicated. Alternatively lysates were taken from cells cultured in adherent conditions either confluent in 0.5% or subconfluent in 10% NCS with doxycycline in DMEM (Lanes 12 and 13). To analyse protein expression levels, cell lysates were separated by SDS-PAGE and analysed by Western blot. The blots were sequentially probed with 287.3, E23 and sc-481 antibodies recognising Cyclin D1, Cyclin A and Cyclin E respectively. Cyclin A and Cyclin E-associated kinase activities were assayed by immunoprecipitation with E72 and sc-481 antibodies and protein G sepharose and they were assayed for their ability to phosphorylate Histone H1.
Figure 4.11

a)

![Graph showing 3H Thymidine Incorporation (cpm) over time (h) with + Dox and - Dox conditions.]

b)

| NCS (10%) | + | + | + | + | + | + | + | + | + | + | - |
| Time in suspension (h) | 14 | 14 | 18 | 22 | 26 | 30 | 34 | 38 | 42 | 46 | 0 | 0 |
| Dox (100 ng/ml) | + | - | - | - | - | - | - | - | + | + | + | + |

- **Cyclin D1 WB**
- **Cyclin E WB**
- **Cyclin E IP Histone H1**
- **Cyclin A WB**
- **Cyclin A IP Histone H1**
Figure 4.12 PI 3-kinase is inhibited by LY 294002 for at least 32 h

a) Confluent cultures of STRAC.C cells were incubated in DMEM with 5 μg/ml insulin and doxycycline. The cells were media changed into DMEM, insulin and the indicated concentration of LY 294002 or its solvent DMSO for 5 h. EGF (50 ng/ml) was added to the indicated dishes for 3 min and then cells were harvested. Cell lysates were separated by SDS-PAGE and analysed by Western blot. Blots were sequentially probed with AKTSer473, 9272 and phospho p42/44 MAP kinase antibodies against phosphorylated AKT, total AKT and phosphorylated p44/p42Erk1/2.

b) Confluent cultures of STRAC.C cells were incubated in DMEM with 5 μg/ml insulin and doxycycline. The cells were media changed into DMEM, insulin and 10 μM of LY 294002 or its solvent, DMSO for the indicated times. EGF (50 ng/ml) was added to the indicated dishes for 3 min and then cells were harvested. Cell lysates were separated by SDS-PAGE and analysed by Western blot. Blots were sequentially probed with AKT Ser473 and 9272 antibodies against phosphorylated AKT and total AKT, respectively.

Figure 4.13 Anchorage-independent growth induced by Rac1Y22 is not dependent on PI 3-kinase activity

Confluent cultures of STRAC.C cells were incubated in DMEM with 5 μg/ml insulin and doxycycline. The cells were media changed into DMEM, insulin, doxycycline and the indicated concentration of LY 294002 or its solvent, DMSO for 1 h prior to trypsinisation. 1x10^5 cells were seeded onto polyHEMA coated dishes in DMEM with 10% NCS in the presence or absence of doxycycline and in the indicated concentration of LY 294002 for 32 h. Tritiated thymidine was added to the cells 6 h prior to harvesting and a thymidine incorporation assay was performed.
Figure 4.12

(a) EGF + - + + + +
LY (µM) - - 2 5 10 50
phospho-AKT
AKT
phospho-ERK

(b) EGF + - + + + +
Time (h) 24 24 8 16 24 32
LY (µM) - - 10 10 10 10
phospho-AKT
AKT

Figure 4.13

[Graph showing thymidine incorporation and fold increase with dox (100 ng/ml) and various LY concentrations (0, 2, 4, 6, 10 µM)]
Figure 4.14 Raf inhibits the ability of Rac1V12 to induce anchorage-independent growth

STRAC.C cells were infected with LXSN RER retrovirus and cells resistant to 2.5 μg/ml puromycin and 1 mg/ml G418 were pooled to form a polyclonal population.

a) STRAC/RER cells were incubated in 0.5% NCS for 48 h then trypsinised and seeded onto polyHEMA coated dishes in DMEM with 10% stripped NCS, insulin and if indicated doxycycline and ethanol or the indicated concentration of 4-OH tamoxifen. Cells were harvested after 36 h (SUSP). In addition cell lysates were taken from subconfluent populations of STRAC/RER cells adhered in DMEM with 10% stripped NCS in the presence of doxycycline (ADH). Cell lysates were separated by SDS-PAGE, using both a 12.5% gel and a MAP kinase shift gel and analysed by western blotting. The MAP kinase blot was probed with 122.2 antibody against p42Erk2 and the other blot was probed with 9E10, then cut into two pieces which were sequentially probed with sc-596 and sc-391 then sc-527 to detect Cyclin D1, Cyclin A, p21Cip1 and p27Kip1 respectively.

b) STRAC/RER cells were incubated in 0.5% stripped NCS for 48 h then trypsinised and seeded onto polyHEMA coated dishes in DMEM with 10% stripped NCS, insulin, BrdU and if indicated doxycycline and ethanol or the indicated concentration of tamoxifen. Cells were harvested after 36 h. Cells were then cytospun onto glass slides and fixed. Their BrdU content was analysed by immunohistochemistry using an anti-BrdU antibody and all cells were marked by staining with Hoechst 33258. Cells were photographed and counted, the total cell number for each point was between 150 and 250. The percentage of BrdU positive cells is represented in the figure.

e) STRAC/RER cells incubated in DMEM with 10% stripped NCS were trypsinised and 1.5x10^5 cells were incubated in MC matrix and if indicated, doxycycline and ethanol or the indicated concentration of tamoxifen for 48 h. Tritiated thymidine was then mixed in and cells were incubated for a further 14 h and then harvested and assayed for thymidine incorporation.

Figure 4.15 RhoAV14 induces anchorage-independent growth

ΔRER/Rho and ΔRER/plink cells cultured in DMEM with 10% stripped NCS were trypsinised and 1x10^5 cells were incubated on polyHEMA coated 6 well plates in DMEM with 10% stripped NCS. 16 h later 100 nM 4-OH tamoxifen or ethanol was added to the indicated cells for 22 h. Tritiated thymidine was added to the cells 6 h prior to harvesting and a thymidine incorporation assay was performed. Each point was performed in triplicate.
5. Discussion
5.1 Regulation of the cell cycle by Ras and Raf

5.1.1 Signal strength determines signal specificity

A possible mechanism by which multiple effects can be generated by activation of the same signal transduction pathway has been identified. We have shown that Raf-1 signals of different strengths are able to differentially induce protein expression, which has consequences for the mitogenic response of the cell. The specificity of ERK signalling is also determined by the duration of activation (Marshall, 1995). For example, in PC12 cells NGF causes cell cycle arrest and differentiation and has been shown to prolong activation of ERK for at least 24 h, these effects can be mimicked by constitutively activated Ras or MEK1 (Cowley et al., 1994; Decker, 1995; Noda et al., 1985). In contrast EGF stimulates ERK activation only transiently and stimulates mitogenesis (Decker, 1995). Interestingly it has been shown recently that although NGF initially stimulates Ras-mediated activation of ERK, the sustained ERK activation is independent of Ras and is mediated by Rap1, a Ras family member through activation of B-Raf and MEK1 (York et al., 1998). B-Raf may be the principal target of NGF stimulation because it is able to activate ERK more strongly than Raf-1 (Pritchard et al., 1995). This implies that regulation of both duration and strength of signals are mechanisms used by the cell to determine signal specificity.

Hormonal activation of ΔRaf:ER constitutively activates ERK and prevents growth factor stimulated DNA synthesis of 3T3 fibroblasts and Schwann cells (Lloyd et al., 1997; Samuels and McMahon, 1994). We have shown that this inhibition can be prevented by reducing the strength of the Raf-1 signal. It would be interesting to observe whether altering the duration of Raf-1 activation would have similar effects, i.e. whether a short intense pulse of Raf-1 activation, to mimic growth factor stimulation, would be sufficient to have a proliferative effect in NIH 3T3 cells, and also whether this would be more mitogenic than sustained Raf-1 activation at a low level.

The finding that a cell can utilise a single pathway to elicit multiple effects has profound implications for the conclusions that have been drawn from studies in which proteins have simply been overexpressed or inhibited. We have demonstrated the value of using an inducible system which allows the level of activation of a signal to be controlled, rather than just switching signals on and off, since additional properties may be revealed.

5.1.2 Signal specificity is regulated by differential protein expression

Cell growth, arrest or death are some of the consequences of Ras or Raf-1 activation of specific signal transduction pathways. We have proposed a mechanism through which Ras may elicit two of these effects: proliferation and cell cycle arrest. We have shown that Raf-1 target proteins have different thresholds of induction by Raf-1. This is
demonstrated by the ability of RafAR to induce Cyclin D1, but not p21^{Cip-1}, at moderate levels of activation. Whereas strongly activated RafAR is able to induce both p21^{Cip-1} and Cyclin D1. Analogous work by Woods et al (1997) has shown that the presence of p21^{Cip-1} in CDK2 and CDK4 complexes and their associated kinase activities is sensitive to the level of activation of ΔB-Raf:ER, and this correlates with the ability of ΔB-Raf:ER to stimulate DNA synthesis.

This ability to differentially regulate protein expression which correlates with the effects on DNA synthesis has led to a model of Raf signal strength determining cellular responses through differential protein induction. Such that in the absence of p21^{Cip-1} induction, Cyclin D1 protein is expressed and CDK2 and CDK4 complexes are active and are able to phosphorylate pRb and drive cell cycle progression. However, induction of p21^{Cip-1} by strong Raf-1 signals inhibits cyclin/CDK kinase activities and pRb remains hypophosphorylated. One might expect that in the absence of Cyclin D1 Raf-1 activation may cause a more profound arrest. However since Cyclin D1 null MEFs are inhibited to a similar extent to wild type MEFs upon activation of RafAR there is probably redundancy with other D-type cyclins. Importantly as Ras^{V12} or RafAR signal intensities increase we observe gradual inhibition of DNA synthesis and a gradual induction of p21^{Cip-1}. This suggests that a delicate and sensitive balance exists between the cyclin-associated complexes and p21^{Cip-1} that controls their kinase activity which regulates the contrasting effects of Raf-1 activation.

Interestingly, recent studies into the development of dorsal appendages on the Drosophila egg have revealed a mechanism where different thresholds of ERK activity, regulated by the EGF receptor, induce expression of different genes which change cell fate. Initially ERK is weakly activated by Gurken, a Drosophila homologue of TGFα, through the EGF receptor. This activates Spitz (another TGFα homologue) which promotes the development of the dorsal appendages. However Spitz further activates ERK through a positive autocrine loop and when ERK activity increases beyond a certain threshold, Argos expression is induced. This protein is inhibitory to the development of the dorsal appendages because it activates a negative feedback loop and downregulates ERK (Wasserman and Freeman, 1998). Thus different strengths of ERK activity induce differential protein expression which has developmental consequences.

5.1.3 Raf-induced cell cycle inhibition is mediated by p21^{Cip-1}

We have demonstrated both genetically and biochemically that p21^{Cip-1} is required for inhibition of DNA synthesis by activated Raf-1. Woods et al (1997) have analogous findings through the use of various Raf family oestrogen receptor fusion proteins. This concurs with the observation in Schwann cells that the expression of antisense p21^{Cip-1} constructs reduced the degree of DNA synthesis inhibition caused by ΔRaf:ER activation.
Lloyd et al., 1997). Suggestive but not conclusive evidence from investigations into NGF-mediated cell cycle arrest of PC12 cells also supports a major role for p21^Cip-1 as a mediator of Raf/MEK/ERK induced cell cycle arrest. NGF stimulation of PC12 cell differentiation and cell cycle arrest involves a MEK dependent translational upregulation of p21^Cip-1 (Pang et al., 1995; van Grunsven et al., 1996). Furthermore, ectopic expression of p21^Cip-1 can arrest the cells and stimulate neurite outgrowth (Erhardt and Pittman, 1998). In addition NGF-mediated cell cycle arrest of NIH 3T3 cells ectopically expressing the NGF receptor, TrkA, upregulates p21^Cip-1 which enters CDK2 and CDK4 complexes which are inactivated (Decker, 1995; Pumiglia and Decker, 1997). We have shown that p21^Cip-1 enters and specifically inhibits the associated kinase activities of G1 cyclin/CDK complexes. Activation of D-type cyclin-associated kinase activity is necessary for the phosphorylation of pRb, which is required for progression through G1. We have shown that pRb becomes hypophosphorylated upon RafAR activation. RafAR activation also inhibits Cyclin E-associated kinase activity which also has a role in pRb phosphorylation, but has additional roles which may involve initiation of immediate early transcription. Thus inhibition of these activities by p21^Cip-1 should be sufficient to prevent G1 to S-phase progression.

5.1.4 p21^Cip-1 upregulation by Raf is independent of p53

We have shown that RafAR activation can increase p21^Cip-1 promoter activity. p21^Cip-1 is a transcriptional target of p53 (el-Deiry et al., 1993) yet we have shown in MEFs that p21^Cip-1 protein levels can be induced by RafAR in the absence of p53. This was also observed by another group who performed similar work using ΔRaf:ER molecules (Woods et al., 1997). Nevertheless, p53 function has been shown to be necessary for growth inhibition and a senescent phenotype that was observed in MEFs that were retrovirally infected with Ras^V12 or with MEK1^P56 (a constitutively active MEK1 mutant). Rather than these oncogenes inhibiting DNA synthesis in p53 null MEFs as we had observed upon RafAR activation; Ras^V12 and MEK^P56 actually promoted DNA synthesis (Lin et al., 1998; Serrano et al., 1997). This response concurred with experiments in which Ras^V12 expression permitted p53 null MEFs to proliferate in the absence of anchorage and form tumours when injected into nude mice (Tanaka et al., 1994).

This difference in p53-dependency is unlikely to involve Ras-mediated, Raf independent pathways since a similar requirement for p53 is observed with constitutively active MEK1. Furthermore Ras^V12 mediates proliferative inhibition through MEK since growth arrest can be partially inhibited by the MEK specific inhibitor, PD 98059 (Lin et al., 1998).

The difference in p53-dependency in MEFs is more likely to be associated with the different kinetics of Raf-1-MEK1-ERK activation. We have observed that different intensities of Raf signals can elicit different responses. The kinetics (and probably the
intensities) of activation of ERK caused by rapid activation of RafAR with hormone will differ from constitutive expression of MEK1^{ps6} or Ras^{v12}. This is probably what contributes to the difference in p53 dependency. Rapid activation of RafAR may induce an additional inhibitory mechanism which is independent of p53 function, for example, upregulation of p21^{cip-1}. This mechanism may not be induced by constitutive expression of Ras^{v12} or MEK1^{ps6} because it is blocked by feedback loops caused by constitutive ERK activation, which are not established in the case of rapid RafAR activation. Indeed ΔRaf:ER cells activate ERK to 20 fold lower levels if they have been selected continuously in the presence of activating hormone compared to ΔRaf:ER activation in cells which were selected in the absence of hormone (Kerkhoff and Rapp, 1998). Thus RafAR may control two mechanisms that lead to growth arrest; a p53-independent upregulation of p21^{cip-1} and possibly a p53-dependent pathway, that may or may not involve p21^{cip-1}, whereas constitutive expression of Ras^{v12} or MEK^{ps6} may induce only the p53-dependent inhibitory pathway. Furthermore, p53-dependency appears to vary between cell types since ΔRaf:ER-mediated inhibition in Schwann cells is p53-dependent, indicating the absence of a mechanism allowing p53-independent upregulation of p21^{cip-1} in response to rapid activation of ΔRaf:ER (Lloyd et al., 1997).

5.1.5 Putative mechanisms for p53 independent induction of p21^{cip-1}

There are many instances of p21^{cip-1} upregulation in cells deficient of p53 function, however the mechanisms of induction have not been fully defined (el-Deiry, 1998). Although we have not categorically demonstrated it, our results suggest that Raf is able to induce p21^{cip-1} transcription in the absence of p53 function. Induction of p21^{cip-1} transcription in the absence of p53 has been demonstrated many times (el-Deiry, 1998; Michieli et al., 1994). The transcriptional co-activator p300 has been implicated in this. A mutant of the adenovirus E1A protein that specifically sequesters p300 is able to block NGF-mediated, p53-independent, upregulation of p21^{cip-1} in PC12 cells in addition to preventing the subsequent cell cycle arrest and neurite outgrowth (Billon et al., 1996). Furthermore, the transcriptional upregulation of p21^{cip-1} that occurs upon keratinocyte terminal differentiation also requires p300 or a protein with similar E1A binding properties (Missero et al., 1995). Since activation of ERK has been implicated in PC12 cell and keratinocyte differentiation it is possible that p300 may have a role in the Raf-mediated growth inhibition we observe.

