Studies on the role of the tyrosine kinase Lyn in B cell receptor signal transduction

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A thesis presented in partial requirement of the University of London for the degree of Doctor of Philosophy

August 1996
To Nick
Abstract

B lymphocytes recognise and respond to antigens via the B-cell receptor (BCR) expressed on the cell surface. Ligation of the BCR leads to apoptosis, receptor endocytosis, antigen presentation, differentiation and proliferation, depending upon the developmental window of the B cell and the specificity of the antigen ligand. Although the BCR has no intrinsic enzymatic activity, tyrosine phosphorylation is the earliest detectable response to BCR stimulation.

Cells of the hematopoietic system differentially express members of the Src family of tyrosine kinases, and these proteins have been shown to be associated with a variety of receptors in these cells. Notably Lck is physically and functionally associated with the T cell coreceptors CD4 and CD8, whilst Fyn is associated with the CD3 component of the T cell receptor. Likewise B cells express the Src family members Lyn, Blk and Fyn, all of which have been shown to associate with the BCR.

The possible role of Lyn in signal transduction through the BCR was investigated. Mutational analysis is the most powerful method of assigning a function to a gene or protein; therefore expression vectors carrying either the wild type Lyn or a kinase disabled mutant form of the gene were made. This was achieved by changing a critical lysine in the ATP binding site to a methionine residue, which it was anticipated would act as a dominant negative mutation when
overexpressed. Seven lines of transgenic mice were generated, carrying a range of copy numbers of the mutant gene under the control of the immunoglobulin heavy chain enhancer. B cell compartments in these mice were analysed for expression of the transgene, and B cell development in the bone marrow was studied.

Also the expression vectors were transfected into a B cell line in vitro in order to study the biochemistry of signalling through the BCR.
Acknowledgements

I would especially like to thank my supervisor Victor Tybulewicz for his support and encouragement during the course of this study, and for making the laboratory such a pleasant place to work. I would also like to thank all the members of his lab, Joe Mee, Marian Quinn, Alice Walters, Patrick Costello and Linda Duddy for their help, particularly Martin Turner for the critical evaluation of this manuscript.

Thanks to Andy Mullord for bunny injections, Andy Mellor for help with generating the transgenic mice, Russell Huby and Steve Ley for antibodies and advice on immunoblotting, Chris Atkins for FACS work, Liz Hurst for transmission electron microscopy and Frank Johnson for help in the preparation of this manuscript.

I am also eternally grateful to my parents and partner Nick for their encouragement and financial support, without which this work could not have been completed.
# Table of Contents

Abstract .................................................................................................................3  
Acknowledgements ...........................................................................................5  
Abbreviations ......................................................................................................9  

Chapter One .......................................................................................................11  
General Introduction .......................................................................................11  
1.1 Structure and function of the B cell antigen receptor .............................12  
1.1.1 The core B cell antigen receptor complex ....................................12  
1.1.2 Structure of the T cell antigen receptor .......................................13  
1.1.3 Genomic organisation of the Ig genes ..........................................13  
1.1.4 Rearrangement of immunoglobulin genes ................................16  
1.1.5 B cell development ..........................................................................17  
1.1.6 The pre-B cell receptor .....................................................................19  
1.1.7 Allelic exclusion ..............................................................................21  
1.1.8 Selection events during development ........................................21  
1.1.9 Tolerance ...........................................................................................23  
1.1.10 Receptor editing .............................................................................24  
1.1.11 The B1 lineage ................................................................................24  
1.1.12 The CD19/CD21/TAPA-1 complex .............................................25  
1.1.13 The germinal centre response.....................................................27  
1.1.14 Costimulatory molecules .............................................................28  
1.1.15 Affinity maturation and terminal differentiation ..................29  
1.1.16 Summary .........................................................................................30  
1.2 Signal transduction through the B cell antigen receptor ................30  
1.2.1 Tyrosine phosphorylation .............................................................30  
1.2.2 The role of Igα and Igβ ................................................................31  
1.2.3 Immunoreceptor Tyrosine-based Activation Motifs ................31  
1.2.4 The Src family of tyrosine kinases................................................33  
1.2.5 The Syk/ZAP-70 family ..................................................................35  
1.2.6 The sequential activation model of TCR signalling .................37  
1.2.7 Sequential activation in signalling through the BCR ..............37  
1.2.8 Signalling through the high affinity IgE receptor .....................39  
1.2.9 Regulation of BCR proximal signalling events .........................39  
1.2.10 Downstream effectors of BCR signalling ...................................42  
1.2.11 Vav ...................................................................................................43  
1.2.12 Btk .....................................................................................................44  
1.2.13 The MAP kinase cascade ..............................................................45  
1.2.14 Phospholipid mediators of signal transduction ......................48  
1.2.15 Trimeric G proteins .......................................................................49  
1.2.16 Lipid kinases ...................................................................................50  
1.2.17 Differential outcomes of BCR signalling ...................................50  
1.2.18 Summary .........................................................................................51  
1.3 Experimental strategies .........................................................................53  
1.3.1 The tyrosine kinase Lyn .................................................................53  
1.3.2 Experimental mouse models .........................................................54  
1.3.3 Creation of mutant mice lacking functional Lyn alleles ..........54  
1.3.4 Overexpression of dominant negative mutations ......................56
1.3.4 Dominant negative mutations versus gene knockouts ........................................................................57
1.3.5 In vitro models of Lyn’s function ........................................................................................................59
1.3.6 Summary ........................................................................................................................................60

Chapter Two ..................................................................................................................................................61
Materials and Methods ..................................................................................................................................61

2.1 Solutions ..................................................................................................................................................61

2.2 Bacterial media .......................................................................................................................................62

2.3 Mammalian tissue culture media ........................................................................................................62

2.4 Bacteriological techniques ..................................................................................................................63

2.4.1 Transformation and culture ..............................................................................................................63

2.4.2 Harvesting of plasmid DNA from E.coli ...........................................................................................63

2.5 Preparation of mammalian genomic DNA ..........................................................................................64

2.5.1 Preparation of genomic DNA from cultured cells ...........................................................................64

2.5.2 Preparation of genomic DNA from mice ..........................................................................................64

2.6 Manipulation of DNA ..........................................................................................................................65

2.6.1 Endonuclease restriction digestion of DNA .......................................................................................65

2.6.2 Dephosphorylation ...........................................................................................................................65

2.6.3 Filling in of 5' overhanging DNA termini .........................................................................................66

2.6.4 Ligation ................................................................................................................................................66

2.6.5 Electrophoresis of DNA ....................................................................................................................67

2.6.6 Recovery of DNA from gels ..............................................................................................................67

2.6.7 Transfer of DNA from agarose gels to membranes ........................................................................68

2.6.8 Labelling of DNA using random priming .......................................................................................68

2.6.9 Hybridisation of Southern blots .......................................................................................................69

2.6.10 Removal of bound probe from membranes ....................................................................................70

2.7 Polymerase Chain Reaction ................................................................................................................70

2.8 Sequencing of DNA ............................................................................................................................71

2.8.1 Sequencing reactions .........................................................................................................................71

2.8.2 Polyacrylamide gels ...........................................................................................................................72

2.9 Transgenic mice .....................................................................................................................................72

2.9.1 Superovulation of mice .....................................................................................................................72

2.9.2 Harvesting of fertilised eggs ............................................................................................................73

2.9.3 Microinjection of fertilised eggs .......................................................................................................74

2.9.4 Transfer of embryos to foster mothers ..............................................................................................75

2.10 Tissue culture ........................................................................................................................................75

2.10.1 WEHI-231 .......................................................................................................................................75

2.10.2 Transfection of WEHI-231 cells .....................................................................................................76

2.10.3 Transfection of COS-1 cells ..........................................................................................................77

2.11 Protein analysis ......................................................................................................................................78

2.11.1 Total cell lysates ...............................................................................................................................78

2.11.2 Coupling antibodies to Protein-A-Sepharose beads .....................................................................78

2.11.3 Immunoprecipitations .....................................................................................................................79

2.11.4 SDS-PAGE .....................................................................................................................................80

2.11.5 Immunoblotting ...............................................................................................................................80

2.11.6 Antibody staining of immunoblots .................................................................................................81

2.11.7 Membrane stripping .......................................................................................................................83

2.11.8 In vitro immune complex kinase assays .........................................................................................83

2.12 Flow cytometry ....................................................................................................................................84

2.13 MTT assay ............................................................................................................................................85

2.14 Tritiated thymidine uptake assay ........................................................................................................86

2.15 Electron microscopy ...........................................................................................................................86
Chapter Three ....................................................................................................87
Generation of anti-Lyn antisera and a kinase inactive mutant of the 56kD isoform of murine Lyn .................................................................87
3.1 Generation of anti-Lyn sera ........................................................................87
3.2 Analysis of sera ..........................................................................................89
3.3 Generation of a kinase defective form of p56 Lyn ......................................92
3.4 Vector design .............................................................................................93
3.5 Expression of wild type and mutant p56 Lyn in COS-1 cells ....................99
3.6 Analysis of the specific kinase activities of wild type and mutant p56 Lyn .................................................................100
3.8 Summary ...............................................................................................102

Chapter Four ....................................................................................................104
Generation and analysis of mice transgenic for mutant p56 Lyn ............104
4.1 Generation of transgenic mice ................................................................104
4.2 Analysis of B cell development in p56 Lyn transgenic mice ...............110
4.3 Analysis of mature B cell compartments in p56 Lyn transgenic mice ....114
4.4 Analysis of T cells in p56 Lyn transgenic mice .....................................116
4.5 Summary ................................................................................................117

Chapter Five ....................................................................................................119
Expression of mutant Lyn in transgenic mice ............................................119
4.1 Transgene expression in lymphoid compartments ..............................119
5.2 Lyn kinase activity in spleen ..................................................................124
5.3 Summary ...............................................................................................126

Chapter Six .......................................................................................................127
Expression of wild type and mutant Lyn in WEHI-231 cells ..................127
6.1 Apoptosis in WEHI-231 .........................................................................127
6.2 Transfection of WEHI-231 with pBIS Lyn and pBISK 275M ..............129
6.3 Transfectants generated with pc Lyn and pcK 275M ..............................133
6.4 Epitope tagging of p56 Lyn .....................................................................136
6.5 Transfection of WEHI-231 with pBLF and pBLKM F ..........................138
6.6 Summary ................................................................................................142

Chapter Seven .................................................................................................143
Discussion ........................................................................................................143
7.1 Lyn knockout mice ....................................................................................143
7.2 LDN transgenic mice ................................................................................145
7.2.1 Factors affecting expression in transgenic mice ...............................145
7.2.2 Locus Control Regions ......................................................................148
7.3 WEHI-231 Transfectants ........................................................................149
7.3.1 Potential toxicity of overexpressed p56 Lyn ......................................150
7.4 Further experiments .................................................................................152
7.5 Summary ................................................................................................153

References .......................................................................................................155
Abbreviations

ATP adenine-5’-triphosphate
BCR B cell antigen receptor
bGH bovine growth hormone
BSA bovine serum albumin
C constant
cDNA DNA complementary to mRNA
Ci Curie
CMV cytomegalovirus
CDR complementarity determining region
cpm counts per minute
D diversity
DMSO dimethylsulphoxide
DNA deoxyribonucleic acid
dNTP deoxynucleotide-5’-triphosphate
DTT dithiothreitol
EDTA (ethylene diamine) tetraacetic acid
ES cell embryonic stem cell
FACS fluorescence activated cell sorting
FceRI high affinity IgE receptor
FCS foetal calf serum
FDC follicular dendritic cell
HA haemagglutinin
HEL hen egg lysozyme
Ig immunoglobulin
IgH immunoglobulin heavy chain
IgL immunoglobulin light chain
IL-2 interleukin-2
IL-4 interleukin-4
IP_3 inositol triphosphate
ITAM immunoreceptor tyrosine-based activation motif
ITIM immunoreceptor tyrosine-based inhibition motif
J joining
kd kilodalton
kb kilobase
MAPK mitogen activated protein kinase
MHC major histocompatibility complex
MOPS 3-[N-morpholino] propane-sulphonic acid
mRNA messenger ribonucleic acid
NP40 nonidet P-40
OD optical density
pBCR pre-B cell receptor
PBS phosphate buffered saline
PCR polymerase chain reaction
PI-3-kinase phosphatidylinositol-3-kinase
PLC phospholipase C
PMA phorbol 12-myristate 13-acetate
rpm revolutions per minute
slg surface immunoglobulin
SCID severe combined immunodeficiency
SDS sodium dodecyl sulphate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
SSC standard saline citrate
SV40 simian virus 40
TCR T cell receptor
TdT terminal deoxynucleotidyl transferase
Tris tris(hydroxymethyl)-amino-methane
V variable
Chapter One

General Introduction

The basis of adaptive immunity is the ability of lymphocytes to distinguish self from non-self and respond appropriately. This is conferred by the expression on the lymphocyte cell surface of antigen receptors capable of recognising and responding to a wide variety of antigens, which is in turn made possible by receptors of different specificity on individual lymphocytes. Burnet's theory of clonal selection originally predicted the monospecificity of antigen receptors, such that each cell expressed receptors with a single specificity (Burnet 1959). This allows only those cells specific for a particular antigen to become activated. The activated cell would then undergo clonal expansion and mediate the appropriate immune response, thus avoiding the wasteful activation and proliferation of non-antigen specific cells. Likewise this monospecificity allows clonal deletion of self-reactive cells in order to prevent autoimmune disease, whilst sparing potentially useful cells. In order to generate the antigen receptor diversity necessary, the immune system has evolved a genomic polygenic complex from which each lymphocyte can construct its own unique receptor. Via somatic recombination of these receptor genes, a large repertoire of receptors is possible. This mechanism poses a problem, as lymphocytes must find a delicate balance between generation of diversity, which is random, and control of the immune response in order to prevent autoimmunity. To achieve this balance, lymphocytes first have to construct a functional receptor, which is potentially capable of binding antigen and signalling to the cell to respond, secondly, the cell's response must be appropriate to the stimulus. Depending upon the developmental window occupied by
the cell, the appropriate response could be allelic exclusion, apoptosis, anergy induction, receptor internalisation and antigen presentation, or proliferation. Therefore the structure and function of antigen receptors are pivotal to the immune response. The precise molecular mechanisms by which the same receptor can signal to the cell to induce such a range of biological outcomes remains one of the most exciting problems in molecular immunology today. As both B and T cell antigen receptors share common features that allow them to fulfil these requirements, they will be compared throughout much of this introduction.

1.1 Structure and function of the B cell antigen receptor

1.1.1 The core B cell antigen receptor complex

The B cell antigen receptor (BCR) is a membrane bound, multimeric complex containing surface-bound immunoglobulin (sIg) of either IgM or IgD isotype. Antigen recognition via the BCR occurs through binding of antigen to the variable regions of both heavy and light chain. Each variable region contains three complementarity determining regions (CDRs) which form peptide loops capable of making contact with the antigen. Also present in the receptor complex are the invariant chains Igα and Igβ (Figure 1.1), which are both members of the immunoglobulin superfamily and contain the characteristic immunoglobulin beta-pleat motif. They form disulphide linked heterodimers which associate with sIg in the membrane. Igα was first discovered as a molecule necessary for IgM expression on the cell surface in the plasmacytoma line J558L, a cell line that lacks sIg but expresses cytoplasmic Ig. When transfected with sIgM, only those clones which also expressed a 34kD protein (Igα) that coprecipitated with sIgM could express membrane-bound IgM (Hombach 1988). Soon
afterwards, the same group and others isolated Igβ, which also associates with both sIgM and sIgD (Campbell and Cambier 1990; Hombach, Tsubata et al. 1990). Igα is the product of the mb-1 gene, whilst Igβ is encoded by the B29 gene, both in mice and humans (Hombach, Lottspeich et al. 1990). Differential N-linked glycosylation of Igα gives rise to two forms, one of which associates with sIgM and the other with sIgD (Wienands, Hombach et al. 1990). Two forms of Igβ also exist, which differ by a truncation of 30 amino acids in the carboxy terminus and in their glycosylation (Cambier and Campbell 1992). The physiological significance of this glycosylation and the two forms of Igα and Igβ is unclear.