In addition, the transcription factor and tumour suppressor protein, IRF-1 has been shown to transcriptionally induce p21^{cip-1} independently of p53 in response to DNA damage or TGF β (Miyazaki et al., 1998; Tanaka et al., 1996). Binding sites for the Ets family and Sp1 family of transcription factors within the p21^{cip-1} promoter have also been demonstrated to be necessary for upregulation of p21^{cip-1} promoter activity in response to factors that are able to mediate p53-independent upregulation of p21^{cip-1} and growth arrest.
(Biggs et al., 1996; Funaoaka et al., 1997; Nakano et al., 1997). These data suggest that a variety of different mechanisms have the capacity to regulate p53-independent induction of p21Cip-1 transcription.

We have demonstrated that p21Cip-1 can be transcriptionally upregulated by Raf-1 activation. However other mechanisms may also regulate p21Cip-1 expression. p21Cip-1 levels can be upregulated independently of transcription, for example induction of p21Cip-1 by okadaic acid is not inhibited by the transcriptional inhibitor, actinomycin D, whereas transcription is required for p21Cip-1 induction by the phorbol ester TPA (Zeng and el-Deiry, 1996). This may occur in part through stabilisation of p21Cip-1 mRNA which has been shown to occur by a mechanism dependent on MEK in response to stimulation by PKC-activating phorbol esters (Akashi et al., 1999). p27Kip1 is also regulated by post-transcriptional mechanisms involving translational regulation and protein stability (Hengst and Reed, 1996; Pagano et al., 1995). Protein degradation may also have a role in regulating p21Cip-1 levels since p21Cip-1 is a target of ubiquitination (Maki and Howley, 1997).

In addition to regulating the activity of p21Cip-1 through control of its expression levels, there is also some evidence suggesting that p21Cip-1 activity may also be regulated by a p21Cip-1 inhibitor protein (Hermeking et al., 1995). This concurs with our observations in which PI 3-kinase activity was able to stimulate DNA synthesis in the presence of high levels of p21Cip-1. However this protein has not yet been identified. It may be the case that multiple proteins are able to sequester inhibitors from their cyclin/CDK targets. For example, Cdc25A is upregulated by c-myc and can compete with p21Cip-1 for Cyclin/CDK binding (Saha et al., 1997). The HPV16 oncoprotein E7 is also able to block inhibition by p21Cip-1 (Funk et al., 1997). An unidentified activity is also able to inhibit p27Kip1 inhibition in response to c-myc to promote cell cycle progression. The effects of the unidentified inhibitor cannot be reproduced by Cdc25A or the G1 cyclins (Vlach et al., 1996).

5.1.6 p21Cip-1 is not necessary for Ras or Raf-mediated cell cycle arrest of human fibroblasts

Interestingly, in contrast to murine fibroblasts, human fibroblasts appear able to arrest and acquire a senescent phenotype in response to activation of the Ras-Raf pathway independently of p21Cip-1 or p53. Activated ΔRaf:ER or constitutively expressed RasV12 were able to inhibit the proliferation of non-immortalised IMR-90 human fibroblasts expressing the human papilloma virus E6 protein (which ablated p53 and p21Cip-1 expression) or a dominant negative p53 mutant, respectively (Serrano et al., 1997; Zhu et al., 1998). The difference in response of the human and murine cells may be due to the presence of additional checkpoint control pathways in human cells. Human cells are much more resistant to immortalisation and transformation than rodent cells. For
example, co-expression of activated Ras, HPV E6 and E7 (which inactivates pRb) and overexpression of telomerase does not permit human fibroblasts to grow in soft agar (Morales et al., 1999). This indicates that human cells are well protected against oncogenic activation and that multiple mechanisms protect the cells. These probably involve multiple cell cycle inhibitor proteins, for example both p16^INK4a and p21^Cip1 are induced by ΔRaf:ER activation in IMR-90 cells (Zhu et al., 1998) and p27^Kip1 is induced in human small cell lung carcinoma cells (Ravi et al., 1998). However, although overexpression of a single inhibitor is sufficient to induce cell cycle arrest (Zhu et al., 1998), the loss of one is insufficient for deregulated growth, for example, neither inhibition of p21^Cip1 or p16^INK4a (through the use of a dominant negative CDK4 mutant) was able to prevent cell cycle inhibition by Ras (Serrano et al., 1997; Zhu et al., 1998). This implies that there are multiple backup mechanisms in place. It would therefore be of interest to inhibit multiple cell cycle inhibitor proteins in human fibroblasts and assess the response of the cells to Ras or Raf activation.

5.1.7 Utilisation of a developmental differentiation pathway as a defence against oncogenic activation

Inhibition of cell proliferation by activation of Ras, Raf and MEK1 has been demonstrated in many mammalian cell types, including: non-immortalised human fibroblasts; human small cell lung cancer cells; rat fibroblastic cell-lines; rat Schwann cells; PC12 cell and, as we and others have shown, primary and immortalised murine fibroblasts (Lin et al., 1998; Lloyd et al., 1997; Noda et al., 1985; Olson et al., 1998; Pritchard et al., 1995; Ravi et al., 1998; Samuels and McMahon, 1994; Serrano et al., 1997; Sewing et al., 1997; Woods et al., 1997; Zhu et al., 1998). Interestingly many instances of Ras or Raf activation result in differentiation. For example, NGF stimulation or activation of Ras or MEK1 causes PC12 cells to differentiate, phenotypically demonstrated as cell cycle arrest and neurite outgrowth (Cowley et al., 1994; Decker, 1995; Noda et al., 1985). Moreover, the importance of regulated Raf signalling in differentiation is demonstrated in vivo by the aberrant differentiation patterns in transgenic mice expressing activated Ras and Raf. For example, expression of activated Raf in the thymus accelerates T-cell differentiation (O'Shea et al., 1996) and activated Ras under the control of the keratin 10 promoter resulted in increased differentiation which led to hyperkeratosis of the skin (Bailleul et al., 1990). Furthermore, the Ras/Raf/ERK pathway has been implicated in differentiation in invertebrates. In Drosophila, Ras and Raf are required for terminal differentiation of the R7 photoreceptor cell and posterior terminal cell fates (Dickson et al., 1992; Lu et al., 1993; Sprenger et al., 1993). In C.elegans the Raf homologue, Lin45, is involved in the transmission of inductive signals that promote migration and terminal differentiation of vulval precursor cells from the homologues of the GGF
receptor and Ras, Let23 and Let60 respectively (Han et al., 1993; Sternberg et al., 1993). The mechanism for cell cycle arrest by MAP kinase cascades through induction of cyclin/CDK inhibitor proteins is also conserved in yeast. Pheromone-mediated G1 arrest in S.cerevisiae is executed via a MAP kinase cascade which activates an ERK homologue, Fus3, which regulates the activity of the transcription factor, Ste12. Ste12 is necessary for proper regulation of the Cln/Cdc28 kinase inhibitor, Far1, which mediates G1 cell cycle arrest (McKinney and Cross, 1995; Oehlen et al., 1996; Peter et al., 1993; Peter and Herskowitz, 1994).

Therefore although transient activation of the ERK MAP kinase pathway is required for controlled cell cycle progression, aberrant, constitutive activation of this pathway has the potential to cause deregulated cell proliferation. However, the cell utilises a developmental mechanism for terminal differentiation as a protective mechanism against sustained oncogenic activation of the ERK MAP kinase pathway. This would lead to cell cycle arrest and thus prevent propagation of a potentially tumorigenic cell. In fibroblasts activation of this pathway has been shown to result in senescence (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998). This may be an alternative to differentiation since fibroblasts are not thought to differentiate.

5.1.8 Physiological relevance of controlling cellular responses by signal strength

We have proposed a mechanism that a cell may use for determining signal specificity based on constitutive activation of signalling pathways which mimics a similar scenario to oncogenic activation. As discussed above the development of dorsal appendages in the Drosophila egg rely on ERK signals of different strengths (Wasserman and Freeman, 1998). Sustained activation of ERK by NGF has also revealed a mechanism in which differentiation is determined by the duration of activation of a signal (Marshall, 1995; York et al., 1998). A multitude of possible physiological mechanisms that could be utilised to regulate the degree of activation of a signalling pathway have already been identified. For example ERK activity has been shown to be regulated by the availability and type of growth factors (Decker, 1995) and the availability of their receptors (Fan et al., 1995); by the attachment of cells to substratum and ligation of integrin receptors (Hotchin and Hall, 1995; Zhu and Assoian, 1995); by the phosphorylation of Sos (Foschi et al., 1997); by the presence of Ras effectors that may compete with Raf for Ras binding and activation (Kikuchi and Williams, 1996); by the different strengths of activation of Raf family members (Pritchard et al., 1995); by the action of specific MAP kinase phosphatases (Duff et al., 1995; Foschi et al., 1997; Sun et al., 1993); by the availability of scaffold/adaptor proteins (Schaeffer et al., 1998; Whitmarsh et al., 1998) and by many other factors which regulate the activities of proteins upstream of ERK (Grammer and Blenis, 1997).
5.1.9 Signal specificity is also affected by the expression of other regulatory proteins

The ability of different strengths of Raf signals to differentially activate downstream pathways provides an explanation for the contrasting abilities of A-Raf and B-Raf oestrogen receptor fusion proteins to induce DNA synthesis at similar levels of hormonal stimulation (Pritchard et al., 1995). Signal specificity can also be altered by the activation of other proteins, for example, c-myc is able to induce sequestration of p21\textsuperscript{Cip-1} and thus inhibit cell cycle arrest by RafAR (Sewing et al., Manuscript in preparation). Similarly, the introduction of v-mil (avian v-Raf) into macrophages is able to co-operate with v-myc and induce DNA synthesis in reduced serum conditions (Graf et al., 1986). Viral oncogenes are also able to change the response of cells to Raf-1 activation, expression of both SV40 Large T Antigen and Adenovirus E1A are able to rescue inhibition of cell cycle progression by ΔRaf:ER activation (Lloyd et al., 1997; Ravi et al., 1998).

5.1.10 RafAR-mediated upregulation of p21\textsuperscript{Cip-1} and growth inhibition is not dependent on Cyclin D1

We and others have shown that activated Ras and Raf-1 are able to upregulate Cyclin D1 protein levels (Filmus et al., 1994; Liu et al., 1995; Lloyd et al., 1997; Winston et al., 1996). Although it is mainly considered an activator of DNA synthesis, Cyclin D1 has also been implicated in cell cycle arrest. For example, ectopic expression of Cyclin D1 in PC12 cells can promote cell cycle arrest and differentiation (Yan and Ziff, 1995). In addition, a p53-dependent arrest in mouse fibroblasts requires Cyclin D1 induction (Del Sal et al., 1996). Moreover, ectopic induction of Cyclin D1 expression in NIH 3T3 cells has recently been shown to induce p21\textsuperscript{Cip-1} and p53. However this did not lead to cell cycle inhibition instead these cells acquired the ability to grow in soft agar (Hiyama et al., 1997). In this case p21\textsuperscript{Cip-1} may have had a role in promoting cyclin-CDK complex assembly or stability (LaBaer et al., 1997). However through the use of Cyclin D1 null MEFs we have demonstrated that the presence of Cyclin D1 is not necessary for the Raf-mediated cell cycle arrest or the upregulation of p21\textsuperscript{Cip-1}. However the participation of other D-type cyclins cannot be discounted.

5.1.11 Activated Ras has greater ability to induce DNA synthesis than activated Raf-1

Ras\textsuperscript{V12} is much more potent at inducing DNA synthesis than activated Raf-1. This concurs with the reported properties of Ras effector domain mutants. The Ras\textsuperscript{V12S35} mutant is able to bind Raf and stimulate ERK activation, however it was unable to stimulate DNA synthesis unless it was co-expressed with another Ras mutant, Ras\textsuperscript{V12G37} (Joneson et al., 1996b). Thus activation of two separate signal transduction pathway by Ras is more mitogenic than Ras-mediated activation of Raf. In addition the capacity of Raf-1 to stimulate DNA synthesis is severely restricted by the narrow window in which
Raf signals are sufficiently strong to induce positive signals such as Cyclin D1 upregulation, but not so strong as to induce p21$^{CIP-1}$. In comparison, high levels of Ras$^{V12}$ efficiently induce DNA synthesis. It would be of interest to determine whether further activation of Ras would also cease to induce DNA synthesis, i.e. whether the mitogenic differences were due to differences in the relative activity of endogenous Raf activated by Ras$^{V12}$ and the Raf-1 mutants. However, Raf-independent downstream targets of Ras may also affect the activity of the ERK MAP kinase pathway. Downstream targets of Ras, for example, Rac and Rho, have been shown to affect activation of Raf and MEK by Ras (Frost et al., 1997; King et al., 1998; Renshaw et al., 1996). Therefore one could not be sure whether differences in ERK activation by Ras$^{V12}$ and Raf-1 mutants were quantitative or due to activation of Raf-independent signal transduction pathways by Ras feeding into the ERK MAP kinase module.

5.1.12 A Ras-mediated pathway inhibits Raf induction of p21$^{CIP-1}$

The differences in the stimulation DNA synthesis by Ras and Raf-1 may be caused by their contrasting abilities to induce p21$^{CIP-1}$ in serum-deprived cells. p21$^{CIP-1}$ is the main factor responsible for Raf-mediated inhibition and when expressed by similar mechanisms of gene induction RafCAAX upregulates p21$^{CIP-1}$ much sooner than Ras$^{V12}$. Importantly, induction of p21$^{CIP-1}$ by RafCAAX occurs prior to S-phase entry, in contrast to induction of p21$^{CIP-1}$ by Ras$^{V12}$ which occurs after S-phase has started. This latter observation is also supported by microinjection data in which p21$^{CIP-1}$ was not induced by Ras$^{V12}$ in serum-deprived NIH 3T3 cells (Olson et al., 1998).

Furthermore, we have demonstrated that Ras$^{V12}$ is able to downregulate p21$^{CIP-1}$ induced by ΔRaf:ER suggesting the participation of an additional Ras effector pathway. Neither constitutive activation of PI 3-kinase nor RasGEF pathways were able to mimic the effect of Ras$^{V12}$. Therefore either downregulation of p21$^{CIP-1}$ is the function of another Ras effector pathway, or our experiments were unsuited to reveal this ability of PI 3-kinase or Rif. The number of putative Ras effectors is large and growing and any of these proteins are potential candidates for downregulation of p21$^{CIP-1}$. In addition, rather than affecting p21$^{CIP-1}$ levels downstream of ERK, Ras may activate another pathway which feeds back negatively into the ERK MAP kinase cascade. PI 3-kinase, conventional PKCs, Rho family GTPases and PAK have been implicated in regulation of ERK activation either independently of Ras or other elements of the ERK MAP kinase cascade. However, we do not observe an effect of Ras$^{V12}$ on ΔRaf:ER-mediated ERK phosphorylation, but changes may occur that are too subtle to be detected by Western blot. Alternatively downregulation of p21$^{CIP-1}$ may also be due to cellular adaptation to constitutive ERK activation by Ras$^{V12}$ which may alter the cellular response to additional ERK activation by
ΔRaf:ER. One could test this by constitutively expressing RafCAAX in cells expressing ΔRaf:ER and testing whether a similar effect is observed.

Viral and cellular oncogenes are able to counteract Ras or Raf upregulation of p21\textsuperscript{Cip-1}. SV40 large T antigen counteracts upregulation of p21\textsuperscript{Cip-1} in Schwann cells, probably through its sequestration of p53 which is necessary for p21\textsuperscript{Cip-1} induction (Lloyd et al., 1997). RhoA activity has also been shown to downregulate p21\textsuperscript{Cip-1} in response to Ras\textsuperscript{V12} and inhibit its transcription (Olson et al., 1998). However the mechanism by which this occurs has not yet been elucidated.

### 5.1.13 Differential regulation of Rho activity by Ras and Raf

Due to the reported ability of Rho activity to inhibit Ras-mediated upregulation of p21\textsuperscript{Cip-1} (Olson et al., 1998), the difference between Ras and Raf-mediated induction of p21\textsuperscript{Cip-1} could be rationalised if they were found to influence RhoA activity differently. It is not yet known whether this is the case or even whether Ras directly activates RhoA. Reagents to test this have very recently been developed. These allow the amount of active RhoA in the cell to be measured by its ability to bind to the Rho-binding domain of one of its specific effectors, Rhotekin (Ren et al., 1999). As previously discussed, many links between Ras and Rho activity have been postulated. Proposed mechanisms for biochemical interaction include the physical interaction of p120\textsuperscript{RasGAP} and p190\textsuperscript{RhoGAP}, RasGEFs which are able to also act as Rho family GEFs or Ras-mediated regulation of RhoGEFs through PI 3-kinase activity. In addition, dominant negative RhoB has been shown to inhibit Ras, but not Raf transformation (Prendergast et al., 1995). However as discussed below regulation of p21\textsuperscript{Cip-1} by RhoA is more complex than initially proposed, and constitutive RhoA activity does not inhibit p21\textsuperscript{Cip-1} induction by Ras, Raf or serum.

### 5.1.14 Co-operative regulation of p21\textsuperscript{Cip-1} induction by Raf-1 and RhoA or PI 3-kinase

We have demonstrated that both PI 3-kinase and RhoA signals can co-operate with ΔRaf:ER to induce p21\textsuperscript{Cip-1}. We also show that the presence of RhoA\textsuperscript{V14} accelerates induction of p21\textsuperscript{Cip-1} by ΔRaf:ER activation. Since neither constitutive activation of PI 3-kinase nor RhoA upregulates p21\textsuperscript{Cip-1} alone they must either upregulate an essential limiting factor necessary for p21\textsuperscript{Cip-1} induction by Raf-1, or downregulate an inhibitory element. This implies that multiple signals can feed into and regulate p21\textsuperscript{Cip-1} expression and that Raf-1 induction of p21\textsuperscript{Cip-1} is not through a linear pathway.

Superinduction of p21\textsuperscript{Cip-1} by co-activation of Raf-1 and RhoA or PI 3-kinase (which acts upstream of Rac) concurs with the observations that Rac and Rho signals feed into the ERK MAP kinase pathway at the level of PAK and permit or assist Raf and Ras activation of ERK (Frost et al., 1997; Frost et al., 1996; King et al., 1998; Renshaw et al., 1996).
Chapter Five - Discussion

We observe a higher level of ERK phosphorylation in serum-deprived confluent RER/Rho cells. This could lead to a greater induction of \( p21^{Gp-1} \) since we have shown that \( p21^{Gp-1} \) induction is sensitive to the level of ERK activation. However neither RhoA nor PI 3-kinase activity upregulates \( \Delta \text{Raf:ER} \)-mediated activation of ERK in cells which are subconfluent, even though \( p21^{Gp-1} \) levels in these cells are still superinduced. Therefore activated RhoA and PI 3-kinase must also affect \( p21^{Gp-1} \) expression through mechanisms independent of ERK activation. Alternatively regulation of ERK may occur through mechanisms independent of its phosphorylation state or in a manner too subtle to detect by Western blot analysis.

The ability of RhoA\(^{V14}\) to superinduce \( p21^{Gp-1} \) upon \( \Delta \text{Raf:ER} \) activation contrasts starkly with the reported ability of RhoA to downregulate \( p21^{Gp-1} \) expression (Olson \textit{et al.}, 1998). However mechanisms are present in cells which allow \( p21^{Gp-1} \) induction in the presence of Rho activity. For example, serum, which activates Rho, also transiently upregulates \( p21^{Gp-1} \) when added to quiescent cells (Michieli \textit{et al.}, 1994). Furthermore, in the presence of serum, Ras and Raf-1 activation are able to induce \( p21^{Gp-1} \) in subconfluent NIH 3T3 cells. Nevertheless contact-inhibited Swiss 3T3 cells in the presence of serum did not induce \( p21^{Gp-1} \) upon microinjection of Ras\(^{V12}\), indeed these cells were induced to initiate DNA synthesis (Olson \textit{et al.}, 1998). This disparity may result from the different proliferative states of the cells. The inhibitory effect of RhoA on \( p21^{Gp-1} \) expression may only occur in response to acute activation of the RhoA pathway through microinjection or treatment with CNF-1 which specifically activates Rho proteins. Cells may respond differently to the sustained activation of the RhoA pathway and may also have adapted to RhoA activation and downregulated certain responses. One could activate Rho activity using CNF-1 in the presence of \( \Delta \text{Raf:ER} \) activation to determine whether the method of Rho activation is responsible for the differences we observe. Alternatively, Ras\(^{V12}\) may co-operate with RhoA to downregulate \( p21^{Gp-1} \) through a Raf-independent pathway which cannot be mimicked by \( \Delta \text{Raf:ER} \). This would fit with our observed differences in the relative abilities of Ras and Raf to induce \( p21^{Gp-1} \).

A further distinction between the experimental methods involved was that microinjection of Ras\(^{V12}\) occurred within a background of non-Ras\(^{V12}\) expressing cells, in contrast to our experiments in which every cell was induced to express Ras\(^{V12}\) or activate Raf-1. Therefore there is a possibility that autocrine signals from normal cells may affect the behaviour of the cells.

However the observation that RhoA\(^{V14}\) in one case represses \( p21^{Gp-1} \) and in another stimulates \( p21^{Gp-1} \) reiterates what we have discovered about signal specificity: the conditions in which a signal is activated can have major implications on the response of the cell to that signal. We have demonstrated an important point, that there is a link
between Rho activity and p21<sup>Cip1</sup> expression which can lead to promotion or inhibition of p21<sup>Cip1</sup> depending on the context of the cell.

5.1.15 PI 3-kinase is able to positively regulate DNA synthesis

Cells expressing a constitutively activated PI 3-kinase mutant were demonstrated to support mitogen-independent growth. This concurs with other data indicating a mitogenic role for PI 3-kinase activity. Recently the ability of PI 3-kinase to induce S-phase has been confirmed. A regulatable PI 3-kinase mutant has been generated that consists of a membrane targeted p110 subunit fused to the hormone binding domain of the oestrogen receptor. Activation of this mutant in a quiescent serum-deprived rat embryo fibroblast cell-line initiated cell cycle entry and also supported growth in soft agar in the presence of serum (Klippel et al., 1998). Furthermore a cell-permeable peptide, based on the p85 binding region of the PDGF receptor that was able to bind p85 and activate PI 3-kinase activity has been demonstrated to stimulate DNA synthesis to the same degree as serum or EGF. This induction was sensitive to inhibition by wortmannin and rapamycin indicating the requirement for PI 3-kinase and p70<sup>S6kinase</sup> activities. Furthermore it was independent of MEK activation and the peptide did not lead to ERK phosphorylation (Derossi et al., 1998). This suggests that the ERK MAP kinase cascade was not involved in this induction. This contrasts with experiments in which PI 3-kinase activity was induced by a monoclonal antibody, which stimulated DNA synthesis. This was sensitive to inhibition of p70<sup>S6kinase</sup>, but also inhibition of Ras and MEK (McIlroy et al., 1997). Thus by consensus it appears that PI 3-kinase requires p70<sup>S6kinase</sup> for its mitogenicity. p70<sup>S6kinase</sup> has been shown to be required for serum-stimulated DNA synthesis however its mechanism of action is unknown. It can directly regulate translation through phosphorylation of the S6 subunit of the ribosomal complex, and furthermore its substrates include transcription factors indicating it can also regulate transcription (Grammer et al., 1996).

It is of interest to note that although the p110-oestrogen receptor fusion protein initiated S-phase in serum-deprived conditions, the cells were unable to complete the cell cycle and underwent apoptosis. Therefore prolonged activation of PI 3-kinase activity to the level induced by the p110:ER protein may have been incompatible with completion of S-phase which caused the cells to die. Our ΔRER/p110 cells may have downregulated PI 3-kinase to a level compatible with cell growth, or may have undergone genetic changes to adapt to constitutive activation of PI 3-kinase. Alternatively strongly activated PI 3-kinase or this particular mutant may have the capacity to directly induce apoptosis in contrast to its recognised role as a survival signal.

The mitogenic ability of PI 3-kinase may be related to the increased levels of Cyclin D1 and downregulation of p27<sup>Kip1</sup>. This inverse relationship of p27<sup>Kip1</sup> and Cyclin D1 also
occurred when quiescent cells were stimulated with serum or Raf, both of which are mitogenic signals. Furthermore, although RhoA$^{V14}$ upregulated Cyclin D1, it did not reduce p27$^{E1}$ levels and also did not induce mitogen-independent growth. It would be of interest to test whether PI 3-kinase induces DNA synthesis through downregulation of p27$^{E1}$ which then allows activation of the G1 cyclin-CDK complexes.

Furthermore, while both RhoA and PI 3-kinase co-operated with Raf to induce p21$^{Cp}$, only PI 3-kinase activity provided resistance to inhibition of DNA synthesis by these high p21$^{Cp}$ levels. Therefore it would be interesting to find out how PI 3-kinase activity is able to overcome this induction of p21$^{Cp}$ and stimulate DNA synthesis. Myc activation is able to abrogate RafAR induced cell cycle arrest in the presence of high levels of p21$^{Cp}$. This is believed to occur through the upregulation of Cyclin D1 and D2 which sequester p21$^{Cp}$ and thus lead to Cyclin E-associated kinase activation (Perez-Roger et al., Manuscript in preparation). PI 3-kinase activity may utilise a similar mechanism, although ARaf:ER induced similar levels of Cyclin D1 in the presence or absence of PI 3-kinase activity. This would require further investigation into the expression and activities of the components of cyclin-CDK complexes.

5.1.16 Ras controlled pathways can work together to induce DNA synthesis

We also demonstrated that multiple Ras effector pathways induce mitogen-independent growth to a larger extent than one alone. This supports the hypothesis that activation of multiple Ras effector pathways control Ras-induced or Ras-mediated cell cycle progression. Further support derives from the ability of any two of the Ras effector domain mutants Ras$^{V12C40}$, Ras$^{V12S35}$ or Ras$^{V12G37}$ to induce focus formation (Rodriguez-Viciana et al., 1997). Furthermore these data also show that different combinations of Ras effector pathways, through co-expression of activated mutants of Ras target proteins can co-operate to induce foci. This is similar to our observation that PI 3-kinase can co-operate with Raf or Rlf to induce DNA synthesis. However the specific combination of Ras effectors appears important since co-activation of Raf-1 and Rlf did not significantly increase DNA synthesis levels. It is possible that Raf-1 and Rlf are components of the same pathway, however, co-expression of Rlf and PI 3-kinase caused a synergistic increase in DNA synthesis, while activation of Raf-1 in the presence of PI 3-kinase only increased DNA synthesis additively. This suggests that Raf and Rlf use different mechanisms to co-operate with PI 3-kinase. A more likely alternative is that Rlf activation is permissive for PI 3-kinase, but not Raf-1, signal transduction. A method of co-inducing PI 3-kinase and Raf-1 or Rlf activity in the same cell would enable a more thorough investigation into the contribution of each of these pathways on DNA synthesis.
5.1.17 Future Directions

It would be of interest to elucidate the mechanism by which Raf upregulates $p21^{Cp-1}$ transcription and to test whether this involves p300. In addition, to further understand the downregulation of $p21^{Cp-1}$ by $Ras^{V12}$ one should determine if it is due to transcriptional downregulation or whether $Ras^{V12}$ alters $p21^{Cp-1}$ mRNA or protein stability. Furthermore, one could try to reproduce the downregulation of $p21^{Cp-1}$ by Ras with Ras effector domain mutants and thus narrow down candidate effector pathways.

The synergistic ability of PI 3-kinase and Raf to induce DNA synthesis would also be worth investigating, especially using regulatable molecules such as the recently developed p110:ER (Klippel et al., 1998). Moreover it would be of interest to determine whether these pathways induce DNA synthesis independently of ERK activation or whether these pathways feed into the ERK MAP kinase module. For example, sustained activation of ERK by PDGF has been shown to be dependent on PKCs and PI 3-kinase activity, but independent of MEK (Grammer and Blenis, 1997).

Investigations into how PI 3-kinase is able to induce DNA synthesis in the presence of high $p21^{Cp-1}$ may reveal novel mechanisms for regulating the inhibitory properties of CKIs. It would be of interest to determine whether the mechanism utilised by PI 3-kinase to overcome $p21^{Cp-1}$ induction by Raf are similar to those of c-myc involving sequestration by D-type cyclins (Perez-Roger et al., Manuscript in preparation)
5.2 Rac and its effect on the cell cycle

5.2.1 Separation of Rac- and Rho-mediated anchorage- and mitogen-independent growth

We and others have demonstrated that activated Rac can support and initiate DNA synthesis in the absence of anchorage (Khosravi-Far et al., 1995; Qiu et al., 1995a; Westwick et al., 1997). RacV12 must therefore be able to propagate signals normally provided by adhesion to substratum. RacV12 has also been reported to induce mitogen-independent growth upon microinjection (Lamarche et al., 1996; Olson et al., 1995). However, since RacV12 does not stimulate mitogen-independent DNA synthesis or colony formation in STRAC.C cells Rac may have two independent roles in DNA synthesis initiation which may be cell type dependent or dependent on Rac signal strength. We observe the same separation in ΔRAR/Rho cells and a similar response is observed upon stable transfection of Rho guanosine exchange factors, Lbc and Dbl, into Swiss 3T3 cells, which could induce anchorage- but not mitogen-independent DNA synthesis (Schwartz et al., 1996). Rac and Rho are linked to adhesion-mediated signalling processes. Rac function has been shown to be necessary downstream of integrins for mammary epithelial cell invasion and collagenase expression in synovial fibroblasts (Keely et al., 1997; Kheradmand et al., 1998). In addition, a requirement for Rho has been demonstrated for integrin-mediated activation of the MAP kinase pathway by the use of Rho specific inhibitors (Renshaw et al., 1996). Integrin and growth factor mediated signals have been shown to converge at the level of ERK activation (Miyamoto et al., 1996).

5.2.2 Rac may be required for DNA synthesis through its activation of PAK

The requirement for Rac, Rho and Cdc42 that has been demonstrated for serum-stimulated DNA synthesis using dominant negative versions of these proteins (Khosravi-Far et al., 1995; Olson et al., 1995; Prendergast et al., 1995; Qiu et al., 1997; Qiu et al., 1995a; Qiu et al., 1995b). Furthermore, there are indications that activation of multiple Rho family proteins are required for DNA synthesis initiation. For instance, although RhoAV14 has been shown to induce mitogen-independent DNA synthesis (Olson et al., 1995), a dominant negative Rac mutant is able to inhibit DNA synthesis in the presence of serum stimulated Rho activity (Khosravi-Far et al., 1995; Nobes and Hall, 1995; Qiu et al., 1995b). This indicates that Rho activity is insufficient to stimulate DNA synthesis in the absence of Rac function. However, it is important to bear in mind that the mode of action and importantly the specificity of these dominant negative mutants is not established. The proposed mechanism of inhibition is through the sequestration of RhoGEFs, however there are multiple RhoGEFs which have exchange activity towards
multiple members of the Rho family. Furthermore, RhoGEFs have additional functions, for example Sos acts as a RasGEF. Therefore S17N-type Rho family mutants may inhibit non-targeted factors that are necessary for mitogenesis and give a false impression of the necessity of Rho family proteins. However, these mutants demonstrate some specificity, since Rac\textsuperscript{N17} does not inhibit LPA stimulation of stress fibres by Rho (Nobes and Hall, 1995). Furthermore, the necessity for Rho activation in DNA synthesis has also been demonstrated with the Rho specific inhibitor, C3 transferase.

Adhesion and mitogens are required for fibroblast proliferation. Thus the requirement for Rho family proteins in serum-stimulated DNA synthesis may utilise their ability to mediate signals from adhesion complexes. Integrin-mediated signals have been demonstrated to feed into the Ras-Raf-MEK-ERK pathway at the level of Ras, Raf and MEK (King et al., 1997; Miyamoto et al., 1996; Nobes et al., 1995; Renshaw et al., 1997; Renshaw et al., 1996). Moreover, adhesion signals have been shown to be necessary for growth factor activation of MEK (Renshaw et al., 1997). Constitutively activated MEK also co-operates with activated Ras to form much larger soft agar colonies than Ras alone, but does not assist Ras\textsuperscript{V12} in adhered low-mitogen conditions. This indicates that MEK is providing signals that are limited by the lack of adhesion (Renshaw et al., 1997). Ras transformation requires Rac and Rho activity, however, since they are unable to mimic the ability of Ras to stimulate mitogen-independent growth, they probably do not act downstream of Ras in a linear pathway. PAK1 is an effector of Rac and was recently shown to phosphorylate MEK1 and Raf-1 and influence their activation (Frost et al., 1997; King et al., 1998). PAK3 activation was also deemed necessary for Raf activation by Ras (King et al., 1998). These pathways may provide a mechanism through which Rac\textsuperscript{V12} could replace the requirement for adhesion-mediated upregulation of MEK through activation of PAK. A requirement for Rac to mediate adhesion signalling could also explain why Rac inhibition prevents serum-stimulation of DNA synthesis and Ras\textsuperscript{V12} transformation (Khosravi-Far et al., 1995; Olson et al., 1995; Qiu et al., 1995a).