1.1.2 Structure of the T cell antigen receptor

Similarly the T cell receptor (TCR) consists of a heterodimeric antigen recognition subunit analogous to sig, with either an α and β chain, or γ and δ chains, associated with the CD3 complex and ζ chains. CD3 consists of an γε heterodimer and a δε heterodimer, whilst ζ exists as homodimer. Like Igα and Igβ, the CD3γ, δ and ε components of the TCR are members of the immunoglobulin superfamily (reviewed in Weiss 1993).

1.1.3 Genomic organisation of the Ig genes

The genomic structure of the immunoglobulin genes encoding the BCR underlies the diversity of receptor specificities. Immunoglobulin consists of two heavy chains (IgH, 50kD) covalently bonded by disulphide bridges, and two light chains (IgL, 25kD), each of which are built of variable, joining and constant regions. Heavy chains also have a diversity region (Early, Huang et al. 1980). All of these regions are encoded by separate gene segments in both mouse and human. The organisation of the heavy chain gene segments at the Ig loci is shown
Figure 1.1. Structure of a) the B cell antigen receptor, and b) the T cell antigen receptor. a) The Ig heavy chains are shown in blue and the light chains in pink. The position of the complementarity regions (CDRs) is indicated. The invariant chains are shown in green. Yellow boxes represent ITAM motifs (see Section). b) The T cell receptor α and β chains are analogous to the light and heavy chains respectively. Again the invariant chains are shown in green and the ITAMs in yellow. See main text for details.

in Figure 1.2 and is reviewed in (Chen and Alt 1993). The IgH constant regions are encoded by the μ, δ, γ, ε and α genes, whose expression determines the class of immunoglobulin expressed (IgM, IgD, IgG, IgE and IgA respectively) and thus the effector function of the immunoglobulin. It is the variable (V), diversity (D) and joining (J) regions which are responsible for the immense repertoire of antigen
specificities, since they encode the antigen binding domains of Ig. For this reason, the V, D and J genes have evolved as large families of related genes. Work in transformed cell lines revealed that the mouse has up to 1500 $V_H$ genes, twelve D genes and four $J_H$ genes. Somatic recombination brings together one of each of these segments in a $V_HDJ_H$ configuration (Early, Huang et al. 1980). The $V_HDJ_H$ unit is linked to the constant region by splicing. Subsequently, following an appropriate stimulus, the locus can undergo recombination-mediated class switching to allow the $V_HDJ_H$ segment to splice to the $\gamma$, $\epsilon$ or $\alpha$ constant regions.

![Diagram of IgH locus](image)

**Figure 1.2.** Organisation of the immunoglobulin heavy chain locus. In the germline configuration (top), the variable ($V_H$) genes are shown in blue, diversity (D) genes in green and joining genes (J) in yellow. During B cell development the germline configuration is rearranged to give a functional heavy chain locus (middle panel). The constant genes are linked to the rearranged VDJ by splicing (bottom panel).

The $IgK$ locus is similar to the $IgH$ locus, except that it lacks D regions. There are hundreds of $V_K$ genes, five $J_K$ genes and one $C_K$ gene in mouse and human, although one of the murine $J_K$ genes is a pseudogene. The murine $Ig\lambda$ genes are organised differently. Two
clusters of \( \lambda \) gene segments exist in the mouse, each consisting of one \( V\lambda \) gene and two \( C\lambda \) genes. Each \( C\lambda \) gene has its own associated \( J\lambda \) region, and the \( J\lambda C\lambda_4 \) segment appears to be a pseudogene, as its expression has not been found in any laboratory strains of mice. The human \( \lambda \) locus has six \( C\lambda \) gene segments and many more \( V\lambda \) regions, and again each \( C\lambda \) region has its own \( J\lambda \) gene. Approximately 60% of human Ig and 95% of murine Ig contain \( \kappa \) light chains.

1.1.4 Rearrangement of immunoglobulin genes

Alt et al. used Southern blot analysis of B cell progenitors transformed by Abelson murine leukaemia virus, which maintain the ability to undergo recombination of their Ig genes in vitro, to show that \( D->J_H \) recombination occurs first, followed by \( V_H->D_J_H \), based on the observation that in the majority of cell lines tested, the rearrangements found were either \( D_J_H \) or \( V_HD_J_H \), but never \( V_HJ_H \) or \( V_HD \) (Alt, Yancopoulos et al. 1984). During the course of this investigation, the same authors also discovered a further mechanism for diversifying the BCR repertoire, namely the addition of N regions. These regions consist of extra nucleotides, not encoded by the Ig genes, that are added in a template-independent fashion during recombination of the IgH locus by the enzyme terminal deoxynucleotidyl transferase (TdT) (Alt and Baltimore 1982). Similarly, the light chain genes are rearranged by \( V_L->J_L \) joining, followed by the production of a primary transcript containing the \( V_LJ_L \) region and the constant region, separated by an intron. The \( \kappa \) locus rearrangement begins first, and if unsuccessful, is followed by \( \lambda \) rearrangement (Hieter, Korsmeyer et al. 1981). Subsequent work has shown that whilst this order of rearrangement, heavy chains before light chains, and \( \kappa \) before \( \lambda \), is not absolute, it is generally favoured during the assembly of the B cell receptor in B cell development (Ehlich, Schaal et al. 1993).
1.1.5 B cell development

The construction of a functional, non-autoreactive antigen receptor is the major goal of B cell development. Mammalian B cell development takes place in the foetal liver and spleen, and adult bone marrow. All cells of haematopoietic lineage are thought to descend from a common precursor which maintains the ability to self-renew, whilst also producing daughter cells that differentiate into either lymphoid or myeloid progenitors. However the exact point at which stem cells become committed to the lymphoid lineage, and the mechanisms controlling this, are still unclear.

One of the biggest technological advances in the study of B and T cell ontogeny was the development of multiple parameter flow cytometric analysis in conjunction with monoclonal antibody technology. This has enabled classification of different stages of differentiation based on the expression of various marker proteins, usually, but not always, expressed on the surface of the cells. This flow cytometric analysis allows the sorting of different populations whose gene expression can be determined by PCR, allowing an intricate description of B cell development. Such a phenotypic analysis is shown in Figure 1.3.

Developing B cells are classed as either pro-B, pre-B, or immature, emerging after ontogeny as mature, recirculating B cells. This distinction is based upon the expression of four markers, B220, CD25, IgM and IgD. B220 is the isoform of CD45 predominantly expressed by B cells, and once switched on in the pro-B cell, its expression remains on throughout development and in mature cells. Interestingly B220 is implicated in the activation of mature B cells through the BCR, but its function in the earliest committed B cells, which have not yet formed a
BCR, is enigmatic. These B220+ pro-B cells begin the process of assembling a BCR by rearranging their heavy chain genes. The recombination-activating genes RAG-1 and RAG-2 are expressed, and mRNA encoding Igα can be detected (Li, Hayakawa et al. 1993). Although the precise function of the RAG genes is uncertain, they are required for VDJ recombination, as mice homozygous for null RAG alleles cannot rearrange their Ig or TCR genes and thus fail to develop B and T cells (Mombaerts, Iacomini et al. 1992; Shinkai, Rathbun et al. 1992). After successful heavy chain rearrangement the cells enter the pre-B cell compartment, where they begin to express CD25, the α chain of the IL-2 receptor (Chen, Ma et al. 1994; Rolink, Grawunder et al. 1994). Pre-B cells rearrange their light chain genes, and if they produce functionally rearranged light chains capable of combining with the heavy chains, they begin to express IgM, thereby becoming immature B cells. Once they express both IgM and IgD they are classified as fully mature.

A more detailed picture of development can be drawn by incorporating further markers into the analysis. Pro-B cells can be subclassified into four fractions, A, B, C and C' according to the experiments carried out in Hardy's laboratory using CD43, BP-1 and HSA (Hardy, Carmack et al. 1991; Li, Hayakawa et al. 1993). Fractions A-C express CD43, distinguishing them from fractions D-F, which do not. Fraction A represents the earliest subset, which becomes fraction B with the expression of heat stable antigen (HSA). At this point the majority of cells show DJH rearrangement, whilst some have VHDJH configurations, and a small proportion VKJk. Fraction C denotes cells that have switched on expression of BP-1 and have completed VHDJH rearrangement. 20% of this fraction also contain rearranged VKJk genes. Correspondingly, the expression of TdT is downregulated in
fraction C. C' represents B220+, BP-1+, CD43lo, HSAhi cells. On entering fraction D, CD43 expression is switched off, and the cells move into the pre-B cell compartment, fraction D. The appearance of IgM marks the passage into fraction E, populated by immature B cells, followed by development into fraction F, mature, recirculating B cells.

1.1.6 The pre-B cell receptor
The heavy chain of IgM (μ chain) is the first component of slg to be expressed, since V_{H}D_{J_{H}} rearrangement occurs first during development. The μ chain is expressed in association with the surrogate light chains V_{preB} and λ5 in pre-B cells, until these are displaced by κ or λ light chains on developing to immature B cells (see Figure 1.3 and reviewed in Rolink, Karasuyama et al. 1994). V_{preB} is analogous to the V_{L} region and λ5 to the J_{L}C region of the light chain. This V_{preB}-λ5-μ complex is referred to the pre-B cell receptor or pBCR, and is necessary for the progression from pro- to pre-B cells (fraction C' -> D), since mice with targeted mutations in either λ5 or μ chain show arrested development at this point, although the λ5 mutant mice do eventually fill up their peripheral B lymphocyte pool after 1-2 years (Kitamura, Roes et al. 1991; Rolink, Karasuyama et al. 1993). Like the BCR, the pBCR is associated with Igα and Igβ. In a recent paper Papavasiliou et al showed that transgenic mice expressing a mutant μ chain incapable of associating with Igβ cannot rescue B lymphocyte development in RAG/- mutant mice, whereas a control transgenic μ chain capable of Igβ association can (Papavasiliou, Misulovin et al. 1995). The addition of the cytoplasmic domain of Igβ to the mutant μ chain is sufficient to restore its ability to drive development, suggesting that Igβ is sufficient for the pBCR-mediated transition of pro-B cells into pre-B cells.
Figure 1.3. Cartoon of B cell development. Pro-, pre-, immature and mature B cells are shown. The pro-B cell compartment is further subdivided into fractions A-C. The status of Ig gene rearrangement during in the different developmental windows is shown. Also shown is the temporal expression of the relevant genes discussed in the main text.
1.1.7 Allelic exclusion

Burnet's theory of clonal selection predicted that antigen receptors on any given lymphocyte would be monospecific, so that only those cells specific for a particular antigen would become activated (Burnet 1959). These activated cells would be selected, on the basis of antigen recognition, to expand. Expression of Ig of more than one specificity per cell is prevented by a process known as allelic exclusion, which allows only one IgH allele to be expressed per cell. Allelic exclusion achieves this by a feedback mechanism for which the pBCR is absolutely required. Once a successful \( V_{H}DJ_{H} \) rearrangement has occurred, the pBCR can be assembled, which signals to the cell to halt further rearrangement of the second allele. This is demonstrated by mouse mutants lacking components of the pBCR such as \( \lambda 5 \), or mice which can only produce secreted rather than membrane-bound IgM, whose B cells can produce functional IgM from both alleles (Kitamura and Rajewsky 1992; Loffert, Ehlich et al. 1996). The mechanism by which the pBCR prevents further heavy chain rearrangement is unclear, although Ig\( \beta \) is again necessary, since the mutant mice described above in which Ig\( \beta \) cannot associate with the \( \mu \) chain also exhibit a breakdown in allelic exclusion (Papavasiliou, Misulovin et al. 1995).

1.1.8 Selection events during development

There are a number of checkpoints during ontogeny past which cells cannot develop unless they fulfil the necessary requirements. The first selection event occurs when the cell displays its pBCR, although whether the pBCR is expressed on the cell surface or is sequestered in an intracellular compartment is unclear, as is the nature of the ligand. Cells that do not have a functional \( V_{H}DJ_{H} \) rearrangement die at this point, whilst those which have been successful in assembling a pBCR...
are selected to expand, as evidenced by up to 85% of fraction C' cells occupying the S and G2 stages of the cell cycle (Karasuyama, Rolink et al. 1994). Since this clonal expansion is pBCR dependent, it represents a form of positive selection.

Further positive selection occurs at the immature B cell stage, when the BCR is expressed. Recent findings from mice in which the cytoplasmic domain of Igα has been removed show that without a competent BCR, the number of mature B cells in the periphery is reduced to one hundredth of that of control animals (Torres, Flaswinkel et al. 1996). The phenotype of the Igα mutant mice points to a positive selection event, such that cells expressing a non-functional receptor are removed. This could be analogous to positive selection in thymocytes, whereby only those cells that can recognise peptides in the context of self-MHC are allowed to complete development.

The pre-B and immature B cell compartments in the mice lacking the cytoplasmic portion of Igα are reduced 2-4 fold, which, when taken in context with the mutant mice in which Igβ cannot associate with IgM, suggests a possible mechanism by which similar receptors mediate different physiological outcomes; Igβ is seemingly sufficient for signalling through the pBCR and thus regulating the pro- to pre-B transition, whilst Igα is necessary for the immature to mature transition, although a function for Igβ at this point cannot be excluded. Mice completely lacking Igβ expression exhibit an early block in B cell development, as heavy chain gene rearrangement is incomplete, with D→JH rearrangement occurring, but not V→DJH (Gong and Nussenzweig 1996). Thus whilst the phenotype of these mice does not allow any inferences for the role of Igβ in signalling through the pBCR, they
suggest that the invariant chains also have receptor-independent functions.

1.1.9 Tolerance

Tolerance of self is necessary to prevent autoimmunity and can be achieved by a variety of means. Central tolerance induction takes place in the bone marrow at the immature B cell stage (fraction E). To account for the possibility that some B cells may develop with specificities for self antigens not present in the bone marrow, tolerance can also be induced in the periphery. Also a tolerant state can be achieved by either clonal deletion of the autoreactive cells or by the induction of anergy, a state in which autoreactive B cells become unable to respond to the antigen for which they are specific. Transgenic technology has allowed mouse models of tolerance to be generated by making mice transgenic for a BCR and then introducing the antigen for which the BCR is specific. Two such models come from Nemazee's laboratory, where the transgenic BCR recognises a particular MHC allele, H2-K^k or H2-K^b, and Goodnow's laboratory, using an anti-hen egg lysozyme (HEL) BCR (Goodnow, Crosbie et al. 1988; Nemazee and Burki 1989). For example, if the anti-H2-k^k BCR is expressed in mice whose genetic background does not include that allele of the MHC, then B cells expressing the transgenic receptor develop and appear in the periphery. However if the transgene is introduced into mice expressing H2-k^k or H2-K^b, then developing B cells are clonally deleted at the immature B cell stage (Nemazee and Burki 1989).

Anergy, rather than clonal deletion, occurs in anti-HEL BCR transgenic mice if soluble HEL is present during development (Goodnow, Crosbie et al. 1988). Thus high valency, multimeric membrane bound antigen
induces clonal deletion, whilst soluble self-antigen results in anergy, suggesting that a threshold of BCR-antigen binding exists that determines whether the outcome will be deletion or anergy.

1.1.10 Receptor editing
During the development of central tolerance, self-reactive cells are able to try and alter the specificity of their BCR by upregulating the RAG genes and further rearranging their light chain genes in a process termed receptor editing. This has been elucidated using the transgenic systems discussed above, by detecting BCRs in the transgenic mice with different idiotypes to the transgene, which represent receptors consisting of the transgenic heavy chain associated with endogenous light chains as a consequence of receptor editing (Gay, Saunders et al. 1993; Tiegs, Russell et al. 1993). Cells unable to generate new, non-autoreactive receptors this way then undergo clonal deletion. However since receptor editing has only been observed in transgenic mice so far, its relevance to normal physiology is unclear.

1.1.11 The B1 lineage
Around 1% of mature B cells in mice and humans belong to a subpopulation referred to as B1 cells. B1 cells differ from B2 cells (conventional B cells) in a variety of ways. Most notably they almost exclusively home to the peritoneal and pleural cavities, tend to express Ig specific for autoantigens and exhibit restricted \( V_H \) gene usage. Originally discovered on the basis of their expression of CD5, which is lacking in conventional B cells (Hardy 1992), they have since been further classified into two distinct subsets; B1a cells, which express CD5, and B1b cells, which do not. However both B1a and B1b share other characteristics missing in the B2 population, such as the expression of
CD43 and Mac-1, they have a higher IgM:IgD ratio and do not express CD23.

B1 and B2 cells may be descended from distinct progenitors, since the early development of B1 cells takes place in the neonate, and then appears to halt, with the mature B1 cells retaining the capacity to self-renew in the periphery (Hardy 1992). The functional significance of this sub-population is poorly understood, as is the relevance of CD5 expression, although they are thought to be necessary for the maintenance of serum IgM that is present without antigenic challenge (Herzenberg, Stall et al. 1986). It has also been suggested that they have evolved as a primary defence against common bacterial pathogens, hence their localisation to the lung and gut.

1.1.12 The CD19/CD21/TAPA-1 complex
There are various mechanisms by which B cells "see" antigen. Cross-linking of the BCR by pathogens with repetitive epitopes, such as structural components of bacterial cell walls like polysaccharides and lipopolysaccharides, can induce B cell activation without the need for T cell help. This allows the animal to respond to non-proteinaceous antigens (T cells can only recognise protein antigens in the context of self-MHC). However to elicit the maximal B cell response, a coreceptor as well as the BCR must be engaged, in a similar fashion to the engagement of both the TCR and CD4 or CD8 with a peptide-MHC complex. Coreceptors facilitate maximal activation by determining the threshold of antigen necessary for stimulation.

The CD19/CD21/TAPA-1 complex is thought to act as a B cell coreceptor and consists of four transmembrane proteins expressed on the cell surface (Figure 1.4 and reviewed in Fearon 1993). CD19 is a
member of the Ig superfamily, containing two Ig motifs in its extracellular domain, whilst the second component of the coreceptor is complement receptor 2 (CR2, or CD21). TAPA-1 is a tetraspan protein preferentially expressed on cells of the haematopoietic lineage, thought to be involved in homotypic aggregation.

![Diagram of the CD19/CD21/TAPA-1/Leu-13 complex](image)

**Figure 1.4.** The CD19/CD21/TAPA-1/Leu-13 complex thought to act as a B cell coreceptor. The Ig-like domains of CD19 are shown in blue, whilst regions of homology to sequences in the cytoplasmic domains of Igα/β are shown as hatched yellow boxes.

The putative role of the complex in determining the threshold necessary for activation through the BCR was suggested by work carried out in Fearon’s laboratory (Carter and Fearon 1992), and is confirmed by studies on the CD19 component using both mice lacking functional CD19 alleles or mice overexpressing the protein. Overexpression results in B cells that appear to be hypersensitive to proliferative signals (Engel, Zhou et al. 1995). Mice lacking CD19 show that conventional B cell development is unperturbed, an unexpected finding considering that CD19 is one of the earliest B cell specific
proteins to be expressed during development, being switched on in cells in fraction A (Uckun, Burkhardt et al. 1993; Engel, Zhou et al. 1995; Rickert, Rajewsky et al. 1995). B1 cells are markedly reduced however, and B2 cells are unable to respond to T-dependent antigens, suggesting that CD19 is crucial for the correct responses to these antigens.

Whilst the ligand for CD19 itself is elusive, the ligand is known for CD21. CD21 is a receptor for the third component of complement, and this may be the mechanism by which the coreceptor exerts its costimulation, via an antigen-antibody-complement complex capable of simultaneous binding to both the BCR and the CD19/CD21TAPA-1 complex (Fearon 1993). CD21 is also able to bind CD23, the low affinity IgE receptor (FceRII), which is expressed on follicular dendritic cells; this may potentiate signalling to B cells through the coreceptor during the germinal centre response. Thus, by binding to either complement or CD23, the coreceptor is able to participate in the activation of B cells.

1.1.13 The germinal centre response

Whilst some B cell responses are T cell independent as mentioned above, class switching to isotypes other than IgG3, affinity maturation and the generation of memory is dependent upon T cell help. Help is recruited in specialised compartments of the secondary lymphoid organs such as spleen and lymph node, firstly in the periarteriolar lymphoid sheath, which surrounds the arterioles of these organs, and then in the lymphoid follicle, where they give rise to germinal centres (for a review, see MacLennan 1994). This process is a complex interplay between at least three cell types, B cells, follicular dendritic cells and T cells. Follicular dendritic cells are interdigitating cells found within the follicles of lymph nodes and which are thought to act by fixing antigen on their surfaces for presentation to, and subsequent activation of, B
Although the precise interaction is unclear, B cell-FDC interactions result in increased expression of B7-1 and MHC class II on the B cell (Koscoe-Vilbois, Gray et al. 1993). Antigen bound to the BCR is internalised by endocytosis, whereupon it is processed into peptides and returned to the cell surface in association with MHC class II, which the T cell can respond to. One proviso for this process is that the T cell is specific for the same antigen as the B cell, although not necessarily the same epitope (Fuller, Kanagawa et al. 1993). This helps to prevent the unnecessary activation of B cells as well as preventing responses to self. In fact, B cells that become activated but do not receive T cell help undergo apoptosis (Lane 1996).

1.1.14 Costimulatory molecules

After activation by ligating the BCR and the coreceptor, the B cell both receives and sends second signals in order to mount the appropriate response. The activated B cell recruits help not only by presenting peptides to T cells but also by upregulating CD80 (B7-1) and CD86 (B7-2), which bind to CD28 on the T cell, thereby providing the T cell with a second signal for activation. The activated T cell in turn upregulates CD40L, the ligand for CD40, constitutively expressed by B cells, and secretes cytokines such as IL-4 which act on B cells and induce them to proliferate.

CD40 is a member of the Tumour Necrosis Factor Receptor superfamily of transmembrane receptors, and CD40L a member of the TNF family which comprise their ligands (reviewed in Armitage 1994). This CD40-CD40L interaction is required for the B cell to class switch from IgM and IgD to either IgG, IgE or IgA during T-dependent responses. Although B cells stimulated by T-independent antigens can class-switch, predominantly to IgG3, the precise mechanism for this is not known.
Evidence for the role of CD40 in class switching comes from a naturally occurring human mutation that leads to hyper-IgM syndrome, and from engineered mouse mutants in which there is a striking deficit of antibodies of any class other than IgM (Allen, Armitage et al. 1993; Aruffo, Farrington et al. 1993; Renshaw, Fanslow et al. 1994). That the defect specifically affects class-switching is demonstrated by the elevated levels of IgM in the sera, suggesting that initial activation of B cells is still possible. Also the possibility that CD40 is necessary but insufficient for class-switching cannot be excluded, as the CD40-CD40L interaction may indirectly, rather than directly, stimulate class-switching. As yet the mechanism by which cells decide to switch to one class or another is unknown, although cytokines are also thought to play a role.

1.1.15 Affinity maturation and terminal differentiation

Apart from clonal expansion and class switching, B cells in the germinal centre undergo affinity maturation by somatic hypermutation. Cells initially responsive to antigen are selected for oligoclonal expansion, during which mutations are introduced in the V regions (Berek, Berger et al. 1991; Jacob, Kelsoe et al. 1991). These mutations are found primarily in the CDR regions of the antibody, and only those cells whose mutations confer a higher affinity for the antigen are allowed to survive, suggesting even further selection. This would seem appropriate, as mutations in the non-antigen binding regions are likely to be useless or deleterious. The precise mechanism by which these mutations occur is as yet unknown, as are the signals necessary to initiate it, although mice injected with antibodies against B7-2 exhibit a lower amount of somatic hypermutation than controls (Han, Hathcock et al. 1995). Consistent with this process, massive cell death is detectable in germinal centres. This represents the removal from the repertoire of cells whose mutations have lead to
autospecificity or a decreased affinity for the original antigen (reviewed in Lane 1996). Of the cells that survive the germinal centre response, some will undergo terminal differentiation into antibody-secreting plasma cells, whilst others will develop into long-lived memory cells. The processes governing these two pathways are still far from clear.

1.1.16 Summary
The BCR is the raison d'etre of a B cell and the key to its function in the immune response. As demonstrated above, the assembly of the BCR from the polygenic complex encoding it is monitored stringently at the pro-B to pre-B transition during B cell development by signals through the pBCR. Once mature, the cell relies on costimulatory signals from molecules such as CD19, CD21 and CD40 to allow it to decide on the appropriate response: apoptosis, class switching, antibody secretion or memory cell formation.

1.2 Signal transduction through the B cell antigen receptor

1.2.1 Tyrosine phosphorylation
Studies in a number of laboratories in both splenic B cells and the B cell line WEHI-231 have shown that tyrosine phosphorylation is the earliest detectable event induced by ligation of the BCR (Campbell and Sefton 1990; Gold, Law et al. 1990). As none of the known components of the BCR have any intrinsic kinase activity, the focus began to shift towards identifying putative kinases responsible for these observations. Four kinases, Lyn, Fyn, Lck and Blk, were found to be associated with the BCR and to become activated following BCR ligation (Burkhardt, Brunswick et al. 1991; Campbell and Sefton 1992; Li, Mahajan et al. 1992; Lin and Justement 1992; Yamanashi, Fukui et al. 1992; Yamamoto, Yamanashi et al. 1993).
1.2.2 The role of Igα and Igβ

Gold et al showed that Igα and Igβ were themselves targets for phosphorylation on ligation of the B cell receptor (Gold, Matsuuchi et al. 1991) and, as mentioned previously, they are both sufficient for selection signals in developing B cells at different stages of differentiation. Biochemical evidence to suggest that Igα and Igβ might mediate different signals from the BCR comes from Cambier's laboratory, who used glutathione-S-transferase (GST) fused to either the Igα or Igβ cytoplasmic domains to probe B cell lysates. Interestingly they found that Lyn and Fyn could be recovered bound to Igα but not Igβ, whilst the 85kD subunit of phosphatidyl-inositol-3-kinase was found bound to both (Clark, Campbell et al. 1992). Further work supporting the participation of Igα and Igβ in BCR signal transduction comes from work using mutants of IgM unable to associate with Igα and Igβ in the membrane (due to amino acid substitutions in the transmembrane region of IgM) to show that the invariant chains were essential for tyrosine phosphorylation and calcium fluxes in response to IgM ligation (Sanchez, Misulovin et al. 1993). Furthermore, when a chimaeric protein consisting of the extracellular and mutant transmembrane domains of μ fused to the cytoplasmic region of either Igα or Igβ was transfected into the same cell line, the rescue of the response was dependent upon whether Igα or Igβ was used. Both were able to reconstitute calcium fluxing, whereas only Igα was able to reconstitute tyrosine phosphorylation (Sanchez, Misulovin et al. 1993).

1.2.3 Immunoreceptor Tyrosine-based Activation Motifs

The discovery by Reth in 1989 that Igα, Igβ, and other antigen receptor invariant components, notably the CD3 and ζ chains of the TCR, contained a conserved motif in their cytoplasmic domains suggested the mechanism by which the invariant chains could act as signal
transduction modules for antigen receptors (Reth 1989). This motif, subsequently termed an ITAM, for Immunoreceptor Tyrosine-based Activation Motif (Cambier 1995), consists of the consensus amino acid sequence YXXL/I(X$_{6-8}$)YXXL/I, where X can be any amino acid. Igα, Igβ, CD3γ, δ, and ε all contain one copy of this sequence, whereas ζ contains three tandem ITAMs (shown as yellow rectangles in Figure 1.1). Experiments in which the tyrosine residues in the ITAMs of ζ or ε have been mutated demonstrate that the integrity of the ITAMs is necessary for signalling through the TCR.

Further experiments from Reth's laboratory using J558Lµm cells, the cell line mentioned previously that does not express endogenous Igα and therefore can only express cytoplasmic Ig, reconstituted with a CD8-Igα chimera, have generated more evidence for the involvement of the ITAMs in BCR signal transduction (Flaswinkel and Reth 1994). Various constructs were used containing mutations in the Igα cytoplasmic domain, notably the replacement of the two tyrosines of the ITAM by phenylalanine residues, or the complete deletion of 52 amino acids containing the ITAM. Their results showed that mutation of one or other of the tyrosines caused a slight decrease in the level of signal transduction through the chimeric receptor as measured by tyrosine phosphorylation after BCR cross-linking. However removal of both tyrosines, or complete removal of the ITAM, drastically reduced this response. They then went on to show in an in vitro assay that the Src tyrosine kinase Fyn was able to phosphorylate the ITAM, and they proposed that on ligation of the B cell receptor, Src kinases are activated and phosphorylate the ITAMs of Igα.
1.2.4 The Src family of tyrosine kinases

The BCR-associated kinases Lyn, Blk, Fyn and Lck are members of the Src family of non-receptor tyrosine kinases, the prototype of which, Src, was first identified as the transforming oncogene in Rous sarcoma virus (referred to as v-Src). After the discovery of v-Src, a mammalian cellular homologue was discovered, c-Src (Stehelin, Varmus et al. 1976), and the Src family now consists of 9 members, whose expression is differentially regulated in various cell lineages. Their structure and expression patterns are shown in Figure 1.5. Each has a unique region at the N-terminal end of the protein, although there is a conserved myristoylation site present immediately after the initiator methionine, by which the protein is anchored into the cytoplasmic side of the plasma membrane. At the carboxy terminus is the kinase domain, which contains several highly conserved residues essential for kinase activity. The lysine at position 295 of Src is absolutely required for kinase activity and its transforming ability (Snyder, Bishop et al. 1985; Kamps and Sefton 1986). This lysine is conserved among all the family members. Also there are two conserved tyrosine residues, at positions 416 and 527 of Src. Phosphorylation of these sites regulates the activity of the proteins, as demonstrated by the finding that v-Src's transforming ability is due to a truncation at the carboxy terminus which removes Tyr 527 and leads to constitutive kinase activity. Furthermore, Kmiecik and Shalloway showed that mutation of Tyr 416 to phenylalanine leads to suppression of its kinase activity and transforming ability, whilst the same amino acid change at Tyr 527 activated the kinase (Kmiecik and Shalloway 1987). Thus the working model of Src regulation is that phosphorylation of Tyr 527, or its equivalent in the other family members, negatively regulates kinase activity, whilst phosphorylation of Tyr 416 increases its kinase activity.
However mutation of both Tyr 527 and Tyr 416 together results in an inactive kinase, thus dephosphorylation of Tyr 527 alone cannot optimally activate Src (Kmieciak and Shalloway 1987). Concomitant phosphorylation of Tyr 416 is necessary to achieve this. The high degree of homology between family members, and also between species, hints at their importance in signal transduction pathways. In fact some features of these proteins are shared with other families of signal transduction molecules, most notably the SH2 and SH3 domains (for a review see Pawson and Gish 1992). These domains mediate interactions between the proteins that contain them.

\[\text{N-terminus} \quad \text{Unique} \quad \text{SH3} \quad \text{SH2} \quad \text{Kinase} \quad \text{C} \]

b)

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Figure 1.5. a) Structure of the Src kinases. The N-terminal myristoylation site is indicated, as are the unique, SH3, SH2 and kinase domains. Y416 represents the positive regulatory tyrosine and Y527 the negative regulatory tyrosine. The position of the conserved lysine at the ATP binding site is also shown. b) Table showing the distribution of Src family members' expression in a number of cell types.