In addition, PI 3-kinase has also been shown to be necessary for integrin-mediated activation of Raf-1, MEK1 and ERK, but not Ras (King et al., 1997; Nobes et al., 1995). We have demonstrated that Rac\textsuperscript{V12} is able to upregulate anchorage-independent DNA
synthesis in the absence of PI 3-kinase activity. However, Ras$^{V12}$ and specific growth factors have been shown to require PI 3-kinase activity for Rac-mediated reorganisation of the actin cytoskeleton (Hawkins et al., 1995; Kotani et al., 1994; Nobes and Hall, 1995; Rodriguez-Viciana et al., 1997; Wennstrom et al., 1994a) indicating that PI 3-kinase can activate Rac. Therefore PI 3-kinase may mediate integrin activation of Rac, which would permit PI 3-kinase to activate ERK through PAK. Indeed we have shown that inhibition of PI 3-kinase activity with LY 294002 reduces ERK activation by EGF.

However upregulation of ERK activity through Rac activation of PAK, cannot be the only mechanism required to induce anchorage-mediated growth, since we have shown that ΔRaf:ER is able to upregulate ERK activity in the absence of anchorage and yet when activated to different levels it does not stimulate DNA synthesis. Indeed the response of the cells to ΔRaf:ER activation appears to be identical to adherent cells, with differential expression of Cyclin D1 and p21$^{Cip1}$ caused by different strengths of Raf-1 signal. It may be that transient activation of the ERK MAP kinase cascade by serum is required for Rac$^{V12}$ to assist in the upregulation of MEK. Especially since we do not observe an increase in ERK phosphorylation upon Rac$^{V12}$ expression.

Reported experiments in which constitutively activated MEK mutants have been expressed in NIH 3T3 cells have yielded contradictory findings. One group showed that MEK activation was sufficient for formation colony formation (Cowley et al., 1994), while other investigators observed no anchorage independent growth (Renshaw et al., 1997), although these groups used different MEK mutants. Furthermore, contrary to our findings with ΔRaf:ER and RafCAAX, an amino terminal truncated constitutively activated Raf mutant, ΔRaf22W has been reported to promote colony formation in soft agar (Oldham et al., 1996). These contradictory findings are probably founded in the different abilities of these mutant proteins to activate ERK which we have seen can stimulate different cellular responses.

5.2.3 Activation through the SRE is unnecessary for anchorage-independent DNA synthesis

We have demonstrated that Rac$^{V12}$ in NIH 3T3 (NIH 1) cells does not induce SRE-mediated transcription indicating that activation of the SRE is not required for Rac$^{V12}$-induced anchorage-independent growth. This concurs with the ability of SRF null embryonic stem cells, in which the major SRE binding protein, SRF is not present, to proliferate normally. This implies that SRF is not necessarily required for proliferation, although it does appear to be required for mesoderm formation in the developing embryo (Arsenian et al., 1998). Nevertheless, experiments using competitive inhibition have suggested that SRF is required for DNA synthesis in some cell types. For example, microinjection of double stranded oligonucleotides encoding the SRE, SRF specific
antibodies or polypeptides containing the DNA binding domain of SRF have all been shown to inhibit DNA synthesis induced by serum or microinjected Ras$^{\text{V12}}$ in Ref52 fibroblasts (Gauthier-Rouviere et al., 1993; Gauthier-Rouviere et al., 1991; Gauthier-Rouviere et al., 1990). In addition, transcriptional induction of anti-sense SRF RNA in myogenic cells has been shown to inhibit cell proliferation (Soulez et al., 1996).

### 5.2.4 Serum-mediated activation of SRF is independent of Rac

As we have demonstrated serum, but not activated Rac, stimulates transcription from the SRE in our NIH 1 cell-line, which implies that serum-mediated activation of SRF does not occur directly through activation of Rac, although a requirement for Rac input cannot be discounted. To our knowledge, although Rho activity has been shown to be necessary for induction of SRF activity by serum through the use of C3 transferase (Hill and Treisman, 1995), no requirement for Rac in serum stimulation of the SRE has been demonstrated. Rac has however been shown to be necessary for polyoma virus middle T antigen to signal to SRF in co-transfection assays using dominant negative Rac mutants with middle T antigen (Urich et al., 1997). Thus it is probable that middle T activates a distinct pathway to serum.

### 5.2.5 Activation of JNK is not required for initiation of DNA synthesis

Since JNK was not activated by Rac$^{\text{V12}}$ in STRAC cells, we can conclude that JNK activation is not required for Rac$^{\text{V12}}$-mediated induction of anchorage-independent DNA synthesis. Furthermore, activation of JNK has been shown to be dispensable for Rac$^{\text{V12}}$ induction of mitogen-independent DNA synthesis in Swiss 3T3 cells. The Rac effector domain mutant, Rac$^{\text{L61C40}}$ was able to stimulate mitogen-independent DNA synthesis upon microinjection, but was unable to activate JNK in transient transfection assays (Lamarche et al., 1996; Westwick et al., 1997). This mutant was also able to induce focus formation in co-operation with RafCAAX. Furthermore, Rac effector domain mutants, Rac$^{\text{L61D43}}$ and Rac$^{\text{L61D26}}$, that were able to activate JNK and the SRF but not bind or activate PAK, had reduced focus formation ability in co-operation with RafCAAX (Westwick et al., 1997). Thus it appears that the ability of activated Rac to overcome constraints on DNA synthesis by loss of anchorage, lack of mitogens or contact inhibition is independent of JNK activation.

### 5.2.6 Rac induction of Cyclin D1 may mediate anchorage-independent growth

We have demonstrated that Rac$^{\text{V12}}$ is able to rescue Cyclin D1 expression upon loss of anchorage. Overexpression of Cyclin D1 in NIH 3T3 and Rat1 cells has been reported to promote DNA synthesis in the absence of anchorage (Resnitzky, 1997; Zhu et al., 1996a). Thus induction of Cyclin D1 by Rac$^{\text{V12}}$ may be sufficient to promote anchorage-independent growth.
independent DNA synthesis. Furthermore, overexpression of Cyclin D1 is also able to upregulate Cyclin A in Rat1 cells in suspension (Resnitzky, 1997) which correlates with Rac$^{V12}$-mediated upregulation of Cyclin A which we observe after Cyclin D1 induction. Recently, ectopic induction of Cyclin D1 in NIH 3T3 cells has been shown to support colony formation in soft agar (Hiyama et al., 1997). Moreover, primary fibroblasts transformed by activated Ras and Cyclin D1 are able to form colonies in soft agar (Lovec et al., 1994).

5.2.7 Rac$^{V12}$ induction of mitogen-independent DNA synthesis may be a secondary effect of actin reorganisation

Induction of DNA synthesis by Rac$^{V12}$ has only been demonstrated in confluent serum-deprived Swiss 3T3 cells. It is of interest to note that the induction of membrane ruffles in NIH 3T3 cells by Rac$^{V12}$ is much less profound than in Swiss 3T3 cells (personal communication P.Rodriguez-Viciana). [This is also suggested from published investigations into Rac function by the change in cell-type from NIH 3T3 cells to porcine aorta endothelial cells for demonstration of actin reorganisation (Rodriguez-Viciana et al., 1997; Westwick et al., 1997)]. The property of actin reorganisation has not yet been separated from the ability of Rac$^{V12}$ to induce DNA synthesis in Rac effector domain mutants (Lamarche et al., 1996). Therefore, induction of DNA synthesis may be a consequence of the disruption of contact inhibition through induction of membrane ruffles induced by the microinjected Rac$^{V12}$, rather than by a direct effect of Rac$^{V12}$ on cell cycle control proteins. Alternatively, the properties of the cell types may vary in other ways. For example the reported properties of activated Rho in rodent fibroblasts contrast greatly. In some instances activated Rho is reported to be able to induce malignant transformation and yet in others, cells display no properties of transformation (Khosravi-Far et al., 1995; Perona et al., 1993; Prendergast et al., 1995; Qiu et al., 1995b). Furthermore, the different methods of protein activation may be responsible, since microinjection transmits a large signal very rapidly in contrast to transcriptional derepression or constitutive expression.

5.2.8 Rac$^{V12}$ can assist DNA synthesis induced by additional factors

Although we do not observe Rac$^{V12}$ induction of mitogen-independent DNA synthesis, we have demonstrated that Rac$^{V12}$ can assist colony formation and increase DNA synthesis in cells that are in the presence of mitogens and therefore not quiescent. Rat1 cells stably expressing Rac$^{V12}$ have been shown to have a higher rate of proliferation in reduced serum conditions than control cells, although both cell populations increase in cell number indicating that the cells are not arrested (Qiu et al., 1995a). Furthermore, another group has stated that NIH 3T3 cells expressing Rac$^{V115}$ are able to proliferate in 1% NCS
but do not show their data (Khosravi-Far et al., 1995). We show that Rac^{V12} expression in STRAC.C cells cultured in 2% NCS supports more dense and numerous colonies than control cells. It may be possible to titrate the concentration of serum added to the cells such that one could suppress growth stimulated by serum, but maintain growth mediated by the combination of Rac^{V12} and serum. Therefore it appears that although Rac activation is unable to replace mitogen-signalling it is able to counteract some limiting factor to cell proliferation.

5.2.9 Future directions for investigations into Rac-mediated DNA synthesis

Many questions remain about the mechanisms utilised by Rac to regulate DNA synthesis. It would be of interest to investigate whether Rac^{V12} mediates anchorage-independent DNA synthesis via PAK, and whether this is dependent on activation of MEK. Furthermore, it would be interesting to test whether stimulation of anchorage-independent DNA synthesis occurs through Rac^{V12} input into ERK activation and whether Rac acts as a mediator between integrins and ERK activation. One should also test whether this involves PI 3-kinase and/or Ras.

Further work using the STRAC cells we have developed is impeded by the low percentage of cells (approx. 25%) which are induced to initiate DNA synthesis. This effect is not large enough to acquire mechanistic biochemical information about Rac^{V12} induction of DNA synthesis since Rac^{V12} stimulated events leading to initiation of DNA synthesis would be masked by the 75% of cells that do not initiate DNA synthesis. Thus another system should be utilised to investigate these effects which would allow identification of the cells that were induced to initiate cell cycle progression. One possibility is to utilise fluorescent activated cell sorting to isolate cells which have initiated progression through G1 by using a green fluorescent protein reporter construct driven by a G1 activated promoter, such as Cyclin D1.
6. Materials and Methods
Chapter Six - Materials and Methods

6.1 Materials

6.1.1 Equipment

Centrifuges
Centaur 2, MSE
Megafuge 1.0 R, Heraeus
Microcentrifuge, MSE
Centra-4R centrifuge, IEC
J-6M/E centrifuge, Beckman

Incubators
Water Jacketed Incubators, Forma Scientific and Heraeus

Agarose gel electrophoresis equipment
Mini gel system, Hybaid Ltd
Midi gel system, Northumbria Biologicals Ltd

Western Transfer Equipment
Trans-blot SD Semi-dry Electrophoretic Transfer Cell, Bio-Rad
Genie blotter, Idea Scientific

Polyacrylamide gel electrophoresis equipment
Mini-protean II, Bio-Rad
Midi gel system, Atto
Sequencing gel system, model S2, BRL

Spectrophotometer
UV-Visible spectrophotometer DU-20, Beckmann

Microscopes
Diaphot, Nikon
TMS, Nikon
Axiophot, Zeiss

PCR machine
PTC 200, MJ Research
Power packs
Power-pac 300, Bio-Rad
DC power supply ISOTECH IPS 302 A, RS Components
Power supply model 500/200, Bio-Rad
Power supply model 3000/300, Bio-Rad
Power supply model 200/2.0, Bio-Rad

Shaking Bacterial Incubators
Controlled Environment Incubator Shaker and G24 Environmental Incubator Shaker,
New Brunswick Science

Miscellaneous
Multi-purpose Scintillation Counter LS6500, Beckman
Phosphor-Imager, Molecular Dynamics
Milli-Q Water system, Millipore
Transilluminator model TL-33, UVP
Transilluminator cabinet model CC-60
Filter disk vacuum manifold, Millipore
Luminometer 1251, Bio Orbit, with LKB wallac pump
R100/TW Rotatest Shaker platform, Luckham Ltd
Rocking platform 3013, Jencons Ltd

6.1.2 Frequently Used Solutions
Phosphate buffered saline A (PBSA)
6.5 mM Na2HPO4, 1.5 mM KH2PO4, 2.5 mM KCl, 140 mM NaCl
Tris-EDTA (TE)
10 mM Tris-HCl pH 8.0, 1 mM EDTA
Tris saline
24 mM Trizma base (Sigma), 0.8% (w/v) NaCl, 0.38% (w/v) KCl, 0.01% (w/v)
Na2HPO4, 0.1% (w/v) dextrose, pH 7.7 (adjusted with HCl)
### 6.1.3 Antibodies

**Table 6.1 Primary antibodies used in this thesis**

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Detected Antigen</th>
<th>Source</th>
<th>Antibody type</th>
<th>Dilution for purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DPERK M8159</td>
<td>Thr 202 &amp; Tyr 204 phosphorylated p42erki and p44erki</td>
<td>Sigma</td>
<td>Mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>sc-474</td>
<td>JNK</td>
<td>Santa Cruz</td>
<td>rabbit polyclonal</td>
<td>5 µl per 100 µg of protein for IP</td>
</tr>
<tr>
<td>anti BrdU</td>
<td>BrdU</td>
<td>Beckton Dickinson</td>
<td>Mouse monoclonal</td>
<td>2 µl per cell pellet for FACS analysis</td>
</tr>
<tr>
<td>anti BrdU</td>
<td>BrdU</td>
<td>Amersham-Pharmacia</td>
<td>Mouse monoclonal</td>
<td>undiluted for immunofluorescence</td>
</tr>
<tr>
<td>sc-198</td>
<td>human Cyclin E</td>
<td>Santa Cruz</td>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>phospho p42/p44 MAP kinase</td>
<td>Thr 202 &amp; Tyr 204 phosphorylated p42erki and p44erki</td>
<td>New England Biolabs</td>
<td>purified mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>AKT Ser473 92715</td>
<td>Ser 473 phosphorylated Akt/PKB</td>
<td>New England Biolabs</td>
<td>purified mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>AKT 9272</td>
<td>Akt</td>
<td>New England Biolabs</td>
<td>purified rabbit polyclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>T4026</td>
<td>β-tubulin</td>
<td>Sigma</td>
<td>purified mouse monoclonal</td>
<td>1:2000 for Western</td>
</tr>
<tr>
<td>sc-163</td>
<td>cdk2</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>sc-260</td>
<td>cdk4</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>sc-596</td>
<td>Cyclin A</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>1:200 for Western</td>
</tr>
<tr>
<td>E23</td>
<td>Cyclin A</td>
<td>T.Hunt, ICRF</td>
<td>purified mouse monoclonal</td>
<td>1:500 for Western</td>
</tr>
<tr>
<td>E72</td>
<td>Cyclin A</td>
<td>T.Hunt, ICRF</td>
<td>purified mouse monoclonal</td>
<td>1 µg per 200 µg of cell lysate</td>
</tr>
<tr>
<td>287.3</td>
<td>Cyclin D1</td>
<td>G.Peters, ICRF</td>
<td>rabbit serum bleed out</td>
<td>1:1000 for Western</td>
</tr>
</tbody>
</table>
### Chapter Six - Materials and Methods