SH2 domains consist of approximately 100 amino acids and bind specifically to phosphotyrosine residues in the context of the appropriate amino acid sequence. Their structure has been solved by crystallography, showing that binding to the phosphotyrosine is by means of an arginine residue deep in the binding pocket of SH2.
Phosphoserine and phosphothreonine residues cannot bind since they do not have long enough side chains to reach into the groove and make contact with the arginine (Waksman, Kominos et al. 1992). The consensus recognition sequence of SH2 domains reveals that the residues at positions +1 and +3 from the phosphotyrosine make contact with the surface of the domain and are thus important determinants of specificity (Pawson and Gish 1992). SH3 domains bind to proline-rich sequences in their target proteins.

1.2.5 The Syk/ZAP-70 family
Syk was first isolated as a novel non-receptor tyrosine kinase of 72kD expressed in spleen, and was later found to be expressed in B cells, mast cells, thymocytes, platelets and neutrophils, whilst the expression of the only other family member to date, ZAP-70, is restricted to the T cell lineage and NK cells. These two proteins share common domains with the Src family, namely the kinase domain at the carboxy terminal end of the protein, and two SH2 domains. Syk and ZAP-70 can associate with the invariant chains of antigen receptors: ZAP-70 with the ζ chain of the TCR and Syk with the invariant chains of the BCR and the β and γ chains of FcεRI (Chan, Iwashima et al. 1992; Hutchcroft, Geahlen et al. 1992; Hutchcroft, Harrison et al. 1992; Shiue, Green et al. 1995). Their tandem SH2 domains can bind phosphorylated ITAMs cooperatively; if only one of the tyrosines in the ITAM is phosphorylated, binding of Syk or ZAP-70 is weaker than if both are phosphorylated. After ligation of these receptors they become phosphorylated themselves and activated.

Signalling through the TCR by ZAP-70 is well documented, particularly since the discovery that mutations in human ZAP-70 are responsible for a form of severe combined immunodeficiency (SCID). Two groups
studying SCID patients observed that in some cases, the CD8+ lineage of T cells was completely absent, whilst the CD4+ T cell compartment had expanded to the extent that the total number of T cells was normal. However the CD4+ T cell population was refractory to stimulation by CD4 and CD3 crosslinking and the pattern of tyrosine phosphorylation seen after T cell activation was altered compared to wild type cells. Further analysis revealed mutations in the kinase domain of ZAP-70 (Chan, Kadlecsek et al. 1994; Elder, Dong et al. 1994). The development of CD4+, but not CD8+ T cells in the absence of ZAP-70 may reflect the ability of Syk to compensate for some ZAP-70 functions. This is supported by two pieces of evidence: Syk appears to be upregulated in thymocytes from a patient lacking ZAP-70 (Gelfand, Weinberg et al. 1995) and ZAP-70 is able to reconstitute BCR signalling events in a Syk deficient B cell line, suggesting a degree of redundancy between the two kinases (Kong, Bu et al. 1995). ZAP-70 expression has been ablated in mice by gene targetting, giving a slightly different phenotype from the human patients (Negishi, Motoyama et al. 1995); ZAP-70-/- mutant mice lack both CD4+ and CD8+ mature T cells.

Syk has also been targeted in the mouse genome, generating evidence for its involvement in both B cell and mast cell antigen receptor signalling. Mice homozygous for null Syk alleles die perinatally, but radiation chimeras made using Syk-/- bone marrow show a block at the pro- to pre-B cell transition, strongly implicating Syk in signalling through the pre-BCR (Cheng, Rowley et al. 1995; Turner, Mee et al. 1995). Also mast cells derived from these mice are unable to respond normally to stimuli through the FceRI antigen receptor (Costello, Turner et al. 1996).
1.2.6 The sequential activation model of TCR signalling

A model has evolved to explain how both the Src and Syk kinases function in signal transduction through antigen receptors based on studies primarily on the TCR, although subsequent work has shown that this model may be equally applicable to both the BCR and the FceRI receptor. Using a COS-1 cell line stably transfected with a CD8-ζ chimeric protein, Iwashima and colleagues were able to show that after stimulation with an anti-CD8 antibody, Lck is required to phosphorylate the tyrosines of the ζ chain ITAMs and that this leads to the recruitment of ZAP-70, which binds to the two phosphotyrosines via its tandem SH2 domains (Iwashima, Irving et al. 1994). Thus the sequential model of tyrosine kinase activation was proposed, in which the Src family kinases become activated first. They phosphorylate the ζ-chain ITAMs, which can then recruit ZAP-70 via its SH2 domains. At this point ZAP-70 becomes activated (Figure 1.6).

1.2.7 Sequential activation in signalling through the BCR

This sequential activation model has been tested by a number of laboratories studying signalling through the BCR and FceRI receptor. There are temporal differences in the activation of the Src kinases and Syk after ligation of the BCR, but this is not sufficient to support the sequential activation model (Saouaf, Mahajan et al. 1994). Evidence that the BCR uses a similar mechanism as the TCR comes from work on Lyn negative B cells, in which the phosphorylation status and activation of Syk are profoundly reduced (Kurosaki, Takata et al. 1994). Also Syk mutants have shown that both SH2 domains are required for efficient BCR signal transduction through the BCR, where they are thought to bind to the ITAMs of Igα and Igβ, in analogy to ZAP-70 binding to the ζ chain of the TCR (Kurosaki, Takata et al. 1994).
Figure 1.6. The sequential activation model of antigen receptor signal transduction. The Src kinases are anchored into the membrane whilst Syk/ZAP-70 reside in the cytoplasm. Occupancy of the antigen receptor leads to activation of Src kinases, which phosphorylate the ITAMs. This allows Syk or ZAP-70 to bind via the tandem SH2 domains, thus recruiting it to the receptor complex, where it becomes phosphorylated, possibly by a Src kinase, resulting in the activation of Syk/ZAP-70. For simplicity, receptor ligation-induced clustering is not shown.
1.2.8 Signalling through the high affinity IgE receptor

The FcεRI receptor, expressed on mast cells and basophils, is structurally and functionally related to the BCR and TCR in that it is a multimeric transmembrane complex containing ITAMs within its cytoplasmic domains. Scharenberg et al. recreated FcεRI in fibroblasts and used mutants of Lyn and Syk, which both associate with FcεRI in mast cells, to ascertain the exact sequence of events after cross-linking of the receptor (Scharenberg, Lin et al. 1995). Their results indicated that Lyn was indeed upstream of Syk in their model system. Similar results were found by Jouvin et al., who determined that Lyn becomes activated upon FcεRI clustering. Lyn then phosphorylates the β and γ chains of the receptor, which mediates the activation of Syk (Jouvin, Adamczewski et al. 1994). Furthermore, after engagement of FcεRI on Syk deficient mast cells, Lyn is still activated, both it and the β and γ subunits become phosphorylated, but downstream targets such as Vav and PLCγ1 fail to become phosphorylated (Costello, Turner et al. 1996).

1.2.9 Regulation of BCR proximal signalling events

If the sequential model of kinase activation holds, then the Src family of kinases and their interactions with the invariant chains of antigen receptors provide the first step in the signal transduction cascade at which regulatory proteins can also act to modulate the response. The phosphorylation of Src kinases at their carboxy terminal regulatory tyrosine, for example, could attenuate signalling. Likewise for them to become activated, this site must be dephosphorylated. Two putative molecules thought to carry out this regulation are the transmembrane phosphatase CD45 expressed in varying isoforms on all haematopoietic lineages and the C-terminal Src kinase Csk. CD45 is a positive regulator and Csk a negative regulator of BCR signalling.
Evidence from CD45 negative cell lines has shown that CD45 is required for signal transduction through the BCR, as cells were unable to flux calcium in the absence of CD45 (Justement, Campbell et al. 1991). Work carried out in T cell lines shows that CD45 is necessary to dephosphorylate the negative regulatory tyrosine of Lck and Fyn, thereby increasing their kinase activity (Hurley, Hyman et al. 1993). In B cells, CD45 is required for determining the threshold necessary to achieve signalling through the BCR, as demonstrated by crossing CD45"/" mice to the HEL transgenics; splenic CD45"/" anti-HEL BCR B cells respond poorly when presented with HEL in vitro. Negative selection does not occur in the presence of soluble HEL, and so these two results suggest that CD45 is required to set the threshold that determines the outcome of BCR signalling in these cells (Cyster, Healy et al. 1996). CD45 has also been shown to dephosphorylate the ITAMs of Igα and Igβ in vitro, although the in vivo significance of this, if any, is unclear (Justement, Campbell et al. 1991).

Csk has been shown to phosphorylate Src family members on the negative regulatory tyrosine, and mouse mutants unable to express Csk have constitutively active Src, Fyn and Lyn, although the effect of this mutation on lymphocytes could not be determined as the mutation is an embryonic lethal (Okada, Nada et al. 1991; Imamoto and Soriano 1993; Nada, Yagi et al. 1993). B cell lines which cannot express Csk because of mutated Csk alleles also exhibit constitutively active Lyn that is hypophosphorylated on its carboxy terminal tyrosine (Hata, Sabe et al. 1994). Furthermore, Syk was also constitutively active in these cells, possibly as a consequence of the activation of Lyn.
The protein tyrosine phosphatase SHP-1 is also involved in the regulation of BCR signalling, although not by directly dephosphorylating Src kinases. SHP-1 is a cytoplasmic tyrosine phosphatase belonging to a widely conserved family of phosphatases whose expression is also found in Drosophila. SHP-1 (previously called PTP1C, HCP, or SH-PTP1) is expressed in all haematopoietic lineages and consists of two SH2 domains and a phosphatase domain (Yi, Cleveland et al. 1992). A naturally occurring mouse mutant defective for SHP-1, motheaten, develops severe autoantibody production and immunodeficiency, with conventional B2 cells missing in these mice and abundant B1 cells. Again the anti-HEL BCR transgenic mice have provided important insights into the function of SHP-1. After crossing them to motheaten mice, Cyster et al. have shown that soluble HEL is sufficient to induce clonal deletion, whereas in SHP-1+/+ mice the B cells are anergised rather than deleted by the presence of soluble HEL (Cyster and Goodnow 1995). Thus in the absence of SHP-1, the threshold necessary for BCR signal transduction is lowered, suggesting that SHP-1 negatively regulates BCR signalling. A number of laboratories have provided good evidence to suggest that SHP-1 functions by binding to phosphotyrosine residues in a recognition sequence similar to ITAMs. This SHP-1-recognition sequence has been named an immunoreceptor tyrosine-based inhibition motif, or ITIM (reviewed in Thomas 1995). The ITIM was first documented in the cytoplasmic tail of the IgG Fc receptor FcγRIIB, as a 13 amino acid sequence containing a conserved tyrosine residue that is critical for its function (Muta, Kurosaki et al. 1994). Binding of IgG has long been known to attenuate BCR-mediated signals and this has recently been attributed to binding of SHP-1 to the ITIM of FcγRIIB (Thomas 1995).
The CD22 molecule, a transmembrane protein with homology to adhesion molecules, also contains an ITIM and is found in the membrane associated with the BCR (Doody, Justement et al. 1995). Although its ligand is unknown it is thought to act as an off switch for BCR signalling by recruiting SHP-1, which binds to the ITIM via one of its SH2 domains (Lankester, van Schijndel et al. 1995).

**1.2.10 Downstream effectors of BCR signalling**

The activation of Lyn and Syk leads to a cascade of downstream signalling events, culminating in a physiological response. Some of these downstream signals require the presence of both Lyn and Syk, but already there is a bifurcation of the signal at this point, as evidenced by experiments in the chicken B cell line DT40. Takata *et al.* removed Lyn and Syk expression by homologous recombination and found that Syk negative cells are unable to mobilise calcium after BCR stimulation as a result of deficient phospholipase Cγ phosphorylation, whilst Lyn negative cells can produce an increase in intracellular [Ca^{2+}] (Takata, Sabe *et al.* 1994). However the calcium release in the Lyn negative cells exhibited slower kinetics than wild type cells, even though IP3 generation appeared normal, suggesting that Lyn and Syk can independently mobilise calcium through different pathways. Tyrosine phosphorylation in these mutant cell lines was also affected, again with different phosphorylated proteins being affected depending on whether the cells were Lyn- or Syk-deficient. The authors point out however that a simple addition of the phosphorylated proteins in both mutant cell lines does not restore the wild type phosphorylation pattern after BCR cross-linking, which may mean that whilst Lyn and Syk phosphorylate unique targets, optimal downstream phosphorylation requires the presence of both.
1.2.11 Vav

The 95kD Vav protein is rapidly tyrosine phosphorylated on B cell antigen receptor stimulation (Bustelo and Barbacid 1992). This rapid tyrosine phosphorylation suggests that it may be a target of BCR associated kinases such as Lyn or Syk. Vav contains two SH3 domains flanking the SH2 region, as well as homology to other molecules known to be involved in signal transduction cascades, such as the GDP-GTP exchange factor Dbl, and a pleckstrin homology (PH) domain. First discovered in pleckstrin, a platelet specific protein, PH domains are approximately 100 amino acids in length, and like SH2 and SH3 domains are found in a wide range of molecules implicated in signal transduction (for a review see Musacchio, Gibson et al. 1993), and are also thought to participate in protein-protein interactions. The significance of these domains has yet to be determined. Vav-deficient mouse mutants have been created, demonstrating that Vav is required for proliferation signals generated through both the B and T cell antigen receptors (Fischer, Zmuidzinas et al. 1995; Tarakhovsky, Turner et al. 1995; Zhang, Alt et al. 1995). Development of Vav−/− lymphocytes is compromised, as thymocytes are not properly positively selected and the resulting mature cells are present in reduced numbers (Fischer, Zmuidzinas et al. 1995). Also the B1 lineage is absent in these mice. Given that B1 cells retain the ability to self-renew in the periphery, and the poor in vitro proliferative responses to antigen-receptor specific stimuli in the T and B cells of the mutant mice, it seems that Vav is necessary for signal transduction through antigen receptors. Further work is necessary to establish exactly what Vav's function is in this process and which kinase phosphorylates it upon receptor ligation.
1.2.12 Btk

Btk is a member of a newly discovered family of non-receptor tyrosine kinases, the Tec family, which have a slightly different structure to the Src kinases; the Tec family have a single SH3 domain, SH2 domain and kinase domain at the carboxy terminus, but they lack the negative regulatory tyrosine present at the carboxy terminus of Src kinases and are not myristoylated. In addition the Tec family have a PH domain N-terminal to the SH3 domain (Tsukada, Rawlings et al. 1994). Mutations in Btk are responsible for congenital immunodeficiencies in both human and mouse: X-linked agammaglobulaemia (XLA) in humans is characterised by an almost complete absence of mature B cells and lack of immunoglobulins of all classes, whilst the xid phenotype in mice is less severe, with around 50% of normal numbers of mature B cells, albeit unable to respond to T-independent type II antigens as evidenced by the failure to produce IgM and IgG3 (IgG3 being the hallmark immunoglobulin of a T-independent antibody response) (Tsukada, Rawlings et al. 1994). Mutations in human Btk which result in XLA have been found in the PH, SH2, SH3 and kinase domains, suggesting that all of these modules are necessary for B cell development, whilst the mutation in xid mice is in the PH domain (Tsukada, Rawlings et al. 1994). Recent work has focused on the function of Btk in terms of its activation on antigen receptor stimulation, as it is known to become phosphorylated and activated 2-5 minutes after BCR, just later than the activation of the Src kinases and before Syk (Saouaf, Mahajan et al. 1994). Interestingly Lyn and Fyn are both able to phosphorylate and activate Btk when expressed together in a cell line, whereas Syk is not (Rawlings, Scharenberg et al. 1996). Using kinase inactive mutants of both Lyn and Btk these authors showed that for optimal activation of Btk both kinases must be functional, and that Lyn first phosphorylates Btk, activating it and allowing it to then autophosphorylate, leading to
increased Btk kinase activity. Very similar experiments with Tec and Lyn give the same result, namely that Lyn can phosphorylate Tec, leading to its activation (Mano, Yamashita et al. 1996). Btk is the only known Tec-kinase expressed in B cells whilst both Btk and Tec are expressed in mast cells. Further evidence for the interaction between Src kinases and Btk comes from studies using the yeast two-hybrid system showing that Lyn, Fyn and Hck can bind to Btk via the SH3 domains of the Src kinases (Cheng, Ye et al. 1994). Also Src kinases are able to activate Btk when cotransfected into COS cells (Mahajan, Fargnoli et al. 1995), although whether this is representative of B cell activation events is not known. At least one target of Btk binding via its SH3 domain has been documented: the 120kD Cbl protein (Cory, Lovering et al. 1995), which becomes tyrosine phosphorylated after antigen receptor ligation (Tanaka, Neff et al. 1995; Panchamooorthy, Fukazawa et al. 1996). This tyrosine phosphorylation is dependent upon Lyn (Tezuka, Umemori et al. 1996).