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Detected Antigen</th>
<th>Source</th>
<th>Antibody Type</th>
<th>Dilution for Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCS 11</td>
<td>Cyclin D1</td>
<td>J.Bartek, ICB, Copenhagen</td>
<td>purified mouse IgG2A monoclonal</td>
<td>1 μg per 200 μg of cell lysate</td>
</tr>
<tr>
<td>sc-481 (M20)</td>
<td>Cyclin E</td>
<td>Santa Cruz</td>
<td>purified mouse monoclonal</td>
<td>0.5 μg/ml for Western, 0.5 μg per 100 μg of protein for IP</td>
</tr>
<tr>
<td>HA</td>
<td>HA tag epitope</td>
<td>Boehringer Mannheim</td>
<td>purified mouse monoclonal</td>
<td>5-10 μg/ml for IP, 0.5 μg/ml for Western</td>
</tr>
<tr>
<td>122.2</td>
<td>MAP kinase</td>
<td>C.Marshall, ICR, London (Leevers and Marshall, 1992)</td>
<td>rabbit serum</td>
<td>1:15000 for Western, 1 μl per 50 μg of protein lysate for IP/kinase assay</td>
</tr>
<tr>
<td>9E10</td>
<td>Myc tag epitope</td>
<td>G.Evan, ICRF</td>
<td>purified mouse monoclonal</td>
<td>1:100 for Western</td>
</tr>
<tr>
<td>sc 757</td>
<td>p21^{Cip-1}</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>1:100 for Western</td>
</tr>
<tr>
<td>sc-6246</td>
<td>p21^{Cip-1}</td>
<td>Santa Cruz</td>
<td>mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>sc-397</td>
<td>p21^{Cip-1}</td>
<td>Santa Cruz</td>
<td>rabbit polyclonal</td>
<td>1:100 for Western</td>
</tr>
<tr>
<td>sc-1641</td>
<td>p27^{Kip-1}</td>
<td>Santa Cruz</td>
<td>mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>sc-528 (C-19)</td>
<td>p27^{Kip-1}</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>0.5 μg/ml for Western</td>
</tr>
<tr>
<td>14001</td>
<td>pRb</td>
<td>Pharmingen</td>
<td>purified mouse monoclonal</td>
<td>1:500 for Western</td>
</tr>
<tr>
<td>sc-227 C-20</td>
<td>Raf-1</td>
<td>Santa Cruz</td>
<td>purified rabbit polyclonal</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>Y13-259</td>
<td>Ras</td>
<td>ICRF</td>
<td>rat monoclonal</td>
<td>1:10 for Western</td>
</tr>
<tr>
<td>pan-ras (Ab-4)</td>
<td>Ras</td>
<td>Oncogene Science</td>
<td>Mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
<tr>
<td>rCD2</td>
<td>ratCD2 for rCD2p110 fusions</td>
<td>D.Cantrell, ICRF</td>
<td>purified mouse monoclonal</td>
<td>1:1000 for Western</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Detected Antigen</th>
<th>Source</th>
<th>Antibody type</th>
<th>Dilution for purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA9340</td>
<td>Rabbit immunoglobulins</td>
<td>Amersham</td>
<td>Donkey anti-rabbit coupled to horseradish peroxidase (HRP)</td>
<td>1:2000 for Western</td>
</tr>
</tbody>
</table>

**Table 6.2 Secondary antibodies used in this thesis**
### 6.1.4 Plasmids

#### Table 6.3 Plasmids and expression vectors used in this thesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p4xPRE TKCAT</td>
<td>2X SRE oligos: CTAG(Xho)CGATGAAACAAACATGAAACGT (Xho) inserted into Xho site of pBLCAT2</td>
<td>R.Treisman, ICRF, unpublished</td>
</tr>
<tr>
<td>p4xSRE TKCAT</td>
<td>2X PRE oligos: TCGA(Xho)GCCCATATATGCGGAGACCAGCCCATATATGCGGAGCG(Xho) inserted into Xho site of pBLCAT2</td>
<td>R.Treisman, ICRF, unpublished</td>
</tr>
<tr>
<td>pBLCAT2</td>
<td>Chloramphenicol acetyl transferase reporter construct for transient transfection into eukaryotic cells. Contains tk promoter for analysis of cis-acting elements in front of a heterologous promoter.</td>
<td>HL (Luckow and Schütz, 1987)</td>
</tr>
<tr>
<td>pBluescript II</td>
<td>cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBluescript RacV12</td>
<td>myc tagged human Rac-1V12 inserted into EcoRI site from pCEXV3 RacV12 and tested for direction by restriction digest.</td>
<td>BW</td>
</tr>
<tr>
<td>pBPSTR1</td>
<td>tetracycline retroviral vector, containing tet operator sequences within a CMV promoter, and encoding tTA (a fusion of the tet repressor protein and the VP16 transactivator) under the control of an SV40 promoter. Also encoding puromycin resistance gene under the control of the 5'LTR.</td>
<td>S. Reeves Harvard MS, Boston (Paulus et al., 1996)</td>
</tr>
<tr>
<td>pBPSTR1 RasV12</td>
<td>RasV12 cDNA was subcloned as a BamHI fragment into pBPSTR1 and then tested for direction by restriction digest</td>
<td>BW</td>
</tr>
<tr>
<td>pBPSTR1 RacV12</td>
<td>BamHI/EcoRV myc tagged human Rac-1V12 fragment from pBluescript RacV12 directionally cloned into BamHI/PmeI sites of pBPSTR1</td>
<td>BW</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>pBPSTR1</td>
<td>RafCAAX from pMT-SM-myc-raf-CAAX blunt-end ligated as a EcoRI (blunted with Klenow fragment of DNA polymerase)/SmaI fragment into the PmeI site of pBPSTR1. Then tested for direction by restriction digest.</td>
<td>BW</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>mammalian expression plasmid with neomycin resistance gene and cytomegalovirus (CMV) promoter driven expression</td>
<td>InVitrogen</td>
</tr>
<tr>
<td>pcDNA3 Rac&lt;sup&gt;V12&lt;/sup&gt;</td>
<td>myc tagged human Rac&lt;sup&gt;-1&lt;/sup&gt; inserted into EcoRI site from pcEXV3 Rac&lt;sup&gt;V12&lt;/sup&gt; and tested for direction by restriction digest</td>
<td>BW</td>
</tr>
<tr>
<td>pcEXV3</td>
<td>mammalian expression vector with SV40 enhancer</td>
<td>R.Treisman, ICRF (Miller and Germain, 1986)</td>
</tr>
<tr>
<td>pcEXV3 Rac&lt;sup&gt;V12&lt;/sup&gt;</td>
<td>human Rac&lt;sup&gt;-1&lt;/sup&gt; inserted into EcoRI site. Rac has myc epitope tag on its amino terminus (MEQKLISEEDL)</td>
<td>A.Ridley, Ludwig Institute, London</td>
</tr>
<tr>
<td>pCMV Ras&lt;sup&gt;V12&lt;/sup&gt;</td>
<td>human Ha-ras&lt;sup&gt;V12&lt;/sup&gt; cDNA inserted as EcoRI fragment into pcDNA3</td>
<td>AS</td>
</tr>
<tr>
<td>pLink RhoA&lt;sup&gt;V14&lt;/sup&gt;</td>
<td>human Rho-1&lt;sup&gt;V14&lt;/sup&gt; inserted into Nco I/EcoRI of pEFpLink</td>
<td>R.Treisman, ICRF</td>
</tr>
<tr>
<td>pEFBos rCD2-p110</td>
<td>chimeric molecule of rat CD2 (extracellular and transmembrane domain) plus the catalytic p110 subunit of PI 3-kinase, with myc epitope tag on the carboxy terminus inserted into mammalian expression vector, pEF-Bos (Mizushima and Nagata, 1990).</td>
<td>D.Cantrell, ICRF (Reifet al., 1996)</td>
</tr>
<tr>
<td>pEFBos rCD2-&lt;br&gt;p110R/P</td>
<td>Same as pEFBos rCD2-p110 except the p110 subunit of PI 3-kinase point mutated in its ATP binding site (R916P in p110 and R1130P in rCD2-p110)</td>
<td>D.Cantrell, ICRF (Reifet al., 1996)</td>
</tr>
<tr>
<td>pLink</td>
<td>mammalian expression vector with myc epitope tag at 5' end of poly linker</td>
<td>R.Treisman, ICRF</td>
</tr>
<tr>
<td>pJ6Hygro</td>
<td>Hygromycin resistance gene inserted into HindII/Clal site of pJ6Ω</td>
<td>HL</td>
</tr>
<tr>
<td>pJ6Puro</td>
<td>Puromycin resistance gene inserted into HindII/Clal site of pJ6Ω</td>
<td>HL</td>
</tr>
<tr>
<td>pJ6Ω</td>
<td>mammalian expression vector with Rat β actin promoter driving expression</td>
<td>HL (Morgenstem and Land, 1990b)</td>
</tr>
<tr>
<td>pLXSN</td>
<td>moloney murine leukaemia virus retroviral vector with neomycin resistance gene</td>
<td>AL (Miller and Rosman, 1989)</td>
</tr>
<tr>
<td>pLXSN3</td>
<td>derived from pLXSN with new poly linker inserted</td>
<td>AS</td>
</tr>
<tr>
<td><strong>pLXSN3 RafAR</strong></td>
<td>A HindII/EcoRI fragment encoding amino acids 305-647 of the human c-Raf-1 protein fused to the 20 carboxy terminal amino acids of K-ras was amplified with the c-RafCAAX encoding sequence in pcDNA3 as a template (primers 1 &amp; 2). This fragment was fused to the fragment encoding the carboxy terminus of the hormone binding domain of the human androgen receptor (amino acids 646-917), which was amplified by PCR (primers 3 &amp; 4) and cloned in frame with a fragment encoding an amino-terminal Myc tag into the retroviral vector pLXSN3 (a modified version of pLXSN)</td>
<td>AS (Sewing et al., 1997)</td>
</tr>
<tr>
<td><strong>pLXSP</strong></td>
<td>moloney murine leukaemia virus retroviral vector derived from pLXSN but with puromycin resistance gene replacing neomycin resistance gene</td>
<td>AS</td>
</tr>
<tr>
<td><strong>pLXSP3</strong></td>
<td>derived from pLXSP with new polylinker inserted</td>
<td>AS</td>
</tr>
<tr>
<td><strong>pLXSP3 RafAR</strong></td>
<td>Same cloning as pLXSN3 RafAR, but pLXSP3 was used as the vector</td>
<td>AS (Sewing et al., 1997)</td>
</tr>
<tr>
<td><strong>pMT-SM-myc-raf-CAAX</strong></td>
<td>RafCAAX inserted into mammalian expression vector, pMT-SM (Kaufman et al., 1989) as Spe I/ EcoRI fragment. RafCAAX is human c-raf-1 tagged with the myc epitope on its amino terminus (MEQKLISEEDL) and with the CAAX motif from K-Ras on its carboxy terminus (Leavers et al., 1994).</td>
<td>C.Marshall, ICR, London</td>
</tr>
<tr>
<td><strong>pMT3-ARlf-CAAX</strong></td>
<td>As pMT2-HA-Rlf-CAAX, but Rlf has catalytic domain deleted (bases 635-983)</td>
<td>J.Bos, University of Utrecht, (Wolthuis et al., 1997)</td>
</tr>
<tr>
<td><strong>pMT3-Rlf-CAAX</strong></td>
<td>Rlf amino terminus (αα1-532) with K-Ras CAAX motif on carboxy terminus inserted as Sal I/Not I fragment into pMT2-SM-HA mammalian expression vector (derived from pMT2 with HA epitope tag at 5' end).</td>
<td>J.Bos, (Wolthuis et al., 1997)</td>
</tr>
<tr>
<td><strong>pCip-1-Luc</strong></td>
<td>Reporter construct with the luciferase gene under the control of the full length human p21&lt;sup&gt;cip1&lt;/sup&gt; promoter.</td>
<td>Xin Lu, Ludwig Inst for Cancer Research, London (el-Deiry et al., 1993)</td>
</tr>
<tr>
<td><strong>pCH110-lacZ</strong></td>
<td>Reporter construct with the b-galactosidase gene under the regulation of SV40 promoter</td>
<td>Pharmacia</td>
</tr>
<tr>
<td><strong>pUC19</strong></td>
<td>cloning vector</td>
<td>HL (Yanisch-Perron et al., 1985)</td>
</tr>
</tbody>
</table>

**AL:** Alison Lloyd  **AS:** Andreas Sewing,  **HL:** Hartmut Land, ICRF
6.1.5 PCR Primers

primer 1: 5' TGTAAGCTTACGGAGTACTCACAGCCGAA3';
primer 2: SP6
primer 3: TGTGGATCCGCA-CCATGACTGAGGAGACACCAACCAGAAG 3';
primer 4: 5'TGTAAGCTTGGCCG-CTGCCTGGGTGTGGAAATAGATGGG3'

6.1.6 Parental cells-lines

Table 6.4 Parental cells and cell-lines used in this thesis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3 (also referred to as. NIH 1 in Chapter 4)</td>
<td>immortalised mouse embryo fibroblast</td>
<td>C. Marshall, ICR, London</td>
</tr>
<tr>
<td>NIH 2</td>
<td>A different line of NIH 3T3 cells</td>
<td>R. Treisman, ICRF</td>
</tr>
<tr>
<td>Rat-1</td>
<td>immortalised rat embryo fibroblast</td>
<td>HL</td>
</tr>
<tr>
<td>GP+E</td>
<td>ecotropic packaging cell-line</td>
<td>HL</td>
</tr>
<tr>
<td>Bosc 23</td>
<td>ecotropic packaging cell-line</td>
<td>HL</td>
</tr>
<tr>
<td>Cyclin D1-/- MEFs</td>
<td>primary fibroblasts from a Cyclin D1/- transgenic mouse embryo</td>
<td>C. Dickson, ICRF</td>
</tr>
<tr>
<td>p53-/- MEFs</td>
<td>primary fibroblasts from a p53-/- transgenic mouse embryo</td>
<td>M.Fried, ICRF</td>
</tr>
<tr>
<td>p21Cip1-/- MEFs</td>
<td>primary fibroblasts from a p21Cip1/- transgenic mouse embryo</td>
<td>T.Jacks, MIT</td>
</tr>
</tbody>
</table>

6.1.7 Generated stable cell-lines

Unless otherwise stated, construction of these cell-lines was performed as part of this thesis.

Table 6.5 Cell-lines used or generated in this thesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Parental cell-line</th>
<th>Vectors stably integrated</th>
<th>Infection / Transfection</th>
<th>Resistance</th>
<th>pool/clone</th>
</tr>
</thead>
<tbody>
<tr>
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AL: Alison Lloyd, PR: Pablo Rodriguez-Viciana, AS: Andreas Sewing, HL: Hartmut Land, ICRF
6.2 Methods

6.2.1 Maintenance of cell stocks

All cells were grown in water jacketed humidified incubators at 37°C with 10% CO₂. Cells were routinely maintained in DMEM (Media production, ICRF) with 10% (v/v) serum, 100 μg/ml kanamycin (Sigma), 2 μg/ml gentamycin (Gibco BRL). NIH-3T3 cells were grown in new born calf serum (Gibco BRL), GP+E (Markowitz et al., 1990) and Bosc 23 (Pear et al., 1993), viral producer cells, Rat 1 cells and mouse and rat embryo fibroblasts (MEFs and REFs respectively) were grown in foetal calf serum (Bioclear UK Ltd). Cells expressing fusion proteins containing the hormone binding domains of steroid receptors were grown as above, but in DMEM minus phenol red (Media production, ICRF) and the serum was charcoal stripped.

6.2.1.1 Selection markers

Cells stably expressing selection markers had antibiotics added to their media thus:
Puromycin resistance: Cell-lines were selected and cultivated in 2.5-4 mg/ml puromycin (Sigma) in dH2O. The selection of primary MEFs and REFs was begun at 1 mg/ml and increased, if necessary, to 2.5 mg/ml. Neomycin resistance: All cells were selected and cultivated in 1 mg/ml G418 geneticin (Gibco BRL) from 100 mg/ml stock in 10 mM Hepes pH 7. Hygromycin resistance: All cells were selected and cultivated in 150 μg/ml hygromycin B (Calbiochem). Selection was always performed against an uninfected / untransfected control.

6.2.1.2 GPT selection

To maintain the viral producing capability of the GP+E and Bosc 23 cell-lines, they were routinely re-selected in GPT selective media (Mulligan and Berg, 1981) for one week. After re-selection the cells were then weaned in HT medium for at least 24 h.

500 ml of GPT selective media: 403 ml DMEM+ antibiotics, 50 ml foetal calf serum, 20 ml 25x Xanthine, 5 ml 100x Glutamine, 10 ml 50x HAT, 5 ml 100x Mycophenolic acid, 1 ml 500x Aminopterin, 1 ml 500x Thymidine.

HT media: Similar to GPT selective media, but HT supplement (Gibco) replaces HAT supplement and it contains no mycophenolic acid.

Stocks
25x Xanthine (Sigma): 6.25 mg/ml; 1.56 g was added to 250 ml of 0.1 M NaOH, filtered and stored at 4°C
100x Mycophenolic acid (Gibco-BRL): 2.5 mg/ml; 0.25 g was added to 100 ml of 0.1 M NaOH, neutralised with 0.1 M HCl (checked with pH paper), filtered and stored frozen in 5 ml aliquots.
50x HAT Supplement (Sigma H-0262): One vial was dissolved in 10 ml of dH₂O, filtered and stored frozen.

500x Aminopterin (Sigma): 1 mg/ml; 0.05 g was added to 50 ml DMEM, filtered and stored frozen.

500x Thymidine: 3 mg/ml; 0.15 g was added to 50 ml DMEM, filtered and stored frozen.

6.2.1.3 Charcoal stripping of serum

0.5% (w/v) activated charcoal (Sigma) and 0.05% (w/v) dextran (Sigma) was mixed into 500 ml of serum and stirred at 37°C for 3 h. The charcoal was then pelleted at 3000 rpm (Beckman J-6M/E centrifuge) for 10 min and then filtered through a 0.45 μM filter unit (Nalgene Ltd or Millipore Ltd) and frozen in 50 ml aliquots.

6.2.1.4 Passaging of cell-lines

NIH 3T3 fibroblasts, GP+E cells and their derivatives were passaged every three days and diluted to a 1:10 ratio.
Rat 1 cells were passaged every three days and diluted to a 1:20 ratio.
REFs and MEFs were passaged every three days and diluted to a 1:6 ratio.
Bose 23 cells were passaged every two days and diluted to a 1:3 ratio.

4 ml of trypsin (2.5 mg/ml in Tris saline) (media production, ICRF) was added to 16 ml of versene (0.2 mg/ml EDTA in PBSA) (media production, ICRF). Cells were washed two times with PBSA and 1 ml of trypsin-verseine mix was added to a 100 mm dish. Cells were returned to the 37°C incubator until they had lifted off the dish, cells were then taken up in normal growth media and diluted (or counted) and seeded on new dishes as required. Cells were counted by adding 200 μl of cells in suspension to 10 ml of Isoton (Coulter Electronics Ltd) and 0.5 ml was counted with a Coulter Counter (Coulter Electronics Ltd).

6.2.1.5 Freezing of cells

Cells were trypsinised and taken up in growth media. They were then pelleted at 1200 rpm (MSE Centaur 2) for 3 min. The supernatant was removed and the cells were resuspended in freeze media (50% (v/v) serum, 40% (v/v) DMEM and 10% (v/v) Dimethyl sulphoxide (DMSO) (Sigma)). One 90% confluent 100 mm dish would be resuspended in 2 ml of cold freeze media and aliquoted into 4 freezing vials (Nunc). Vials were then put into polystyrene freezing boxes (Whatman) and frozen at -80°C for two days before putting into liquid nitrogen storage.
6.2.1.6 Retrieval of cell stocks from liquid nitrogen

Vials were removed from liquid nitrogen storage and immediately incubated at 37°C until defrosted, then added immediately to normal growth media without antibiotic selection. When the cells had adhered and started to spread, their media were changed to remove DMSO from the cells. Antibiotics used for selection were not added to resistant cells until at least 24 h after retrieval.

6.2.1.7 Quiescence of cells

To arrest cells in the G0 phase of the cell cycle, the cells were seeded to a confluent density (for example 5 x 10^5 NIH 3T3 cells in a six well dish) in normal growth media and left to settle overnight in an incubator. The next day the cells were washed twice with DMEM and cultured in DMEM containing 0.25% (v/v) serum (unless otherwise stated) and usual antibiotics and incubated for 24-48 h.

6.2.1.8 Crystal violet staining of colonies

To stain colonies of cells, dishes were washed twice with PBSA, then a 1% (w/v) crystal violet (Sigma) solution in 10% (v/v) methanol was poured onto cells, then very gently washed away with tap water until no more colour was released. Dishes were then air-dried and colonies were counted, or photographed.

6.2.2 Growing cells in anchorage independent conditions

6.2.2.1 PolyHEMA

10 mg/ml polyHEMA (poly(2-hydroxyethylmethacrylate)) (Sigma) solution was prepared by adding the crystals to 100% (v/v) ethanol and stirring at 37°C overnight. The solution was then stored at 37°C. Dishes were coated by adding half the normal media volume for the dish size (e.g. 5 ml per 100 mm dish) of polyHEMA solution and leaving the dish open in a tissue culture hood for approximately 1 h, until dish had dried and then recoating. When dishes were coated twice they were subjected to 30 min UV irradiation. Whereupon they were stored at 4°C for a maximum of 2 weeks. Unless otherwise stated, cells were quiesced, trypsinised and counted. Then the required number of cells were added to a polyHEMA coated dish in growth media containing 10% (v/v) serum and insulin (5 μg/ml) (Sigma) and usual antibiotics.

To harvest, media containing the cells was transferred to a polypropylene tube and dishes or wells washed out twice with PBSA and added to the tube. Cells were pelleted at 1500 rpm in a Heraeus Megafuge 1.0 R for 5 min and if required, transferred to an Eppendorf tube and washed twice in PBSA. If a single cell suspension was required cells were resuspended in 250 μl of PBSA and 250 μl of Accumax (TCS Biologicals) and incubated...
at 37°C for 10 min. Cells were then forced through a syringe with a 25 G x 2" needle 3 times.

6.2.2.2 Methylcellulose

To prepare methylcellulose a 1000 ml sterile erlenmeyer was weighed and 225 ml of sterile dH₂O was added and brought to the boil on a hot plate. 10 g of methylcellulose, which had been UV sterilised for 30 min, was vigorously stirred into the water, until no lumps were present. The suspension was then returned to the hot plate and brought to the boil once more. Immediately on boiling the suspension was cooled to approximately 50°C under running water, then 250 ml of 2x DMEM (Media production, ICRF) was added and mixed well by swirling. The erlenmeyer was then weighed and dH₂O was added so the weight of the suspension minus the original weight of the erlenmeyer was 503 g. The suspension was stirred strongly overnight in the cold room and then aliquoted and frozen at -20°C. (Note: the erlenmeyer was covered with aluminium foil when out of the tissue culture hood, all ingredients were added in the hood in sterile conditions and an aliquot was tested for contamination before use)

Cell suspensions were made by adding methylcellulose drop-wise to serum and antibiotics and any other necessary reagent and gradually mixing them until a uniform solution was obtained containing 10% (v/v) serum. The required number of quiescent cells was then put into the bottom of a 50 ml polypropylene tube in 1/10th final volume of normal growth media and the required amount of methylcellulose growth media was added drop-wise and gradually mixed into the cell containing media. Tubes were capped loosely and put into a 37°C incubator.

To harvest: ice-cold PBSA was gradually added to the tube and mixed thoroughly, tubes were centrifuged at 3500 rpm (Heraeus Megafuge 1.0 R) for 25 min at 4°C, the PBSA was removed and the procedure was repeated at least two more times, until all methylcellulose was removed. Cells were then harvested as required.

6.2.2.3 Soft agar

2x stocks of agarose in dH₂O were prepared in advance [1.8% (w/v) and 1% (w/v) low gelling temperature agarose (Seaplaque agarose (FMC Bioproducts))] and autoclaved. These stocks were re-heated in the microwave and when completely melted, cooled to 37°C. The other ingredients were heated to 37°C before addition to the melted agarose.

60 mm dishes were coated with 4 ml of bottom agar (see below) and allowed to set at room temperature. Then 4 mls of top agar (see below) was mixed with the specified number of cells, poured on top and allowed to solidify at room temperature. After which, the dishes were put into the incubator. Every 3-4 days 0.5 ml of normal growth media was added. (In the case of cells growing in the presence of doxycycline, top agar was
prepared with doxycycline (100 ng/ml) added and on feeding doxycycline of 500 ng/ml was added to the media).

After 2-4 weeks in culture, when colonies were easily visible to the naked eye, the cells were stained, by addition of 1 ml of 0.1% (w/v) Neutral Red (Sigma) solution in PBSA, at 37°C for 3 h. Dishes were then photographed by the photography department (ICRF).

Bottom agar: 0.9% (w/v) agarose, 10% (v/v) serum, 40% (v/v) 2x DMEM (Media Production, ICRF), 100 µg/ml Kanamycin (Sigma), 2 µg/ml Gentamycin (Gibco BRL).

Top agar: 0.5% (w/v) agarose, 10% (v/v) serum, 40% (v/v) 2x DMEM (Media Production, ICRF), 100 µg/ml Kanamycin (Sigma), 2 µg/ml Gentamycin (Gibco BRL).

6.2.3 Transfection

6.2.3.1 LipofectAmine transfections

The day before transfection, 1.25 x 10^5 cells were seeded in a 6 well dish. On the day of transfection 0.7-1 µg of DNA was mixed with 100 µl of OptiMEM media (GibcoBRL) and 7-10 µl of LipofectAmine (GibcoBRL) (for a 1:7-10, DNA : LipofectAmine ratio) was mixed separately with 100 µl of OptiMEM. The two mixtures were then combined and incubated at room temperature for 30 min. In the meantime, the cells were washed once with OptiMEM and incubated in 800 µl of OptiMEM in the incubator. After 30 min incubation the DNA/LipofectAmine mixture was added to the cells which were then returned to the incubator for 5-6 h. After this incubation the cells were washed once with their normal growth media, before having 3 ml normal growth media added and were then returned to the incubator. This method is shown for transfection in 6 well dishes, but was scaled up for larger dishes.

6.2.3.2 Calcium phosphate transfections (after Wigler et al., 1979)

Cells were seeded to 60-80% confluency the day before transfection. On the evening of transfection the cells were media changed 30 min before transfection into 6 ml of normal growth media. 5-20 µg of DNA in polystyrene 6 ml tubes (Falcon) was mixed with 500 µl of 1x HEPES buffered saline (HBS) (25 mM HEPES pH 7.1, 140 mM NaCl, 1.5 mM Na₂HPO₄) then 20-25 µl* of 2.5 M CaCl₂ was added and immediately shaken. Tubes were incubated at room temperature for 10 min until a fine white precipitate had formed: this solution was then added drop-wise onto the cells which were then incubated in the incubator overnight. Next morning cells were media changed into fresh growth media.

* The amount of CaCl₂ was optimised for each batch of HBS.
6.2.3.3 Transient transfections

For transient transfections the cells were grown for 24-48 h after transfection before the relevant assay was performed. If the assay required low serum conditions; the morning after transfection the serum conditions were reduced and the cells maintained in those conditions for 36-48 h before the assay was performed.

6.2.4 Retroviral infections

6.2.4.1 Preparation of retroviral supernatants

6.2.4.1.1 GP+E cell-lines

GP+E cell-lines were grown to 90% confluency on a 100 mm dish. They were then washed with DMEM to remove selective antibiotics and 4 ml of fresh media (containing the serum requirements of the cells to be infected) was added to the cells. 36-48 h later the media were collected from the cells and filtered through a 0.45 mM filter and either used immediately or frozen in aliquots on dry ice in methanol and stored at -80°C.

6.2.4.1.2 Transient transfection of Bosc 23 cells

Bosc 23 cells were seeded or grown to a density of 90% confluency on a 100 mm dish 24 h before transfection. Cells were then transiently transfected using the calcium phosphate method with retroviral DNA, using a similar method to that described above. However, in addition, 25 μM chloroquine (Sigma) was added to the media prior to transfection and the precipitate was only applied to the cells for 8-10 h. After the precipitate was removed the cells had fresh growth media added overnight and the next morning 4 ml of fresh media (containing the serum requirements of the cells to be infected) was added. The virus was harvested as described above.

6.2.4.2 Retroviral infection of cells

Cells to be infected were seeded 24 h prior to infection at a confluency of 70-80%. Viral supernatants were mixed with 8 μg/ml polybrene (Sigma) (from a 100x stock solution of 0.8 mg/ml kept at 4°C). 2 ml of this mix was applied to one 100 mm dish and incubated at 37°C for 2 h. After which the viral supernatant was aspirated from the cells and the cells were incubated with normal growth media for 24-48 h.

6.2.5 Selection of stably transfected or infected cells

Newly infected or transfected cells were split into selective growth media containing relevant selective antibiotics 36-48 h post-infection or -transfection. The dilution to which cells were split depended on whether pools were required or individual colonies. If pools were required cells were split at two times the dilution they were normally passaged. If
individual colonies were required the cells were split much more harshly at titrated dilutions. The selective media were changed every 3 days until equivalent cells, not expressing selection markers, all died. Cells were then pooled or ring-cloned and maintained in selective media.

**6.2.5.1 Pooling of cells**

If pooled populations of cells were required, dishes containing selected colonies were trypsinised and resuspended in selective media, pelleted at 1200 rpm (MSE Centaur 2) for 5 min and resuspended in selective media. They were then plated at densities of approximately $1 \times 10^6$ cells per 15 cm dish, grown to sub-confluency and either expanded further or frozen down as cell stocks.

**6.2.5.2 Ring cloning**

If individual clones were required, dishes that were sparsely populated with colonies were examined and colonies were circled on the underside of the dish with a marker pen. The cells were washed twice with PBSA. The edge of autoclaved clean glass cloning cylinders (V.A. Howe) were dipped into sterile (autoclaved) silicon grease and placed around the required colonies, forming a seal. 100 μl of trypsin-versene was then added to each and after 3 minutes the cells were transferred to fresh selective media in 24 well dishes. Clones were cultured in these dishes until they had grown to almost confluency and were then split on to larger and larger dishes until there were enough cells to utilise or freeze down.

**6.2.5.3 Tritiated thymidine incorporation assay**

All thymidine assays (unless otherwise stated) were performed in triplicate. Cells were incubated with 1-2 μl per ml of media with tritiated thymidine (185 GBq/m mole 5.0 Ci/m mole) (Amersham) for the stated amount of time. If the cells were attached, the media were removed and the cells were lysed in 1-2 ml 1% (w/v) SDS and added to a polypropylene tube. The wells were then washed out with 1-2 ml of PBSA which was also added to the polypropylene tube. If the cells were in suspension, the cells were collected by centrifugation and lysed in the polypropylene tube with 2 ml SDS after which 2 ml of PBSA was added.

An equal volume of freshly made, ice-cold, 15% (v/v) Tricholoroacetic Acid (TCA) (BDH) (stored as a 100% (w/v) solution at 4°C) was added to the lysed cells in PBSA. The lysates were vortexed and incubated on ice for 10 min. Lysates were then filtered through glass microfibre filters (Whatman 2.5 cm circles) on a Millipore vacuum manifold, the tubes were washed out with 8 ml ice-cold 5% (v/v) TCA which was poured through the filters. Filters were then washed with 20 ml ice-cold 5% (v/v) TCA and then
5 ml 100% (v/v) ethanol. The filters were dried and then put into a scintillation vial with 5 ml scintillation fluid (Ultima Gold). The radioactive decay was measured on a scintillation counter (Beckman LS6500).

6.2.5.4 Cell cycle analysis by FACS

Cells were treated with 10 μM BrdU (from 2 mM stock in dH2O stored at -20°C in the dark) for the required amount of time. Cells were then trypsinised and resuspended in DMEM containing 10% serum. The cells were then pelleted at 1200 rpm (MSE Centaur 2) for 5 min and the supernatant was removed. Cells were washed twice with PBSA and resuspended in 250 μl of PBSA. The cells were then vortexed whilst 700 μl of ice-cold ethanol was gradually added to the cells, which were subsequently incubated on ice for 30 min. Cells were then pelleted and washed twice in PBSA, prior to the addition of 2 M HCl for 20 min at room temperature with frequent mixing. After which cells were washed twice in PBSA and once with PBS-BT (0.5% (v/v) Tween 20 and 0.05% (w/v) BSA in PBSA). 2 μl of anti-BrdU antibody (Beckton-Dickinson) was added directly to the cell pellet for 15 min at room temperature, then washed twice in PBS-BT. 50 μl of FITC-conjugated rabbit-anti-mouse immunoglobulins (DAKO) was added for 15 min at room temperature and cells were washed once in PBS-BT. 100 μl of 100 μg/ml Ribonuclease (Sigma) and 400 μl of propidium iodide (PI) (50 μg/ml) was added to the cells which were analysed on a fluorescence activated cell scanner (FACScan) by flow cytometry, using pulse processing to gate out cell doublets and clumps.