1.2.13 The MAP kinase cascade
One of the best documented signal transduction pathways is that of the mitogen-activated protein kinases, or MAPKs, whose mode of action is known to directly connect events at the cell surface to those in the nucleus. These pathways have been highly conserved during evolution, being found in yeast, Drosophila, mouse and human, underlying their fundamental importance in mediating transduction of extracellular signals. Casillas et al showed in 1991 that cross-linking IgM on the surface of a variety of B cell lines, or treating them with the phorbol ester PMA activated a MAP kinase (Casillas, Hanekom et al. 1991). There are a number of different MAPK cascades which appear to be activated by separate extracellular stimuli, and the two most studied in lymphocytes are the ERK and JNK pathways. ERK and JNK are
activated by phosphorylation on tyrosine and threonine residues, by MEK1/2 and SEK respectively, which are MAP kinase kinases (MAPKK). MEK1/2 are in turn activated by Raf, a MAP kinase kinase kinase (MAPKKK) (see Figure 1.7 and reviewed in Marshall 1994). The upstream activators of the ERK pathway, Ras, Raf and MEK, are again ubiquitous throughout various species and are the best understood to date. Tyrosine kinase activity is coupled to the MAPK cascade by adaptor proteins lacking enzymatic activity but containing SH2 and SH3 domains. This allows receptors such as the epidermal growth factor receptor, a transmembrane tyrosine kinase receptor, to input signals into the Ras-Raf-MEK-ERK transmission module.

The key players in funnelling signals via the Src family into the MAPKs are Shc and Grb2, two widely expressed adaptor proteins. Phosphorylation of Shc allows binding of Grb2 via its SH2 domain, followed by Grb2-mediated recruitment of Sos, a nucleotide exchange factor that acts on Ras (Egan, Giddings et al. 1993; Gale, Kaplan et al. 1993; Li, Batzer et al. 1993; Rozakis Adcock, Fernley et al. 1993). Grb2 binds Sos through its two SH3 domains. The subsequent activation of Raf, a MAP kinase kinase kinase (MAPKKK) is dependent on activation of Ras. The precise details upstream of the JNK pathway have not been determined, though it has been suggested that the small G proteins Rac and cdc42 may be involved (Su and Karin 1996).

Whilst some investigators have found that Shc is involved in connecting signals from cytoplasmic tyrosine kinases to the MAP kinase cascade after BCR ligation (Kumar, Wang et al. 1995), others have stated that this is not the case in signalling through the TCR (Osman, Lucas et al. 1995). The recently cloned SLP-76 may also be involved based on its ability to associate with Grb2 (Jackman, Motto et al. 1995), although this is speculative.
The end result of these pathways is the translocation to the nucleus of MAPKs, where they phosphorylate, and thereby activate, transcription factors which initiate transcription, for example of Fos and Jun. ERK
phosphorylates components of ternary complex factor (TCF), such as Elk-1 and SAP-1, allowing TCF binding to the serum response element in the Fos promoter. Similarly JNK phosphorylates Elk-1 and also Jun, which is then able to activate transcription via binding to a response element in its own promoter (Figure 1.7, and reviewed in Su and Karin 1996).

1.2.14 Phospholipid mediators of signal transduction

The breakdown of phosphoinositides in the cell membrane by phospholipases to generate second messengers after ligation of a cell surface receptor is a well-documented phenomenon and plays an integral part in signal transduction from a variety of receptors. This event is catalysed by members of a family of phospholipases classified on the basis of their specificity. For example, phospholipase A2 is responsible for the generation of arachidonate, the precursor of leukotrienes, prostaglandins and thromboxanes. Phospholipase C (PLC) catalyses the formation of inositol-1, 4, 5-trisphosphate (IP$_3$) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol-4,5-bisphosphate during signal transduction through a variety of receptors. IP$_3$ mediates the release of intracellular stores of Ca$^{2+}$ by binding to an IP$_3$ receptor on the endoplasmic reticulum, whilst DAG binds to some members of the protein kinase C family of serine/threonine kinases, thereby activating them. Phospholipase D is involved in the breakdown of phosphatidyl choline.

The PLC-mediated production of IP$_3$ and DAG is utilised by both the BCR and TCR in signalling through the antigen receptors. PLC$\gamma$ isoforms are phosphorylated on antigen receptor ligation and this is thought to stimulate their activity. B cells use primarily the PLC$\gamma$2
isoform and T cells PLCγ1, although the significance of this is unclear (Hempel, Schatzman et al. 1992; Takata, Homma et al. 1995).

1.2.15 Trimeric G proteins
Tyrosine phosphorylation of PLCγ is not the only mechanism by which B cells can generate phosphoinositol second messengers however, as there is evidence for the involvement of a heterotrimeric G protein in BCR signal transduction (Harnett and Rigley 1992). This family of GDP-GTP exchange proteins regulate IP₃ production by acting as activators of PLCβ, rather than PLCγ. Originally timeric G proteins and receptor-associated kinases were thought to initiate mutually exclusive signalling pathways, possibly by the use of different phospholipases. However the discovery that Shc can act as a common link between trimeric G protein- and tyrosine kinase-mediated activation of the MAPK pathway suggests that there is far more interplay between the two than was previously thought. Two groups have independently shown this link. The first determined that a newly cloned tyrosine kinase, PYK2, which is activated by a trimeric-G protein, presumably through PLCβ, phosphorylates Shc, thus leading to the activation of the MAP kinase cascade (Lev, Moreno et al. 1995). The second group demonstrated that overexpression of the Gβ and Gγ G-protein subunits in COS-7 cells could activate the MAP kinase cascade (van Biesen, Hawes et al. 1995). Recently Lyn has been implicated in the transmission of signals from trimeric G-proteins into the MAPKs in neutrophils, in a process also involving Shc (Ptasznik, Traynor et al. 1995). Furthermore, a second group have published similar data after discovering that activation of MAPKs via G-protein-coupled receptors is blocked in mutant cells lacking Lyn expression (Wan, Kurosaki et al. 1996). This work raises the possibility that G-protein orchestrated events in B cells may involve Lyn, although much more work is
necessary before any definitive conclusions about this prospect can be made.

1.2.16 Lipid kinases
Apart from protein kinases such as the Src, Syk and MAPK families, lipid kinases are heavily implicated in signal transduction, again by an evolutionarily conserved mechanism involving members of the large family of phosphatidylinositol-3-kinases (PI-3-kinases). These proteins are classified by a conserved region in the kinase domain and consist of two subunits, an 85kD regulatory subunit and a 110kD kinase subunit. Src kinases, including Lyn, Lck and Fyn, are capable of binding to the 85kD subunit of PI-3-kinase via their SH3 domains (Pleiman, Hertz et al. 1994) and are thus thought to activate PI-3-kinase on antigen receptor ligation. Once activated, PI-3-kinase phosphorylates phosphatidylinositol, which is known to act as a second messenger. However the precise details of PI-3-kinase mediated signalling remain to be established.

1.2.17 Differential outcomes of BCR signalling
As mentioned previously, pBCR or BCR ligation can result in the upregulation of light chain rearrangement, allelic exclusion, positive and negative selection, clonal expansion, affinity maturation and terminal differentiation. However the differential signalling necessary to determine which outcome the cell chooses is far from understood. The affinity of the antigen receptor for antigen is likely to be important, as is the concentration of antigen, as evidenced by work in transgenic mouse models suggesting that a threshold exists that determines the response to BCR ligation. The expression of multiple kinases able to interact with the BCR may provide the first level at which differential signalling can occur. For example, in T cells both Fyn and Lck are
expressed, but they appear to be important for signalling at different developmental timepoints, as evidenced by mutant mice in which their expression has been ablated. In mice lacking Lck, there is a severe block in thymocyte development at a point when signals through the TCR are thought to be crucial, and the number of mature T cells in the periphery is reduced (Molina, Kishihara et al. 1992). Mice lacking Fyn, on the other hand, do not suffer from this block, but Fyn<sup>−/−</sup> thymocytes show markedly reduced calcium fluxing after TCR-CD3 ligation (Stein, Lee et al. 1992). This suggests that different cellular outcomes of antigen receptor ligation may be mediated by different kinases. In B cells, different Src family members are expressed in a developmentally restricted fashion. Blk and Lyn are expressed early in development right through ontogeny, and Blk expression is switched off on differentiation to plasma cells, whilst Fyn and Fgr are upregulated in mature splenic B cells (Wechsler and Monroe 1995). Lyn may be important for signalling during negative selection, as Lyn<sup>−/−</sup> mice exhibit autoantibody production. Also the number of mature B cells that develop in these animals is reduced, suggesting that Lyn may be important for positive selection of B cells or their survival in the periphery (Hibbs, Tarlinton et al. 1995; Nishizumi, Taniuchi et al. 1995). Thus Lyn's major role appears to be at the immature to mature stage of B cell development, although the precise role is unknown. The existence of a further kinase, with a function analogous to that of Lck in T cells, cannot be excluded given that signalling is necessary earlier on in development, and that this signalling does not appear to be dependent upon Lyn.

1.2.18 Summary

Initial events on ligation of the BCR include phosphorylation of Igα and Igβ, the activation of the Src kinases Lyn, Blk and Fyn, and the
activation of Syk. These kinases mediate downstream effects such as the activation of PLCγ, the MAP kinase cascade, PI-3-kinase, and the phosphorylation of tissue specific proteins such as Btk. The culmination of these signalling pathways depends upon the developmental window occupied by the cell, plus the presence or absence of costimulatory signals through CD19, CD40, and cytokines. A diagrammatic summary of BCR signal transduction is shown in Figure 1.8.

Figure 1.8. Cartoon of signal transduction through the B cell antigen receptor (BCR). Molecules known to be involved are shown: the MAPK cascade, a putative trimeric G-protein, Src family kinases, PLC, Vav, Btk and Cbl, amongst others. Putative interactions between proteins are shown as arrows accompanied by question marks.
1.3 Experimental strategies

1.3.1 The tyrosine kinase Lyn

To study signal transduction through the BCR I have focused on the Src family kinase Lyn. At the start of this project Blk had not been cloned, unlike Fyn and Lck, and so Lyn was the newest member of a growing number of haematopoietic cell-specific proteins implicated in antigen receptor signalling. Work on Lck in T cells was beginning to yield exciting insights into signal transduction through the TCR. Lyn was a good candidate for a protein playing a similar role in BCR signalling. Furthermore Lyn expression is distributed widely throughout the immune system, being expressed not only in B cells but in macrophages, monocytes, neutrophils, platelets and mast cells, suggesting that it is part of a conserved signalling system in haematopoietic cells (Stanley, Ralph et al. 1991; Yi, Bolen et al. 1991).

Lyn differs from the other B cell specific Src kinases in that two isoforms are present in all the lineages in which Lyn is expressed. The two isoforms are generated by alternative splicing, resulting in 53kD and 56kD forms of the protein, which differ by 21 amino acids in the unique region (Stanley, Ralph et al. 1991; Yi, Bolen et al. 1991). Src and Fyn mRNA are also differentially spliced, but this is regulated in a developmental fashion in the case of Src, and in a tissue specific manner in the case of Fyn, such that only one isoform is expressed in a given cell. The significance of Lyn's differential splicing remains enigmatic, although the interactions of the two isoforms with the BCR may differ because their different unique domains. Evidence to support this comes from the demonstration that the 56kD isoform
(p56^{lyn}) is downregulated more rapidly than p53^{lyn} on BCR ligation (Yamanashi, Miyasaka et al. 1991).

As expected, considering its breadth of expression in haematopoietic cells, Lyn is implicated in signalling through many receptors other than the BCR. In B cells it is also found associated with the CD19 component of the B cell coreceptor and with CD40 (van, Lankester et al. 1993; Ren, Morio et al. 1994)

1.3.2 Experimental mouse models
Mice have long been used as a model system by biologists due to their relatively small size, cost and short generation time. The continuing development of haematopoietic lineages throughout mammalian life makes them a suitable system for studying lymphocytes in vivo. The current lack of a model of B cell development equivalent to foetal thymic organ cultures used to examine T cell development in vitro means that studies of B cell development have to be carried out in the context of the whole animal. Likewise the ability to mount an appropriate immune response when challenged necessitates in vivo experiments and for these reasons we decided to use mice as one of our model systems.

1.3.3 Creation of mutant mice lacking functional Lyn alleles
Mutational analysis is a powerful tool in ascribing a function to a gene and the protein that it encodes as evidenced by naturally occurring mouse mutants such as the motheaten and xid mice. With the advent of gene targetting technology came the opportunity to disrupt genes almost at will, thereby ablating their expression and using the resulting phenotype to gain insight into the gene's function. Therefore the original aim of this project was the creation of mice lacking functional
Lyn alleles. The generation of such mutant mice would result in a reagent that could be used to glean an enormous amount of information about the role of a protein. Not only would it enable us to look at the possible role of Lyn in B cell development and activation, but its function in other lineages could also be studied.

Such mutant mice are created by harnessing the ability of embryonic stem (ES) cells to remain undifferentiated and pluripotent in culture, where they can be genetically modified before being microinjected into host blastocysts; the ES cells then colonise the developing embryo giving rise to a chimaeric animal. If the ES cells have successfully colonised the germline of the chimera the genetic modification can be transmitted to its offspring, allowing the formation of a colony of mice carrying a stable, heritable mutation. The most common gene modifications are those which completely ablate expression from the locus of choice, referred to as a "knockout" mutation in which the locus is disrupted by the insertion of a selectable marker such as the neomycin resistance gene. After screening a mouse genomic library and isolating a number of clones containing sequences derived from the Lyn gene, I discovered from others working on Lyn that the locus is duplicated in the mouse genome (E. Stanley and A. Dunn, personal communication). The duplication of the Lyn gene has subsequently been published and is extensive: of the 13 exons of Lyn, exons 2-10 are present in the duplication (Hibbs, Stanley et al. 1995). Analysis of the human, sheep, pig, rabbit and rat genomes shows that only the mouse genome contains a Lyn duplication, suggesting that it is a recent evolutionary event. However it is not a unique phenomenon, as a similar duplication of the Src gene exists in the human genome (Parker, Mardon et al. 1985). Also the family of Src kinases are thought to have evolved from a single primordial Src-like gene, based on their
sequence homologies and the similarities in the genomic organisation of the genes (Bolen, Rowley et al. 1992). Hibbs et al. cloned the sequences common to both the Lyn gene and the psuedogene and sequenced them, thus determining that the psuedogene contains a number of mutations, both in the exons and introns. Even though none of these mutations would be deleterious to transcription, they failed to find any mRNA specific for the psuedogene (Hibbs, Stanley et al. 1995).

At the beginning of my project the extent of the duplication was unknown and whilst it would not have been impossible to carry on with the knockout strategy, it would have been technically much more demanding. In view of the time constraints imposed upon the project, the knockout strategy was abandoned.