6.2.5.5 Cytospin

Cells in suspension were harvested and a single cells suspension was obtained. Cells were either counted or an estimate was made at the cell number (from known seeding density) and three dilutions of cells (5x10^5, 10x10^5, 20x10^5) were added to 100 μl of PBSA. Frosted end slides (washed once in methanol and dried) and a Cytospin filter (Shandon) were slid into the cytospin funnel apparatus. Cells in PBSA were added to the funnel and centrifuged at 500 rpm for 5 min in a Cytospin 2 (Shandon). Cells were then air dried for 5 min, circled with a Dako immunohistochemistry pen (Dako), and fixed. The slide with optimal cell distribution was used for immunofluorescence.

6.2.5.6 BrdU staining for immunofluorescence

Cells were fixed in methanol at -20°C for 10 min, washed twice with PBSA, and then incubated for 5 min with 2 M HCl and 0.01% (v/v) Triton X-100 in PBSA, washed twice in PBSA, and then blocked for 30 min in blocking solution (3% (w/v) BSA, 10% (v/v) serum (foetal bovine or goat (Gibco-BRL)), 5% (w/v) fat-free dried milk powder, 0.01% (v/v) Tween-20 in PBSA). Cells were then washed twice in PBSA and incubated with 60
μl of undiluted anti-BrdU antibody (Amersham-Pharmacia) for 45 min in the dark. Cells were then washed 5-6 times with 0.01% (v/v) Tween-20 in PBSA. A fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) was diluted 1:300 in blocking solution with 0.1 μg/ml Hoechst 33258 and incubated on the cells for 30 min. Cells were then washed 5-6 times and mounted in mounting medium under a coverslip.

Mounting medium was prepared by mixing 6 g of glycerol and 2.4 g of MOWIOL (Harlow Chemical Company) to 6 ml of dH2O with occasional agitation for 2 h at RT. Then 12 ml of 200 mM Tris-HCl (pH 8.5) was added and medium was heated to 50°C for 10 min with occasional mixing and then spun at 5000 rpm (Heraeus Megafuge 1.0 R) for 15 min. The supernatant was then aliquoted and stored at -20°C, a working solution was kept at 4°C for 1 month and brought to RT before use.

6.2.6 SDS-polyacrylamide electrophoresis (after Laemmli, 1970).

A resolving gel of 7.5-12.5% (w/v) acrylamide:bisacrylamide (37.5:1)* (Anachem 30% (w/v) stock), 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.12% (w/v) ammonium persulphate (APS) was prepared. This was poured between the glass plates of a gel apparatus, to approximately 1 cm below the reach of the comb. Butan-2-ol-saturated TE was layered on top of the gel, which was left to polymerise. After which the gel border was washed three times with dH2O and blotted dry with Whatman 3MM paper. A stacking gel of 3-5% (w/v) acrylamide:bisacrylamide (37.5:1), 0.12 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.167% (v/v) TEMED and 0.22% (w/v) APS was then prepared and poured on top of the resolving gel. A comb was inserted into the gel and the gel was left to polymerise, upon which the comb was removed.

The gel was put into the electrophoresis apparatus and 1x SDS-PAGE running buffer (20mM Tris base, 190 mM glycine (Sigma), 0.1% SDS, pH 8.3) was added. SDS-PAGE sample buffer to a 1x concentration (3% (w/v) SDS, 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 3.2% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue) was added to the cell lysate which was heated to 95°C for 3 min and then loaded onto an SDS polyacrylamide gel using a Hamilton syringe. Molecular weight markers (Amersham Rainbow Markers) were also loaded. The gel was subject to electrophoresis at 80 v until the samples had entered the resolving gel, then the voltage was increased to 150-250 v.

* MAPK shift gels: Resolving SDS polyacrylamide gels for analysing ERK phosphorylation had a different acrylamide to bisacrylamide ratio: 15% acrylamide (from 30% acrylamide solution, Scotlab) and 0.075% bisacrylamide (from 2% N,N'-methylene bisacrylamide solution, Scotlab), the stacking gels was made as described above. MAPK shift gels were run until the 30 kD marker had just run out of the gel.
6.2.7 Western blotting

After electrophoresis 1000 ml of blotting buffer (20 mM Tris base, 15 mM glycine, 20% (v/v) methanol) was prepared, a PVDF membrane (Immobilon, Millipore) was cut to the size of the resolving gel, soaked for 2 min in methanol then washed with dH₂O and equilibrated in blotting buffer for 5 min. The electrophoresis apparatus was dismantled, the stacking gel was discarded and the resolving gel was equilibrated in blotting buffer for 5 min. 3 sheets of 3MM paper cut to size and soaked in blotting buffer, were placed onto the pads (Scotch-Brite, boiled in 0.1% SDS for 1 h and washed well, before initial use) of the blotting apparatus (Genie blotter, Idea Scientific) which was filled with blotting buffer. The gel was placed on the 3MM paper, the polyvinylidene difluoride (PVDF) membrane (Millipore) placed on top and 3 more pieces of 3MM paper placed on top. At each stage, bubbles were excluded from the 'sandwich'. Blotting pads were then placed on top of the 'sandwich' and the apparatus was put together and subject to 25 v for 45 min at 4°C. Then apparatus was dismantled and the membrane washed twice in PBSA. If required the membrane was then stained for total protein with Ponceau S solution (Sigma) to look at efficiency of transfer and then washed 5-6 times with PBSA.

The membrane was then blocked in western blocking buffer (5% (w/v) fat-free milk powder (Mikrobiologie) and 0.01% (v/v) Tween-20 in PBSA) for at least 30 min to prevent non-specific binding. Primary antibody was then added, diluted in western blocking buffer, for 1-4 h room temperature or over-night at 4°C, with agitation (on a shaking or rocking platform or on a rotating wheel). The membrane was then washed five times for 5 min periods in western washing buffer (0.01% (v/v) Tween-20 in PBSA). Secondary antibody conjugated to horseradish peroxidase was then added, diluted in blocking buffer for 45 min at room temperature, then washed 5 times for 5 min. The membrane was then rinsed in PBSA and ECL detection reagent was added (ECL (Amersham) for 1 min exactly or ECL-kPlus (Amersham) for 5-10 min). The membrane was then wrapped in Saranwrap and exposed to Kodak X-OMAT AR film.

6.2.7.1 Membrane stripping

Western blot membranes were stripped of bound antibodies to allow detection of other proteins on the same filter. Filters were rinsed in PBSA and then incubated with rocking for 20 min in 200 mM glycine (pH 2.5) and 0.4% SDS at room temperature. The membrane was then rinsed in 1 M Tris pH 7.5 and then washed 5-6 times with PBSA. The membranes were then blocked and reprobed as described above.
6.2.8 Cell lysate preparation

6.2.8.1 Lysis buffers

For most SDS-PAGE, SDS lysis buffer was used: 2% (w/v) SDS, 60 mM Tris-HCl pH 6.8, 16 mM dithiothreitol (DTT) (Boehringer Mannheim), 1% (v/v) (19 μg/ml) aprotinin (Sigma).

For immunoprecipitation, Cyclin A and E kinase assays and some SDS-PAGE, Triton X-100 lysis buffer was used: 1% (v/v) Triton X-100, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) aprotinin, 100 μg/ml phenyl-methyl-sulphonyl fluoride (PMSF) (10 mg/ml stock in isopropanol), 10 mM sodium fluoride (NaF) (0.5 M stock), 1 mM sodium orthovanadate (Na₃VO₄) (20 mM stock; Na₃VO₄ is dissolved in dH₂O and pH adjusted to 10.0 with NaOH, solution becomes yellow and is then boiled until it becomes colourless, stored at room temperature) and 1 mM DTT.

For some kinase assays NP40 lysis buffer was used: 0.5% (v/v) NP40, 50 mM Tris-HCl pH 7.8, 200 mM NaCl, 25 mM NaF, 1% (v/v) aprotinin, 100 μg/ml PMSF, 1 mM Na₃VO₄, 1 mM DTT.

For JNK kinase assays JNK lysis buffer was used: 20 mM Hepes, 2 mM EGTA, 50 mM β-glycerol-phosphate, 1 mM DTT, 1 mM Na₃VO₄, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 400 μM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin.

6.2.8.2 Cell lysate preparation for Western blot

Cell monolayers were washed twice with ice-cold PBSA. Cells were then scraped with a rubber policeman and transferred to an Eppendorf tube. Cells were pelleted by a 3 min spin at 6500 rpm in an MSE Microcentrifuge and the PBSA was removed. Cells grown in suspension were collected and washed as described above.

Cell pellets were lysed in 50-100 μl of lysis buffer, vortexed, then heated to 95°C for 5 min and spun at 13000 rpm at 4°C for 10 min. The supernatant was transferred to a new Eppendorf tube and the protein concentration was determined (see below). SDS-PAGE sample buffer was then added and if not used immediately, lysates were stored at -20°C.

6.2.8.3 Protein concentration determination

In most cases the Bio-Rad Protein Assay (Bio-Rad) was used according to the manufacturer’s instructions. 1 μl of lysate was added to 1 ml of Bio-Rad reagent (diluted 1:5 in dH₂O) in a spectrophotometer cuvette. These were mixed and incubated at room temperature for 5 min. Its absorbance was then read at 595 nm using a spectrophotometer, against a blank of diluted Bio-Rad reagent. Concurrently a BSA standard curve was made. 0, 1, 2, 4, 6 and 8 μg of BSA in lysis buffer were each added to Bio-Rad reagent and the absorbances were determined after 5 min. This standard curve was then used to calculate the protein concentrations of the cell lysates.
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In cases where the lysis buffer was incompatible with this system, the Bio-Rad Dc assay was used. Reagent S was diluted with reagent A in a 1:50 ratio to make reagent A/S. In the following order, 10 μl of lysate, 50 μl of reagent A/S and 400 μl of reagent B were added to the spectrophotometer cuvette, mixed and incubated at room temperature for 15 min. The absorbance of the samples was measured at 750 nm and concentrations were calculated against a BSA standard curve as above.

6.2.9 Immunoprecipitation

Cell pellets were collected as described above. Cells were then lysed in 500-800 μl of lysis buffer and vortexed, incubated on ice for 5 min, vortexed again and then centrifuged at 13000 rpm (IEC Centra-4R centrifuge) at 4°C for 10 min. The supernatant was removed to a new Eppendorf tube and the protein concentration determined. Samples were either used immediately or flash frozen on dry-ice in methanol and stored at -80°C. 30 μl of protein A or protein G sepharose beads (4 Fast Flow, Pharmacia) for each immunoprecipitation (IP) were washed twice in lysis buffer and then blocked with 3% BSA in PBSA for 30 min on a rotating wheel at 4°C. They were then washed twice in lysis buffer. Primary antibody and the blocked protein sepharose beads were added to 10-200 μg of cell lysate in 800 μl of lysis buffer. Tubes were then incubated on a rotating wheel at 4°C for 2 h to overnight. Beads were then pelleted at 6000 rpm (MSE Microcentrifuge) and washed 3 times with lysis buffer (if a kinase assay was to be performed the samples were taken to this point and then the kinase assay protocol was followed). Beads were then pelleted a final time and resuspended in 25 μl of 1x SDS-PAGE sample buffer, heated to 95°C for 5 min, the beads were pelleted again and the supernatant was loaded on an SDS-PAGE gel.

6.2.10 Kinase assays

6.2.10.1 Cyclin E and Cyclin A

Proteins were immunoprecipitated in Triton X-100 lysis buffer with 1 μg of antibody (anti-Cyclin E (Santa Cruz, sc-481), anti-Cyclin A (monoclonal E72, ICRF)). After washing with lysis buffer, the protein bound beads were then washed three times with kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM DTT) and then pelleted and all buffer removed with a 25 G needle and syringe. The beads were resuspended in 30 μl kinase reaction mix [50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 30 μM Adenosine triphosphate (ATP), 0.5 μg/μl Histone H1 (Boehringer Mannheim), 0.1 μCi/μl [32P] γATP (Amersham)], vortexed briefly and incubated at 30°C for 30 min in a shaking heating block. 7 μl of 5x SDS-PAGE sample buffer was added to stop the reaction and the samples were heated to 95°C for 5 min. The beads were then pelleted and the supernatant was loaded onto an SDS-PAGE gel. After the gel was run, it was fixed in gel fixative (10% methanol and 10% acetone) for 5 min. The gel was then dried.
for 45 min at 85°C on a gel dryer (Bio-Rad) onto Whatman 3MM paper and then exposed to X-OMAT film (Kodak) at -80°C.

6.2.10.2 Cyclin D1 (after Carlson et al., 1996)
Cells were lysed by addition of Cyclin D1 IP buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1mM EDTA, 2.5 mM EGTA, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 10 mM b-glycerophosphate, 0.1 mM PMSF, 10 μg/ml leupeptin, 20 units/ml aprotinin, 1 mM NaF and 0.1mM Na₂VO₄). Samples were then subject to 15 sec sonication on ice. 300 μg of protein was subject to immunoprecipitation with 1.5 μg of DCS 11 anti-Cyclin D1 monoclonal antibody and 30 μl of protein G sepharose beads. After washing with lysis buffer, the protein G bound beads were then washed three times with Cyclin D1 kinase buffer (50 mM Hepes pH 7.5, 1mM NaF, 10 mM MnCl₂, 2.5 mM EGTA and 1 mM DTT) and then pelleted and all buffer removed with a 25 G needle and syringe. The beads were resuspended in 40 μl kinase reaction mix (kinase buffer with 20 μM ATP, 0.4 mg of GST- pRb and 0.1 μCi/μl [³²P] γATP ), vortexed briefly and incubated at 30°C for 30 min in a shaking heating block. 7 μl of 5x SDS-PAGE sample buffer was added to stop the reaction and the samples were heated to 95°C for 5 min. The samples were then subject to SDS-PAGE. The gel was fixed, dried and exposed to film as above.

6.2.10.3 MAP kinase assay
Proteins were immunoprecipitated in NP40 lysis buffer with 1-2 μl 122.2 MAPK kinase rabbit polyclonal antibody (a gift from Chris Marshall). After washing with lysis buffer the beads were washed three times with kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT) and then pelleted and all buffer removed with a 25 G needle and syringe. The beads were resuspended in 25 μl kinase reaction mix (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.1 M adenosine triphosphate (ATP), 15 μg myelin basic protein (MBP) (Sigma), 1 μCi [³²P] γATP), vortexed briefly and incubated at 30°C for 20 min in a shaking heating block. 6 μl of 5x SDS-PAGE sample buffer was added and the samples heated to 95°C for 5 min. The beads were then pelleted and the supernatant was subject to SDS-PAGE. The gel was fixed, dried and exposed to film as above.

6.2.10.4 JNK kinase assay
Proteins were immunoprecipitated in JNK lysis buffer with 5 μl of JNK-1 antibody (sc-474). After washing with lysis buffer, the beads were washed three times with LiCl wash (500 mM LiCl, 100 mM Tris-HCl pH 7.5, 0.1% (v/v) Triton X-100, 1 mM DTT) and then three times with JNK kinase buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1 % (v/v) Triton X-100), pelleted and all buffer was removed with a 25 G needle and syringe. The beads were resuspended in 20 μl kinase buffer, 7.5 μl of Mg-ATP reaction mix (50 mM MgCl₂, 100 μM ATP, 2 μCi [³²P] γATP) and 10 μg
of GST-jun protein, vortexed briefly and incubated at 30°C for 20 min in a shaking heating block. 6 μl of 5x SDS-PAGE sample buffer was added and the samples heated to 95°C for 5 min. The beads were then pelleted and the supernatant was subject to SDS-PAGE. The gel was fixed, dried and exposed to X-OMAT film as described above.

6.2.11 Reporter assays

6.2.11.1 CAT assay

Cells were washed twice with PBSA. The plates were then drained and the residual PBSA aspirated away. 150 μl of CAT lysis buffer (0.1% (v/v) Triton X-100, 0.25 mM Tris (pH 8.0)) was added drop-wise to the plates. The plates were swirled gently and then incubated on ice for 3-5 min (until only the nuclei were visible under the microscope). The lysates were scraped off the plates with rubber policeman and were transferred to Eppendorf tubes. The collected lysates were then frozen on dry ice and then freeze thawed twice more, then vortexed and spun at 13000 rpm in a MSE Microcentrifuge for 3 min. The supernatants were transferred to fresh Eppendorf tubes and stored at -80°C.

30 μl of extract was heated to 68°C for 15 min, cooled down to room temperature and added to a fresh mix of 1 μl chloramphenicol (Sigma) stock solution (5 mg/ml), 2 μl 14C Acetyl CoA (54 mCi/ mmole) (Amersham), 1.6 μl acetyl CoA (Sigma) stock solution (5 mg/ml), 7.5 μl 1 M Tris-HCl pH 8.0 and 38.5 μl of dH2O.