1.3.4 Overexpression of dominant negative mutations
The ability to create kinase defective mutants of Src kinases is well documented (Kamps and Sefton 1986; Kemiecik and Shalloway 1987). Autophosphorylation of Src kinases is a prerequisite for their activation, and so mutants lacking kinase activity can neither become activated nor phosphorylate their targets. A kinase inactive mutant of Lck has been successfully overexpressed in transgenic mice, yielding interesting results about Lck's role in T cells (Levin, Anderson et al. 1993). These mice exhibit a maturational block during thymocyte development, so that mature T cells fail to appear in the periphery. The severity of the phenotype, as measured by the number of thymocytes present in the thymus, correlates with the level of expression of the transgene, with the highest expressors exhibiting a phenotype similar to Lck-/- mutant mice (Molina, Kishihara et al. 1992). Such kinase defective mutations are thought to act as dominant
negative mutants, competing with the endogenous wild type protein for binding to substrates and/or upstream proteins (Herskowitz 1987). Thus proteins that require phosphorylation by Lck, for example, in order to participate in TCR signal transduction, will be sequestered from the signal transduction pathway, resulting in altered signalling. This allows a function for the wild type protein to be inferred from the phenotype.

Thus after discarding the knockout strategy we decided to study the role of Lyn in B cells by expressing a kinase inactive mutant of Lyn in transgenic mice. As the significance of the two Lyn isoforms is unknown, we chose to express only mutant p56\(^{lyn}\) in an attempt to functionally distinguish the two. By limiting expression to B cells we hoped to make the system as simple as possible. Expression of a mutant Lyn in other lineages may have affected B cells in trans, rather than cis, making analysis of any phenotype more difficult.

1.3.4 Dominant negative mutations versus gene knockouts

The use of a kinase inactive mutant has advantages and disadvantages over the knockout technique. Complete ablation of a gene may result in lethality, making the analysis of the immune system difficult. For example, this is the case in the Syk\(^{-/-}\) mice, which die perinatally, although by generating radiation chimeras with Syk\(^{-/-}\) bone marrow it has been possible to glean useful information about the role of Syk in B cells (Turner, Mee et al. 1995). Lethality at an earlier stage of development, such as in the pre-implantation embryo, would make analysis of the immune system impossible. Conditional knockouts in which the mutation is induced in a tissue-specific and developmentally regulated manner in order to avoid this potential problem are possible, though this technology is less efficient and more
risky than conventional gene targetting. In contrast, problems with lethality in transgenic mice are unlikely when transgene expression is directed exclusively to the lymphoid compartment.

Also there is possibility that in mice in which expression of a gene is ablated a related protein steps in to compensate. As previously mentioned, Syk is able to reconstitute ZAP-70 function in ZAP-70 negative cells, but this does not appear to happen \textit{in vivo} as evidenced by the severe block in T cell development in ZAP-70^{-/-} mice, although expression of human ZAP-70 in thymocytes from the mutant mice allows reconstitution of CD4 and CD8 single positive T cells (Negishi, Motoyama et al. 1995). The expression of multiple Src kinases in B cells means that the possibility that Fyn, Blk or Lck could compensate for the lack of Lyn in knockout mice, at least in some functions, cannot be excluded. In Src knockout mice, for example, the phenotype caused by the mutation was less severe than expected, considering the wide tissue distribution of Src expression (Soriano, Montgomery et al. 1991). It may be that other Src family members are compensating for the lack of Src in this case. Certainly Fyn and Src have some overlapping functions as demonstrated by the analysis of cell lines lacking Csk and either Src or Fyn (Thomas, Soriano et al. 1995). These workers demonstrated that the hyperphosphorylation of cellular substrates was caused by either constitutively active Src or Fyn due to the absence of Csk. Moreover, some of the hyperphosphorylated proteins were common to both Csk^{-/-}, Src^{-/-} cells and Csk^{-/-}, Fyn^{-/-} cells, indicating that there is some redundancy between the two.

One of the advantages that knockout strategies have is that any phenotype can be directly related to the lack of expression of the gene in question. Whilst some compensation may occur, functions that cannot
be compensated for will be apparent. This is not necessarily the case when interpreting the phenotype resulting from the over-expression of a dominant negative mutant. In the case of Lyn, the presence of wild type SH2 and SH3 domains in the mutant protein may mediate unpredictable effects, which could wrongly be ascribed to the lack of kinase activity. An example of how this might happen is that whilst the mutant Lyn would bind to targets of Lyn phosphorylation, it could also bind to proteins that regulate Lyn's activity via its SH2 and SH3 domains, thereby preventing the binding of endogenous Lyn and potentially resulting in the artefactual altered activity of the wild type Lyn. There may also be functions of Lyn that depend solely upon its capability to interact with other proteins via its unique, SH2 and/or SH3 domains. In the presence of exogenous mutant Lyn these functions may be enhanced, complicating the analysis. That this is possible is borne out by the discovery of a kinase-independent function of Lck (Xu and Littman 1993).

1.3.5 In vitro models of Lyn's function

Whilst mouse models constitute a good method by which to study the role of a specific protein in the context of the whole immune system, an in depth biochemical analysis can be more conveniently carried out using transformed cell lines. Therefore a B cell line, WEHI-231, was used to carry out in vitro work investigating Lyn's role in signalling through the BCR. WEHI-231 is a murine B cell lymphoma, once considered to be a model of immature B cells due to the apoptosis induced on ligating the functional BCR expressed on the cell surface (Benhamou, Cazenave et al. 1990; Hasbold and Klaus 1990). However unlike immature B cells, WEHI-231 express IgD on their surface (Haggerty, Wechsler et al. 1993), and if stimulated through the BCR these cells can be rescued from apoptosis via a Bcl-2 mediated pathway.
induced by the concomitant addition to the media of antibodies against CD40 (Choi, Boise et al. 1995). Thus although the exact developmental window in which this lymphoma is arrested is unclear, it provides a good model for studying apoptotic signalling pathways through the BCR in vitro, since the induced apoptosis is quantifiable and can thus be used as a biological readout of signals passing through the BCR. Therefore we decided to overexpress both kinase inactive p56^{lyn} and the wild type p56^{lyn} in WEHI-231 cells to ascertain whether or not the cells would become more or less susceptible to apoptosis via BCR ligation. Furthermore, we also hoped to be able to analyse the patterns of phosphorylation observed after BCR ligation in these transfectants and to compare them to those seen in parental cell lines. This would allow us to determine which proteins were phosphorylated either directly by p56^{lyn} or by targets of it. Finally the ability of the transfectants to activate a variety of signalling pathways such as calcium fluxing and the activation of MAP kinases could also be studied.

1.3.6 Summary
To gain insights into Lyn's function in B cells, I have generated a kinase inactive mutant of p56^{lyn} and an antibody that can recognise both it and the two wild type Lyn isoforms. The making of these is discussed in Chapter Three. I have then employed two main strategies using these reagents: Chapters Four and Five show the generation and analysis of transgenic mice harbouring the kinase mutant, whilst Chapter Six documents the transfection of WEHI-231 cells with both the mutant and wild type p56^{lyn} cDNAs.
Chapter Two

Materials and Methods

2.1 Solutions

TAE: 0.04M Tris-HCl, 0.02M glacial acetic acid, 1mM EDTA (pH 8.0)
SSC: 0.15M NaCl, 0.015M tri-sodium citrate (pH 7.2)
TBE: 0.1M Tris-HCl, 0.09M boric acid, 5mM EDTA (pH 8.0)
TE: 10mM Tris-HCl pH 8.0, 1mM EDTA
PBS: 150mM NaCl, 2mM KCl, 8mM Na$_2$HPO$_4$, 1.5mM KH$_2$PO$_4$
PBS-Tween: PBS containing 0.05% Tween-20
CAPS: 10mM 3-cyclohexylamino-1-propanesulphonic acid, pH 11.0
TNES: 10mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM EDTA, 1% SDS,
       0.25mgml$^{-1}$ Proteinase K
Tail Mix: 50mM Tris-HCl, pH 8.0, 100mM NaCl, 100mM EDTA, 1%
          SDS, 0.25mgml$^{-1}$ Proteinase K
Hybridisation Mix: 0.2M NaPO$_4$, 1mM EDTA, 1% BSA, 7% SDS, 15%
                formamide
TBS: 25mM Tris-HCl pH 7.4, 140mM NaCl, 5mM KCl
SDS-PAGE upper buffer: 0.125M Tris-HCl, 0.1% SDS, pH 6.8
SDS-PAGE lower buffer: 0.375M Tris-HCl, 0.1% SDS, pH 8.8
2.2 Bacterial media

Luria broth:  Bacto-tryptone  10g  
             Yeast extract  5g  
             NaCl  10g  
per litre adjusted to pH 7.2

Luria agar: as above with the addition of 15g Difco agar per litre

Antibiotics: Ampicillin was added to a final concentration of 
             100μgml⁻¹ immediately before use when needed

2.3 Mammalian tissue culture media

WEHI-231: This cell line was grown in suspension in Dulbeco's 
           Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% 
Foetal Calf Serum (TCS, batch no. 38221), 50μM β-mercaptoethanol, 50 
unitsml⁻¹ of penicillin, 50μgml⁻¹ streptomycin and 2mM L-glutamine 
(Gibco BRL). FCS was routinely heat inactivated by incubating at 57°C 
for 30min to prevent complement-mediated lysis of the cells.

COS-1: COS-1 cells were grown in DMEM supplemented with 10% 
FCS, penicillin and streptomycin as above. The same batch of FCS was 
used as for WEHI-231 cells.
2.4 Bacteriological techniques

2.4.1 Transformation and culture

Competent *E.coli* (Subcloning Efficiency DH5α) were purchased from Gibco BRL and transformation was carried out according to the manufacturer's instructions. *E.coli* were grown in liquid culture in Luria broth containing the appropriate antibiotic. Small scale cultures (2ml) ("minipreps") were grown in sterile Universals and 500ml cultures ("maxipreps") grown in 2 litre conical flasks, overnight at 37°C with shaking at 225rpm, in order to aerate the media. Stocks of *E.coli* for short-term use (1-2 weeks) were kept as single colonies on agar plates containing antibiotics, whilst long-term stocks were kept at -70°C in 12% sterile glycerol.

2.4.2 Harvesting of plasmid DNA from *E.coli*

Both small and large scale cultures were harvested and the plasmid DNAs recovered using standard techniques (Maniatis). Plasmid DNA from maxipreps was purified by centrifugation in a caesium chloride gradient by standard procedures. All DNA preparations were stored in TE; "miniprep" DNA at -20°C, "maxiprep" DNA at 4°C. Quantification of DNA was accomplished by measuring the optical density of a 1 in 200 dilution of the sample at a wavelength of 260nm using a spectrophotometer and assuming that an \( \text{OD}_{260\text{nm}} \) of 1.0 represents a concentration of 50\( \mu \text{g} \cdot \text{ml}^{-1} \).
2.5 Preparation of mammalian genomic DNA

2.5.1 Preparation of genomic DNA from cultured cells

10⁷ cells were washed once in PBS before being resuspended in 100μl PBS. After addition of 400μl of TNES with 0.5mgml⁻¹ Proteinase K, cells were incubated at 37°C overnight. The following day an equal volume of TE-equilibrated phenol was added and the sample extracted by vortexing. The aqueous layer was removed and a further extraction performed using an equal volume of chloroform. Again the aqueous layer was removed and the DNA collected by precipitating with 2 volumes of ethanol and spooling out with a sealed Pasteur pipette. After washing the DNA with 70% ethanol and air-drying, it was redissolved in 250μl of TE.

2.5.2 Preparation of genomic DNA from mice

At weaning, and again at the time of the experiment if necessary, approximately 1cm of was cut off from the end of each mouse's tail and placed in 700μl of Tail Mix. The samples were rotated at 55°C overnight before being extracted twice with an equal volume of phenol:chloroform (1:1) and once with chloroform. After the final extraction, 0.6 volumes of isopropanol was added to the aqueous layer and the sample vortexed to generate a pellet of DNA, which was then spooled out using a flame-sealed Pasteur pipette. After washing in 70% ethanol, the DNAs were left to dissolve overnight at 4°C in 100μl TE.
2.6 Manipulation of DNA

2.6.1 Endonuclease restriction digestion of DNA
DNAs were digested using restriction enzymes (New England Biolabs) in the buffer recommended by the manufacturer. Typically 1-2μg of plasmid DNA was digested in a volume of 10μl, with the enzyme constituting no more than 5% of the volume, approximately 1hr at 37°C. 10μg of genomic DNA per digest was incubated in a volume of 25μl overnight, again with the enzyme at less than 5% of the total reaction volume. Digestions were terminated by the addition of 1/10 volume of DNA sample buffer (20% glycerol, 100mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol).

2.6.2 Dephosphorylation
In some cases, in order to minimise recircularisation of vectors during cloning, the terminal 5' phosphate groups were removed from linearized plasmid DNA prior to by using calf intestinal phosphatase (CIP). Appropriately digested plasmid DNA was ethanol precipitated before being resuspended in 20μl of 0.05M Tris-HCl pH 9.0, 1mM MgCl₂, 0.1mM ZnCl₂, 1mM spermidine and 0.2U of CIP (Boehringer Mannheim). After incubation at room temperature for 20min, EDTA was added to a final concentration of 5mM and the reaction heated to 75°C for 10min and the DNA purified on an agarose gel.
2.6.3 Filling in of 5' overhanging DNA termini

Some subcloning required that the 1.5kb MluI-BamHI p56lyn cDNA be ligated into the EcoRV site of pcDNA3. EcoRV generates blunt ended termini, and so the 5' overhangs of the insert were "filled in" using the Klenow fragment of E. coli DNA polymerase I. A typical reaction consisted of 1µg of insert in 20µl of distilled water with 10mM Tris-HCl pH 7.5, 5mM MgCl₂, 7.5mM dithiothreitol, 0.5mM dNTPs and 4 units of Klenow fragment (New England Biolabs). The reaction was incubated for 20min at room temperature and stopped by the addition of EDTA to 5mM plus heating to 75°C for 10min.

2.6.4 Ligations

Vector and insert that had been fully digested with the appropriate restriction endonucleases and dephosphorylated or blunted as required were purified by electrophoresis on a preparative gel, followed by elution from the gel by the "geneclean" method (see later this section). Typically around 300ng of insert DNA was ligated in a reaction with a vector to insert ratio of 1:3 moles. Ligations were carried out in a volume of 5µl, which included 50mM Tris-HCl pH 7.8, 10mM MgCl₂, 10mM dithiothreitol, 1mM ATP, 25µgml⁻¹ bovine serum albumin and 200U bacteriophage T4 DNA ligase (New England Biolabs). The reactions were incubated at 15°C for 4 hours (cohesive termini) or overnight (blunt termini). Ligation reactions were then transformed into E. coli.
2.6.5 Electrophoresis of DNA

For routine analysis, preparative gels and Southern blotting, DNA was separated in 1% w/v molecular biology grade agarose gels (SeaKem™, FMC Bioproducts) whilst PCR products and fragments smaller than 0.5kb were analysed using 1% SeaKem™, 3% NuSeive™ (FMC Bioproducts) gels. In all cases 1μgml⁻¹ ethidium bromide was added to both gel and TAE for visualisation and the gels were run in TAE. DNA sample buffer was added prior to loading the samples. A voltage field of 10Vcm⁻¹ was applied and the gel photographed under UV.

2.6.6 Recovery of DNA from gels

Electrophoresis was carried out as above and the band of interest cut out using a razor blade. The following protocol was then carried out: 2-3 volumes of NaI solution (6M NaI, 0.1M Na₂SO₃) were added to the gel slice and the sample incubated at 50°C with occasional vortexing until the gel was completely dissolved (around 10min). Between 2-5μl of a silica/water slurry (1:1) was added and the mixture incubated on ice for 17min with occasional vortexing. The glass, to which the DNA adheres, was spun out in a microfuge for 15s and the pellet washed once with 300μl NaI solution and twice with ethanol wash solution (50% ethanol, 0.1M NaCl, 10mM Tris pH 7.5 and stored at -20°C). After the last wash, the sample was spun briefly in a microfuge and the remaining last drops of ethanol wash solution removed with a microcapillary. The glass was then resuspended in the desired amount of TE and incubated at 50°C for a further 17min to allow the DNA to
elute, after which the glass was spun out (15s in a microfuge) and the DNA solution recovered from the liquid phase.

2.6.7 Transfer of DNA from agarose gels to membranes

Genomic DNA was digested with the appropriate restriction endonuclease as described earlier and separated according to size on a 1% agarose gel containing 1µgml\(^{-1}\) ethidium bromide in TAE. Gels were run at between 1-2 Vcm\(^{-1}\) for 14-16 hours and photographed. The DNA was denatured by twice soaking in denaturing buffer (0.5M NaOH, 1.5M NaCl) for 20min with gentle rocking. The DNA was then transferred to a Hybond N+ (Amersham) nylon membrane by capillary action using 20X SSC as the transfer buffer and standard techniques (Sambrook, Fritsch et al. 1989). The use of a positively charged nylon membrane allows the DNA to become covalently bonded to the filter under alkaline conditions, negating the need to bake the filter or expose it to UV; therefore after the transfer was completed (12-48 hours) the DNA bound to the membrane was fixed by placing the filter onto 2 sheets of Whatman paper soaked in 0.4M NaOH for 20min.

2.6.8 Labelling of DNA using random priming

Labelling of DNA with which to probe Southern blots was achieved using a kit from Boehringer Mannheim and α\(^{-32}\)P dCTP (~ 6000Ci mmol\(^{-1}\), Amersham). The basis of the kit is the incorporation of \(^{32}\)P-dCTP into the DNA fragment to be used as a probe using a mixture of random hexanucleotides to prime from one strand; the Klenow fragment of \textit{E. coli} DNA polymerase I is then used to synthesise the
complementary strand, incorporating the $^{32}$P-dCTP as it does so. 25-50ng of the probe fragment in a volume of 9μl of water was boiled for 5min to generate single stranded DNA. The reaction was set up according to the kit instructions. The reaction mixture was vortexed, spun briefly (15s) in a microfuge, and incubated at 37°C for 30min. Unincorporated nucleotides were removed using a Sephadex G-50 column (Pharmacia) according to the manufacturer's instructions. In order to calculate the specific activity of the probe, 1μl was removed before passing it down the Sephadex column and a further 1μl removed afterwards. These two samples were then measured in a beta counter (Pharmacia). A typical specific activity was of the order of $10^8$cpmμg$^{-1}$.

2.6.9 Hybridisation of Southern blots

Filters carrying the target DNA were wetted in 2X SSC and inserted into a hybridisation bottle (Hybaid). 10ml of Hybridisation mix were added and the bottle rotated in a hybridisation oven (Hybaid) at 65°C for a minimum of 15min. After the prehybridization, the probe was boiled for 5min to denature the DNA and was added to the hybridisation bottle along with 0.1mgml$^{-1}$ herring sperm DNA, also boiled for 5min. To remove non-specifically bound probe from the membrane so that only the homologously bound probe remained, the filters were washed three times in 0.3% SSC, 0.5% SDS for 20min at 65°C, followed by a final wash in 0.1% SSC, 0.5% SDS for 1 hour at 65°C. The washes carried out here are high stringency, as the human β-globin probe (see
Chapter Four) hybridises strongly to murine globin genes and other related sequences, causing high levels of background. After washing the membrane was sandwiched between two sheets of Saran Wrap (Dow) and exposed to film at -70°C using intensifying screens to strengthen the signal.

2.6.10 Removal of bound probe from membranes

This was achieved by incubating the filter in 0.4M NaOH at 45°C for 30min, then washing with 0.1X SSC, 0.1% SDS, 0.2M Tris-HCl pH 7.5 at 45°C for 15min. The filter could then be rehybridised.

2.7 Polymerase Chain Reaction

All polymerase chain reactions (PCR) were carried out using Pfu polymerase (Stratagene), as this has a 12-fold higher fidelity than Taq. A typical PCR mix contained 10ng of template DNA, 50pmoles of each primer, 200μM dNTPs, 10mM KCl, 10mM (NH₄)₂SO₄, 20mM Tris-HCl pH 8.8, 2mM MgSO₄, 0.1% Triton X-100 and 10μgml⁻¹ BSA, and 1.25U Pfu polymerase in a reaction volume of 50μl. The reaction was overlaid with paraffin oil and started with 1 incubation at 94°C for 150s to ensure that the template was fully denatured, followed by 25 cycles of: 94°C for 30s; 50°C for 60s; and 72°C for 120s. A final extension reaction at 72°C for 10min was then carried out. To verify that the PCR had been successful, 5μl of the reaction was run on a NuSieve agarose gel alongside the appropriate size markers and controls. PCR products were then purified by extracting the reaction mix 1X with TE-
equilibrated phenol, 1X with phenol:chloroform (1:1) and 1X with chloroform before being precipitated with 2 volumes of ethanol and resuspended in 20µl of TE. 1µl of this was kept to use as a control for digestion and the remainder digested with the appropriate restriction endonucleases (Pfu polymerase generates blunt-ended products). 1µl of the digest was analysed alongside the aliquot of undigested product on a NuSieve gel. After complete digestion the PCR products were cloned into the appropriate vector. Ligation reactions with fragments derived from PCR were transformed into MAX Efficiency DH10B™ E. coli (Gibco BRL) according to the manufacturer's protocol.

2.8 Sequencing of DNA

2.8.1 Sequencing reactions
Sequencing of plasmid DNA was achieved using a Sequenase version 2.0 kit from United States Biochemicals. 5µg of DNA in 20µl of water (for a plasmid of 10kb; more or less DNA was used for larger or smaller plasmids) was denatured by the addition of 2µl of 2M NaOH at room temperature for 5min. Then the DNA was precipitated with 9µl of 5M NH₄OAc pH 7.5 and 90µl ice-cold ethanol on dry ice for 10min. From this point onwards reactions were kept on ice unless otherwise stated. The sequencing reactions were then carried out according to the manufacturer's instructions, using ³⁵S-dATP, 1000Ci mmol⁻¹ (Amersham).
2.8.2 Polyacrylamide gels

Gels were poured using 70ml Sequagel (National Diagnostics), and adding 140μl of 25% w/v ammonium persulphate solution. After polymerisation the gels were pre-run at 1.5kV and 60W constant power in TBE 30min. The sequencing reactions were heated to 80°C by floating in a tray of water heated from underneath by a Bunsen burner for 5min before being loaded onto the gel. Samples were run at 1.5kV and 60W constant power for 3hr; the heat generated by this allows the DNA to remain denatured throughout the run. After electrophoresis, gels were immersed for 10min in 10% methanol, 10% acetic acid, in order to leach out the urea so that they could be dried for autoradiography. Drying was accomplished by heating to 80°C under vacuum for 40min. Once dry, the gel was exposed to film without an intensifying screen overnight. Autoradiographs of sequence were analysed manually.

2.9 Transgenic mice

2.9.1 Superovulation of mice

In order to harvest enough fertilised eggs for microinjection, 20 CBA/Ca (CBA) female mice were routinely induced to superovulate by the administration of gonadotropins. Sexually immature, three week old females were used. At 3pm on day -3 prior to the day of microinjection, the mice were injected intraperitoneally with 0.1ml (5IU) of pregnant mare's serum (PMS, available from Intervet
Laboratories Ltd. as Folligon), which mimics follicle-stimulating hormone. The timing of the hormone injections is crucial, as the effects of the hormones are dependent on the light-dark cycle of the mice. On day -1, the mice were given 5IU (in 0.1ml sterile PBS) of human chorionic gonadotropin (Chorulon, Intervet Laboratories Ltd.), again intraperitoneally, at 1pm, and each female placed in a cage with one stud male (CBA) overnight. Stud males were pre-mated to irrelevant females before being used for fertilising the eggs of superovulated females; a record of their performance was kept, and males failing to mate on two consecutive occasions were replaced.

2.9.2 Harvesting of fertilised eggs
The next morning, the females were removed from the studs and checked for the presence of vaginal plugs as an indicator of successful mating. Only those with a plug were harvested for eggs. Mice were sacrificed by cervical dislocation and the ovaries, with oviducts attached, removed and placed in a 35mm Petri dish containing M2 medium. Under 20X magnification the eggs were removed from the ovaries. The ampullae of the oviducts are swollen at this stage and the eggs can be seen clearly through the membrane, which was ripped using watchmakers forceps to release the eggs. The eggs were then removed from the ovaries and debris with a pipette made from capillary tubing flamed and pulled to give a diameter of approximately 200µm; this was attached to a rubber tube and manipulated by mouth. Cumulus cells surrounding the eggs were removed by placing them into a second Petri dish containing M2 medium plus 300µgml⁻¹ of
hyaluronidase; this digests the sticky proteins that cause the cummular cells to cluster around the eggs. The eggs were rinsed in M2 medium to remove the hyaluronidase and placed in M16 medium in a 37°C incubator whilst the microinjection equipment was set up.

2.9.3 Microinjection of fertilised eggs
DNA was prepared by digesting with NotI and purifying the transgene fragment on a 1% agarose gel as described above, and quantified by running dilutions on a second gel against a fragment known to have successfully generated transgenic mice previously. The purity of the DNA used in microinjections is important, as contamination with enzymes or agar may confer toxicity and thus affect embryo survival; also particulate matter will block the injection needle. Therefore the DNA was suspended in sterile TE and further purified by passing it through an Elutip-d column (Schleicher and Schuell) according to the manufacturer’s instructions. This highly purified DNA, at a concentration of approximately 1-2μgml⁻¹, was stored in 100μl aliquots at -20°C until needed.

Microinjections were carried out according to Hogan et al. (Hogan, Constantini et al. 1986) using Leitz micromanipulators and a Nikon microscope assembled on a hydraulic table to minimize vibrations; 30 eggs at a time were removed from the incubator and transferred into M2 medium. Any eggs that were unfertilised, misshapen, or had already divided were discarded and the remainder injected with approximately 1-2μl of DNA, which corresponds to 200-400 molecules
per pl for a 5kb fragment. Typically the DNA was injected into the male pronucleus, as this is larger than the female pronucleus and thus easier to target. After injection the eggs were placed back into M16 and incubated overnight.

2.9.4 Transfer of embryos to foster mothers

Embryos were transplanted into pseudopregnant foster mothers the day after injection, as this allows any embryos that have lysed or failed to develop as a consequence of injection to be discarded. Thus only those eggs that had divided into 2-cell embryos were reimplanted. (B6 X CBA) F1 females were used as fosters as inbred mice have lower reproductive capabilities and are therefore less efficient. The foster mothers were mated to vasectomized males the night before use, and those females with a vaginal plug the following morning were anaesthetised using 17μl of 2.5% Avertin per gram of body weight (typically 350μl). 15 embryos were transferred to each oviduct of the recipient via the infundibulum (Hogan et al) and the mice allowed to recover under a heat lamp. The resulting pups were screened for the presence of the transgene by Southern blotting of tail DNA.

2.10 Tissue culture

2.10.1 WEHI-231

Cells were maintained in the logarithmic phase of growth, which corresponds to between 5x10^4 and 5x10^5 cells per ml. Stocks of WEHI-231 were maintained by supplementing the media with 12% v/v
DMSO and aliquoting cells into freezing vials (Sarstedt). The vials were then placed at -70°C to allow the cells to freeze slowly before being moved to liquid N₂ for long-term storage.

2.10.2 Transfection of WEHI-231 cells

The DNA to be transfected (10µg per electroporation for a 10kb plasmid) was linearized with the appropriate restriction endonuclease and ethanol precipitated. After spinning in a microfuge and removing the ethanol, the DNA pellet was washed with 70% ethanol, which was allowed to evaporate away from the pellet in the tissue culture hood, ensuring that the DNA was sterile. Cells at 5x10⁵ml⁻¹ were pelleted at 1200rpm in a benchtop centrifuge and washed twice in electroporation buffer (1mM MgCl₂, 1mM CaCl₂ in TBS) filter sterilised and stored at 4°C. For each electroporation 5x10⁶ cells were resuspended in 0.5ml electroporation buffer and left on ice. The DNA was resuspended in sterile TE and added to the cells, which were transferred to an electroporation cuvette (0.4cm electrodes, Biorad). Electroporations were carried out at 300V and 500µF using a Biorad Gene Pulser. These conditions gave a discharge time constant of between 10-15ms. After electroporation 1ml of serum free medium at 4°C was added dropwise and the cells left at room temperature for 5min, before being placed in a flask containing 9ml of complete medium and incubated overnight. The next day 10ml of WEHI-231 media containing 4mgml⁻¹ of G418 (Geneticin, Sigma) was given to each flask of electroporated cells, resulting in 20ml of cells in medium containing 2mgml⁻¹ G418. These
cells were then plated out into 96-well tissue culture plates, 100μl of cells per well. After a week of incubation, cells were fed with 100μl fresh medium containing 2mgml⁻¹ G418 and left for a further 2 weeks in the incubator.

After three weeks clones representing stable transfectants were clearly visible. The contents of those wells containing clones, excepting those with multiple clones present, were moved into 48-well plates and given 0.8ml of fresh media containing G418. Clones were expanded in this fashion until they could be cultured in 75ml flasks. Aliquots of each clone were frozen in liquid nitrogen for long-term storage.

2.10.3 Transfection of COS-1 cells

The day before transfection, COS-1 cells at approximately 70% confluency were trypsinised and plated out onto 10cm plates. The following day the cells were washed twice serum-free medium and a mixture consisting of 1.5ml serum-free medium containing 5μg DNA plus 1.5ml of 1mgml⁻¹ DEAE-dextran (Pharmacia) in TBS was added and the cells incubated at 37°C for 45min. The DNA/dextran mix was replaced by medium containing 10% FCS and 100μgml⁻¹ chloroquine (Sigma) and the cells incubated for a further 3hr. Finally the chloroquine media was aspirated off and the cells fed with their usual medium before being returned to the incubator until harvesting.
2.11 Protein analysis

2.11.1 Total cell lysates

Transformed cell lines (WEHI-231 and COS-1) and primary murine cells were lysed in 1% NP40, 150mM NaCl, 20mM Tris pH 7.0, 10mM iodoacetamide and 1μg/ml of each of the peptide inhibitors chymostatin, leupeptin and pepstatin. The peptide inhibitors were kept as 1ml aliquots of a 1000X stock in DMSO at -20°C and added just before use.

Cells (2x10^6 for 100μl of lysate) were pelleted at 1200rpm for 3min in a benchtop centrifuge and washed twice with PBS. 50μl of NP40 lysis buffer was added and the samples rotated at 4°C for 15min. After the lysis, the samples were spun in a microcentrifuge for 15min at 4°C to remove the nuclei, which remain intact, and membranous debris. The supernatant was transferred to a fresh tube and an equal volume of Laemmlli reducing sample buffer (10% glycerol; 3% SDS; 0.5X SDS-PAGE upper buffer; 5% β-mercaptoethanol; 0.005% bromophenol blue) added and the sample was heated to 100°C for 5min to denature the proteins before loading onto the gel.

2.11.2 Coupling antibodies to Protein-A-Sepharose beads

Equal volumes of the anti-Lyn serum and Protein-A-Sepharose beads were mixed together and rotated gently at room temperature for 1 hr. The beads were pelleted by spinning at 2000rpm for 5min in a benchtop
centrifuge and washed twice with 10 volumes of 0.2M sodium borate pH 9.0. The beads were resuspended in 10 volumes of sodium borate and 10μl of the slurry removed. Dimethylpimelimidate was added to 20mM to covalently crosslink the antibodies to the protein A and the beads rotated for 30min. After the removal of a further 10μl of beads, the crosslinking reaction was terminated by pelleting the beads and washing in 0.2M ethanolamine, followed by rotating at room temperature in 10 volumes of ethanolamine for 2 hours. The beads were then washed in PBS and stored at 4°C in PBS containing 0.05% azide. The two 10μl aliquots were analysed by SDS-PAGE and immunoblotting with horseradish peroxidase linked protein A to check that the crosslinking had been successful.