The reaction was incubated at 37°C for 1-4 h. At the end of the incubation the reaction tubes were transferred to ice. 200 μl of ice-cold ethyl acetate was added to the tube, vortexed vigorously for 30 sec and spun at 13000 rpm in a MSE Microcentrifuge for 3 min. From the phase separated mixture, 150 μl of the upper organic phase was transferred to a scintillation vial containing 5 ml scintillation fluid (Ultima Gold). A blank control of untransfected extracts was used as a negative control.

6.2.11.2 β-galactosidase assay

Lysates were prepared as for CAT assay or luciferase assay (but without heating). 150 μl of lysate with 150 μl of β-gal reaction buffer (120 mM Na2HPO4·7H2O, 80 mM NaH2PO4·H2O, 1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma), 2 mM MgCl2, 0.27% (v/v) β-mercaptoethanol (Sigma)) were mixed and then incubated at 37°C for 15 min-4 h. When an obvious yellow colour developed, the reaction was stopped with 500 μl of 1 M Na2CO3. The Eppendorf tubes were then spun for 10 min at 13000 rpm (microcentrifuge), then 400 μl of the supernatant was transferred to a spectrophotometer cuvette and its absorbance was read at 420 nm against a mock extract as negative control.
6.2.11.3 Luciferase assay

If the assay was to be done in conjunction with a CAT or β-gal assay, a 100 mm dish was washed twice with PBSA and drained. 100 µl of 0.25 M Tris-HCl pH 7.5 was added and the cells were scraped off the dish using a rubber policeman. Cells were transferred to an Eppendorf tube where they were pipetted up and down vigorously. The cells were then put through three freeze-thaw cycles and then spun at 13000 rpm (microcentrifuge) for 10 min. The supernatant was assayed for luciferase activity. Alternatively, if only the luciferase activity was required, the cells were washed twice with PBSA, the plate drained and then cells lysed in 150 µl of luciferase lysis buffer (0.65% NP40, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 150 mM NaCl) on ice for 5 min. The lysate was then scraped from the dish and transferred to an Eppendorf tube, centrifuged at 13000 rpm (microcentrifuge) for 1 min and assayed for luciferase activity.

To assay for luciferase activity 20 µl of lysate was added to 350 µl of luciferase reaction buffer (25 mM glycylglycine pH 7.8 (Sigma), 5 mM ATP pH 8.0, 15 mM MgSO4) in a luminometer cuvette and the samples were loaded into the luminometer. The machine then injected 33 µl of 3 mM luciferin (sodium salt, Sigma) into the reaction mixture and measured the peak luminescence.

6.2.12 Preparation of glutathione-S-transferase (GST)-fusion proteins

Plasmid DNA coding for the required GST fusion protein under the control of an isopropyl β-D-thiogalactoside (IPTG) inducible promoter was transformed into BL21 (DE3) competent bacteria and a 50 ml overnight LB culture was grown. This was used to inoculate a 450 ml culture, which was grown until its absorbance at 600 nm was 0.8-1.0. The bacteria were then induced with 1 mM IPTG for 3-4 h shaking at 37°C. The bacteria were then spun down and resuspended in 8 ml of ice-cold lysis buffer (1% (v/v) Triton X-100, 10 mM EDTA pH 8.0, 0.5 mg/ml PMSF, 0.08 mg/ml aprotinin) with gentle pipetting. Lysates were then transferred to 6x 2 ml Eppendorf tubes and lysed further by sonication for two 10 sec bursts. The tubes were centrifuged at 13000 rpm at 4°C (Centra-4R centrifuge) for 10 min and supernatants were passed through a 0.45 µM filter to remove unlysed bacteria and DNA. Supernatants were then applied to the prepared column (see below) at 4°C and the run-through collected and put through the column two more times. The column was then washed twice with one column volume of 1% (v/v) Triton X-100 in PBSA then once with PBSA. Five protein fractions were eluted, each by addition of 500 µl of elution buffer (50 mM Tris pH 8.0 and 5 mM glutathione (Sigma)). Each fraction was assayed for protein content. A sample of each fraction was also subject to analysis by SDS-PAGE. The gel was coomassie stained in 0.1% (w/v) Coomassie Blue (Bio-Rad), 41% methanol and 7% glacial acetic acid and destained in 41% methanol and 7% glacial acetic acid then dried on a gel drying frame (Idea Scientific).
Columns (Bio-Rad) were prepared in advance and constantly kept at 4°C throughout the procedure: 1 ml of glutathione sepharose 4B slurry (Pharmacia), was added to the column to make a bed of approximately 500 µl, which was washed with one column volume of PBSA and then one column volume of 1% (v/v) Triton X-100 in PBSA. The column was not allowed to dry out.

### 6.2.13 Restriction enzyme digestion

Reactions were carried out in 20 µl total volume with 10x reaction buffer (NEB) and 5 units of enzymes (NEB unless otherwise stated) on 0.5-2 µg DNA, at 37°C for 1 h. The reactions were stopped by addition of 5x DNA loading dye (50 mM EDTA pH 8.0, 100 mM Tris pH 8.0, 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue) and were subject to agarose gel electrophoresis.

### 6.2.14 Agarose gel electrophoresis

The percentage of agarose used to make the gels depended on the size of the fragments analysed, but varied between 0.75% (w/v) for fragment over 2 kbp to 5% (w/v) for fragments of around 100 bp. In addition the type of agarose used also depended on the application. In most instances SeaKem le agarose (FMC Bioproducts) was used. For retrieval of DNA from low gelling temperature agarose SeaPlaque agarose (FMC Bioproducts) was used and for resolving fragments less than 500 bp, Nusieve GTG agarose (FMC Bioproducts) was used.

To form the gel, agarose was added to 100 ml of 1x TAE buffer (40 mM TrisOAc, 2 mM EDTA pH 8.0) and heated on a high setting in a microwave until the agarose had fully dissolved. Upon cooling, ethidium bromide to a concentration of 0.01 µg/ml was added and the gel poured into a gel forming tray of the appropriate size with a teflon comb. When set, the gel was submerged in 1x TAE buffer, in an appropriate gel electrophoresis tank. The samples in DNA loading buffer were carefully loaded and subject to electrophoresis at a constant voltage of 80-100 v, depending on the size of gel used. A molecular weight marker (1 kb ladder Gibco BRL) was added to one lane.

DNA bands were visualised on a long wave UV transilluminator and if necessary bands were excised using clean razor blades.

### 6.2.14.1 Extraction of DNA from agarose gels

Various method of DNA extraction were used:

1) - Low gelling temperature agarose was used for the electrophoresis and the required band was excised in as small an amount of agarose as possible. The volume was increased to 100 µl with dH2O and the mixture was heated to 68°C to melt the agarose and then maintained at 37°C, before addition to the ligation reaction.

2) - Excised band from normal agarose were dissolved in 3 volumes of 6 M NaI at 50°C. 5 µl of glassmilk solution (silica matrix in dH2O)(Geneclean II Kit, Bio 101 Inc) was
added and incubated on ice for 5 min. The mixture was then spun at 13000 rpm (MSE Microcentaur) and the glassmilk pellet washed three times in 500 µl of chilled 'New Wash' (Geneclean II Kit, Bio 101 Inc). The pellet was then resuspended in 5 µl of TE and incubated at room temperature for 5 min to dissolve the DNA, spun again and the supernatant removed to another tube. The pellet was again resuspended in 5 µl of TE, incubated, spun and the supernatant added to the previous one. This supernatant was used for the ligation reaction.

3) -100 µl of dH₂O was added to a spin column (GenElute Agarose spin columns (Supelco Inc)) and spun for 1 min (Eppendorf centrifuge 5414), the column was removed to a fresh Eppendorf tube and the excised band was added to the column and spun for 10 min. This eluate was used for the ligation reaction.

6.2.15 Ligation reactions

Ligation reactions were carried out in 20 µl total volume, with 10x ligation buffer (NEB), 10 mM ATP and 0.4 units of ligase (NEB), using 20-50 ng vector DNA and a vector to fragment ratio of 1:2-5. Reactions were carried out at room temperature for 2 h or overnight.

6.2.16 Dephosphorylation of vector DNA

Restriction digestion reactions were incubated at 68°C for 20 min to inactivate the restriction enzymes*. Then 1 unit of calf intestinal alkaline phosphatase (CIP) (NEB) was added to the reaction and incubated at 37°C for 30 min. Reactions were incubated with 5 mM EDTA at 68°C for 20 min to inactivate the CIP.

Alternatively, vectors were dephosphorylated with shrimp alkaline phosphatase (SAP) (Boehringer Mannheim). Restriction digests were inactivated then 50 pmol of DNA from the digest was added to 10x SAP reaction buffer (Boehringer Mannheim), dH₂O and 1 unit of SAP in a total volume of 20 µl. Vectors with 5' or 3' overhangs were incubated at 37°C for 5 min and blunt ended vectors were incubated at 37°C for 1 h. Reactions were incubated at 68°C for 20 min to inactivate the SAP.

*If the restriction enzymes were not heat inactivatable, then EDTA to a concentration of 5 mM was added to stop the reaction and the reaction was phenol-chloroform extracted and the DNA precipitated (in the presence of 1 µg of glycogen) and resuspended in 10x CIP reaction buffer (NEB), dH₂O and 1 unit of CIP.

6.2.17 Phenol-Chloroform extraction of DNA

The volume of the sample was increased, if necessary, to 100 µl with dH₂O. An equal volume of phenol-chloroform (1:1) (Sigma) was added. The reaction was then vortexed for 10 sec and spun at 13000 rpm in an MSE Microcentrifuge for 1 min. The aqueous upper phase was transferred to another tube and the DNA was precipitated.
6.2.18 Ethanol precipitation of DNA

To precipitate DNA out of a sample, 10% of the sample volume of 3 M sodium acetate was added to the sample (if the quantity of DNA was very small (less than 1 µg) 1 µg of glycogen was also added to the sample). Then twice the new sample volume of 100% (v/v) ethanol was added and mixed. The sample was then incubated on dry-ice for 10 min. The sample was then centrifuged at 13000 rpm (MSE Microcentrifuge) for 10 min. The supernatant was removed and 500 µl of 70% (v/v) ethanol was added. The DNA was pelleted for 5 min at 13000 rpm. The supernatant was removed and the pellet was dried and resuspended in dH₂O or TE.

6.2.19 Transformation of DH5α E.coli

To prepare competent bacteria, a single colony of DH5α was picked from a fresh LB agar plate into 3 ml of LB media (10 g/l Bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl, adjusted to pH 7.5 with 5 M NaOH) and grown overnight. 100 ml of LB was inoculated with 1 ml of overnight culture and left to grow with vigorous shaking at 37°C until the absorbance of the culture at 600 nm reached 0.5. The flask was transferred to ice and rapidly cooled down. The bacteria were pelleted at 2000 rpm (Heraeus Megafuge 1.0 R) for 10 min at 4°C and the supernatant discarded. The pellet was resuspended in 15 ml of sterile ice cold 50 mM CaCl₂ by gentle pipetting on ice. After incubating 30 min on ice the bacteria were pelleted again and the supernatant discarded and the pellet resuspended in 3 ml sterile ice cold 50 mM CaCl₂ and 20% (v/v) glycerol by gentle pipetting on ice. The bacteria were then frozen on dry ice in 100 µl aliquots and stored at -80°C.

To transform bacteria with DNA, 100 µl aliquot of competent DH5α, thawed on ice, was mixed with 10 µl of a 20 µl ligation or 50 ng plasmid DNA and incubated on ice for 20 min. The bacteria were then heat-shocked at 37°C for 2 min and incubated on ice for a further 2 min. They were then incubated in 700 µl of LB at 37°C for 45 min. After which the bacteria were pelleted at 6500 rpm (MSE Microcentrifuge). The cell pellet was resuspended in 100 µl of LB and plated onto LB/Amp agar plates containing 100 mg/ml ampicillin (Sigma) in 1.5% (w/v) agar (DIFCO laboratories). Plates were incubated at 37°C overnight. Bacterial colonies were grown up in 2 ml LB containing 100 µg/ml ampicillin overnight, shaking at 37°C and miniprepped.

6.2.20 Mini-preps by Alkaline Lysis

A bacterial colony was picked and grown in 2 ml LB overnight with shaking at 37°C. 1.5 ml of the mini-prep was pelleted at 13000 rpm (MSE microfuge) for 1 min in an Eppendorf tube and the pellet was resuspended in 100 µl of solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). Cells were then lysed by addition of 200 µl of solution 2 (0.2 M NaOH, 1% (w/v) SDS). The tubes were then inverted gently to mix
and incubated on ice for 5 min. Then 150 µl of ice-cold solution 3 (5 M KOAc pH 4.8 [made by mixing 2 volumes of 5 M acetic acid (1 volume of glacial acetic acid diluted with 2.5 volumes of dH2O) with 1 volume of 5 M potassium acetate] was added and mixed immediately by gentle inversion. Lysates were incubated on ice for 5 min, then spun at 13000 rpm (MSE microfuge) for 1 min and the supernatant was transferred to another tube. The supernatant was then phenol-chloroform extracted and ethanol precipitated. The DNA was then resuspended in 50 µl of TE. 100 µg/ml ribonuclease (Sigma) (prepared as a 10 mg/ml stock and boiled for 10 min, then stored at -20°C) was added and samples were incubated at 37°C for 15 min. Mini-prep DNA was then analysed by restriction digest.

6.2.21 Qiagen Spin-prep Mini-preps
Bacteria were pelleted and resuspended in 250 µl of P1 buffer. 250 µl of P2 buffer was added and tube was inverted gently for lysis, then incubated on ice for 5 min. 350 µl of N3 buffer was then added and the tube inverted gently to mix and then incubated on ice for 10 min. The tube was then spun at 13000 rpm (MSE microfuge) for 1 min and the supernatant was transferred to a Qiagen spin-prep column. It was spun through the column at 13000 for 30 sec into a 2 ml Eppendorf tube and the spin-through was discarded. The column was then washed with 750 µl of buffer PE with a 30 sec spin and the spin-through discarded. The column was then spun for a further 30 sec to remove all liquid. 50 µl of TE was then applied, allowed to absorb for 30 sec, then the column was spun for 1 min into a fresh tube. DNA was analysed by restriction digest.

6.2.22 Qiagen Maxi-prep
1 ml of a mini-prep culture were added to 100 ml of LB and incubated at 37°C with shaking overnight. 50 ml of the culture was pelleted in polypropylene tubes at 5800 rpm (Heraeus 1.0 R) for 8 min. The pellet was resuspended in 10 ml of P1 buffer, then lysed in 10 ml of P2 buffer and mixed by gentle inversion. It was then incubated at room temperature for 5 min. 10 ml of ice-cold P3 buffer was added, mixed by gentle inversion and tubes were incubated on ice for 20 min. Tubes were then spun at 5800 rpm (Heraeus 1.0 R) for 30 min. In the mean-time Qiagen Maxi columns were equilibrated with 10 ml of QBT buffer. The bacterial supernatants were then applied to the columns through gauze to filter out the white precipitate. After the supernatants had run through the columns were washed with 30 ml of QC buffer, then the columns were transferred to fresh 50 ml polypropylene tubes and 15 ml of QF elution buffer was applied. When run through, 10.5 ml of isopropanol was added to the eluted DNA, mixed and the tubes were spun at 5800 rpm (Heraeus 1.0 R) for 30 min. The DNA pellet was then washed with 1 ml 70% (v/v) ethanol, dried and resuspended in 250 µl of TE.
6.2.23 Polymerase chain reaction

To extract genomic DNA from mammalian cells, approximately $2 \times 10^4$ cells were lysed in 50 μl of digestion buffer (50 mM Tris pH 8.0, 20 mM NaCl, 0.1% (w/v) SDS and 1 mg/ml Proteinase K), vortexed and incubated at 55°C for 3 h. 500 μl of dH₂O was then added and samples were boiled for 5 min, then spun for 10 sec in a microfuge at 13000 rpm. 1 μl of the supernatant was used per PCR reaction.

Reactions were prepared on ice in 30 μl total volume with 10x PCR reaction buffer (Promega), 200 μM dNTPs, 1 μM of each primer and 50 ng of template DNA or 1 μl of cell lysate and 1 μl of Taq polymerase (PICTAQ, ICRF). For each reaction, control reactions were performed with either template DNA or primers absent.

5 μl of the reaction was subject to agarose gel electrophoresis to analyse the PCR products and the remaining 25 μl was purified using a PCR purification column (Qiagen), prior to digestion with restriction enzymes.


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