2.11.3 Immunoprecipitations

10⁷ cells were pelleted, washed and lysed in 1ml of lysis buffer as above. After spinning out the nuclei, 20μl of packed Protein-A-Sepharose beads (Pharmacia) were added and the sample rotated at 4°C for 30min to clear the lysate of any proteins that bind non-specifically. The preclear was repeated a second time before 30μl of packed anti-Lyn Protein-A-Sepharose beads were added to the supernatant. The beads and lysate were rotated for four hours at 4°C. Occasionally the immunoprecipitations were carried out overnight, which increased the amount of protein recovered, but for most purposes this was not necessary. Protein was recovered by spinning samples in a microfuge for 15s to pellet the beads and the supernatant was removed by
aspiration. The beads were washed 4 times in 1ml of lysis buffer before being resuspended in 100μl of Laemmli sample buffer (10% glycerol, 3% SDS, 0.5X upper buffer, 0.05% bromophenol blue, 5% β-mercaptoethanol). The samples were then boiled for 5min and the beads pelleted by centrifugation. The sample was removed to a fresh tube and was either loaded directly onto a polyacrylamide gel for SDS-PAGE or stored at -20°C for later use.

2.11.4 SDS-PAGE

40ml gels were poured using 10ml of Protogel (National Diagnostics), which results in a 10% acrylamide gel, in SDS-PAGE lower buffer. 100μl of 25% ammonium persulphate and 20μl of TEMED were added to polymerise the gel. Stacking gels were cast after polymerisation of the resolving gel; these were also 10% acrylamide cast using SDS-PAGE upper buffer. Gels were run in SDS-PAGE running buffer (0.025M Tris base; 0.19M glycine; 0.1% SDS) for either 4 hours at 200V or overnight at 50V. Pre-stained molecular weight protein markers (16kD-175kD) were run alongside the samples (New England Biolabs).

2.11.5 Immunoblotting

Proteins separated by SDS-PAGE were immobilised on a polyvinylidene fluoride (PVDF) membrane (Millipore) for autoradiography and immunoblotting. The PVDF membrane was soaked in methanol to pre-wet it before being immersed in CAPS. Transfers were carried out at pH 11.0 as proteins are negatively charged
at this pH and this increases the efficiency of the transfer. The blot was then assembled in the transfer tank cassette using filter papers soaked in CAPS and with the gel nearest the cathode of the tank. The tank was filled with CAPS and the buffer was recirculated throughout the tank during transfer. Blotting was carried out at 100V and 300mA constant current for between 6-12 hours at 4°C. The PVDF membrane was either dried on Whatman paper and exposed to film or placed in blocking solution in preparation for antibody staining.

2.11.6 Antibody staining of immunoblots

Proteins of interest were detected on immunoblots using enhanced chemiluminescence (ECL™) reagents (Amersham). This is a rapid, non-radioactive method that uses an enzymatic reaction to generate light, which is then captured on film. Oxidation of chemicals such as luminol, a cyclic diacylhydrazide, by horseradish peroxidase (HRP) in the presence of hydrogen peroxide and chemical enhancers, results in a product in an excited state; the decay of this product to a stable ground state occurs via the release of photons. The blot is first stained with an antibody specific for the protein of interest; this primary antibody bound to the membrane is then targeted by a secondary antibody (or in the case of the anti-Lyn antibody, protein A) conjugated to HRP. The protein is then visualised by incubating the blot with the ECL™ reagents and exposing it to film.

Blotting began with incubation at room temperature and gentle rocking in 100mls of 5% low-fat Marvel in PBS-Tween (PBS containing
0.05% Tween-20) for 1 hour. The blot was then washed once in 100mls PBS-Tween by rocking vigorously for 5min. To detect p53/56lyn, the anti-Lyn antiserum was diluted three thousand fold in PBS-Tween with 2% Marvel and incubated with the blot at room temperature for 1hr. After washing 4 times in PBS-Tween the anti-Lyn antibody was detected using HRP-protein A at the retailer’s recommended dilution (Amersham). Vav was detected using a mouse anti-human monoclonal antibody (Upstate Biotechnology Inc.) followed by a goat anti-mouse IgG (UBI) conjugated to HRP. Alpha-tubulin was detected using the mouse monoclonal TAT-1 (a kind gift from Dr. S. Ley) followed by protein A-HRP. The 12CA5 antibody was detected with goat anti-mouse-HRP (UBI). In all cases, antibodies were diluted according to the manufacturer’s instructions. Blots were incubated for a further 30min with the secondary layer, again at room temperature and with gentle rocking. The blots washed 6 times for 5min with PBS-Tween and finally 10ml of the ECL reagent were added to the blot. After 1min the ECL solution was discarded and the blot sandwiched between two sheets of Saran Wrap. Excess ECL reagent was removed by placing the wrapped blot onto a sheet of Whatman paper and using a roller to squeeze out any remaining liquid. The blot was placed into a cassette and exposed to film for 1min. This film was immediately developed in order to determine the correct exposure time. Typically an exposure of 15-60s was sufficient.
2.11.7 Membrane stripping

Blots were stripped by incubating in a solution of 10mM β-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl pH 6.7 at 50°C for 30min. After washing 4 times in PBS-Tween for 10min per wash in a fume hood, the blot was re-blocked in 5% Marvel for up to 12 hours.

2.11.8 In vitro immune complex kinase assays

The kinase assay protocol is a modified version of that of Burkhardt et al. (Burkhardt and Bolen 1992). To assay Lyn’s kinase activity in WEHI-231 and transfected COS-1 cells, 10⁷ cells were washed twice in ice-cold PBS and lysed in NP40 lysis buffer containing protease inhibitors and 1mM sodium orthovanadate. Splenocytes are physically smaller therefore when analysing Lyn’s activity in murine spleens, 5x10⁷ cells were lysed in RIPA buffer, again with 1mM sodium orthovanadate. RIPA buffer (150 mM NaCl, 50mM Tris pH 8.0, 5mM NaF, 1% NP40, 1% deoxycholate, 0.1% SDS) was empirically determined to be the most suitable for lysing primary cells prior to performing Lyn kinase assays; NP40 lysis buffer led to only partial solubilisation of proteins and the formation of mixed micelles, as evidenced by ladders of phosphorylated proteins on the autoradiograph. The preclear, immunoprecipitation and washes were all carried out in the appropriate lysis buffer with orthovanadate and protease inhibitors. After immunoprecipitating for 1 hour, the Sepharose beads were washed 4 times in lysis buffer and once in tyrosine protein kinase (TPK) buffer (20mM MOPS, 5mM MnCl₂). Samples were kept on ice during the washes. The kinase reaction was
carried out by resuspending the beads in 25μl of TPK with 1μM ATP and 2.5μl γ-32P ATP, (5000 Cimmol⁻¹, Amersham) at room temperature for 10min. The incubation time was determined empirically (see Chapter Three), as was the divalent cation used in the TPK buffer (Mn²⁺ gave more consistent results than Mg²⁺). Reactions were terminated after 10min by the addition of an equal volume of 2X Laemmli sample buffer and left at room temperature for 30min so that the proteins could elute off the beads. The samples were then boiled for 5min and the Sepharose beads removed by spinning at 14,000 for 15s and transferring the supernatant to a fresh tube. Samples were loaded directly onto a 10% acrylamide gel for SDS-PAGE.

SDS-PAGE and transfer to a PVDF membrane were carried out according to the protocols described above. When the transfer was complete the filter was washed once in PBS-Tween and dried by blotting between 2 sheets of Whatman 3MM paper, before being sandwiched in Saran Wrap and exposed to film for 24 hours. After autoradiography, the filter was stained with the anti-Lyn antibody to determine the relative amounts of protein present between lanes; this also allows the specific activity of the protein to be calculated.

2.12 Flow cytometry

For analysis of cell populations within murine tissues, the organ or tissue of interest was removed from the animal and single cell suspensions in air-buffered IMDM made by mechanical disaggregation.
Cells were then counted using Trypan blue exclusion to distinguish dead cells and 1x10⁶ live cells were used per stain. The cells were washed once in FACS buffer (PBS containing 0.5% BSA and 0.05% sodium azide). After washing the pellets were resuspended in 50μl of the antibody diluted in FACS buffer containing 10% rat serum to block non-specific binding and incubated on ice for 45min in the dark. Antibody concentrations were determined by titration in advance. Excess unbound antibody was removed by washing the cells in FACS buffer. If a secondary stain was necessary, this was added after the washing step, again in a volume of 50μl. Secondary reagents were incubated with the cells for 30min before a final wash in FACS buffer. Typically cells were stained and then fixed in 1% paraformaldehyde in PBS for 5min at room temperature. This fixative was removed by washing and the cells resuspended in 0.5ml FACS buffer. Once fixed, samples could be stored at 4°C for up to 72 hours before being analysed. All samples were analysed on a FACStar Plus (Becton Dickinson) flow cytometer and the data analysis carried out using CellQuest software.

2.13 MTT assay

Metabolic activity was studied using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma). MTT is a water-soluble yellow compound that is metabolised by mitochondrial dehydrogenases to an insoluble blue compound which can be dissolved by the addition of 10% SDS/0.01M HCl allowing the colour change to be quantified spectrophotometrically. To assess the correct concentration
of antibody b.7.6 needed to induce apoptosis in WEHI-231 cells, cells were plated out at $10^4$ in 100μl WEHI-231 media per well in 96-well plates. Antibody b.7.6 diluted in media was added to each well at a range of concentrations and the cells incubated for 48 hours. Metabolic activity was then measured by incubating the cells for a further 4 hours in the presence of 20μl per well of 5mgml$^{-1}$ MTT in PBS. 100μl of 10% SDS/0.01M HCl were then added and the plates returned to the incubator overnight before the absorbance at 560nm was measured in an ELISA plate reader.

2.14 Tritiated thymidine uptake assay

Cells were cultured with the b.7.6 antibody as above. 0.5μCi of $^3$H-thymidine (5Ci mmol$^{-1}$, Amersham) was added per well and the cells incubated for a further 4hr before the supernatants were harvested on a Wallac Betaplate cell harvester. Incorporation of $^3$H-thymidine was measured using a betaplate counter.

2.15 Electron microscopy

For each sample, $10^4$ cells were prepared by the addition of 25% gluteraldehyde to give a final concentration of 2%. The cells were then spun gently at 3,500rpm in a microfuge for 3min. The supernatant was aspirated and 2% gluteraldehyde was layered onto the pellet and the samples analysed by Liz Hurst (NIMR) using transmission electron microscopy.
Chapter Three

**Generation of anti-Lyn antisera and a kinase inactive mutant of the 56kD isoform of murine Lyn**

To detect the overexpression of the 56kD isoform of murine Lyn (p56<sup>lyn</sup>) in transgenic mice and cell lines, an anti-Lyn antiserum which would work both in immunoblotting and immunoprecipitations was required. Secondly, vectors were needed that expressed either wild-type or a kinase disabled mutant of p56<sup>lyn</sup>. This chapter describes the generation and characterisation of these reagents.

### 3.1 Generation of anti-Lyn sera

An anti-peptide rabbit antiserum was generated rather than a monoclonal, for a variety of reasons. The polyclonal nature of anti-peptide sera allows the recognition of multiple epitopes by antibodies with different specificities during both immunoblotting and immunoprecipitation, thereby increasing the efficiency of detection; also within such a pool of antibodies there are likely to be some with a high affinity for the antigen. The generation of polyclonal antibodies is less labour intensive than that of monoclonal antibodies, as there is no screening procedure involved other than the immunoblotting shown in Figure 3.2.

That the antiserum could detect both isoforms of Lyn was fundamental to the experiments in which it was to be used. B lymphocytes express both isoforms of Lyn in equal amounts (by immunoblot), so
overexpression of the 56kD isoform should be detectable by immunoblotting as an increase in the intensity of the 56kD band compared to the 53kD band. Thus if the antisera detects both isoforms, the 53kD isoform can be used as an internal control during analysis of expression of the transgene in mice and cell lines. Not only was it necessary therefore to be able to detect both forms of Lyn by immunoblotting, but also to immunoprecipitate the two, in order to carry out immune complex kinase assays. These assays were important in determining the specific activity of the mutant protein compared to the wild type.

Anti-Lyn antisera were generated by immunising rabbits with a peptide corresponding to amino acids 44-63 of murine p56 lyn (Figure 3.1a) which had previously successfully generated anti-Lyn antisera (Yi, Bolen et al. 1991). This peptide is located in the unique region of the protein which, it was hoped, would generate an antiserum that did not cross-react with other members of the Src family of tyrosine kinases that are expressed in B cells, such as Blk and Fyn. Furthermore, this peptide sequence is common to both the 53kD and 56kD isoforms of Lyn, so that the antiserum was expected to recognise both.

The peptide had a cysteine residue added to its N-terminus which allowed it to be coupled to a carrier protein, keyhole limpet hemocyanin (KLH). KLH is a commonly used hapten carrier, as it is extremely immunogenic and therefore also promotes a good response to the hapten. The peptide was synthesised using an in-house service at
NIMR. After synthesis, the peptide was coupled to KLH by V. L. J. Tybulewicz. Two rabbits were injected with the coupled peptide in complete Freund's adjuvant by A. Mullord according to the schedule shown in Figure 3.1b.

(a)

\[
\begin{align*}
p_{56} &\text{lyn} & SH3 & SH2 & \text{Kinase} \\
p_{53} &\text{lyn} & SH3 & SH2 & \text{Kinase} \\
& & H_3N-\text{CPVPEFHLLPGQRFQTKDPEE-COOH} & \\
\end{align*}
\]

(b)

<table>
<thead>
<tr>
<th>Date</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.11.93</td>
<td>Prebleed (serum A)</td>
</tr>
<tr>
<td>23.11.93</td>
<td>Primary immunisation</td>
</tr>
<tr>
<td>21.12.93</td>
<td>Boost injection</td>
</tr>
<tr>
<td>31.12.93</td>
<td>Bleed (serum B)</td>
</tr>
<tr>
<td>4.1.94</td>
<td>Boost injection</td>
</tr>
<tr>
<td>11.1.94</td>
<td>Bleed (serum C)</td>
</tr>
<tr>
<td>25.1.94</td>
<td>Bleed (serum D)</td>
</tr>
<tr>
<td>8.2.94</td>
<td>Bleed (serum E)</td>
</tr>
<tr>
<td>22.2.94</td>
<td>Bleed (serum F)</td>
</tr>
<tr>
<td>8.3.94</td>
<td>Bleed (serum G)</td>
</tr>
<tr>
<td>5.4.94</td>
<td>Bleed (serum H)</td>
</tr>
</tbody>
</table>

Figure 3.1(a). Schematic diagram showing the structure of the two isoforms of Lyn, the region from which the peptide was taken (pink) and the amino acid sequence. Note the addition of the N-terminal cysteine, shown in pink. (b). Rabbit injection schedule showing bleeds and boost injections carried out to increase antibody titre and facilitate affinity maturation. The boost injections were carried out in incomplete Freund's adjuvant.

3.2 Analysis of sera

Sera obtained from the bleeds were analysed by immunoblotting for their ability to detect the two isoforms of Lyn. Total cell lysates were prepared from the cell line WEHI-231, which expresses both isoforms.
of murine Lyn. An immunoblot consisting of multiple lanes of this lysate was cut into strips, and each strip was incubated with serum from a different bleed to determine which, if any, could recognise Lyn. As expected, the pre-immune serum failed to detect Lyn even after an exposure time of ten minutes. However all of the post-immune bleeds show two bands with molecular weights of between 50-60kD consistent with the expected sizes of p53/56\(\text{lyn}\) (Figure 3.2).

Figure 3.2. Immunoblots stained with the antisera A-H generated in section 3.1 and described in Figure 3.1.(b) A shows the preimmune serum and B-H correspond to the bleeds in Figure 3.1. Each strip contains two lanes of WEHI-231 total cell lysate.

The ability of an antiserum to immunoprecipitate the protein that it is directed against does not necessarily correlate with its ability to recognise that protein on an immunoblot. This is because on an immunoblot, the protein immobilised on the membrane is likely to
retain little of its secondary or tertiary structure, that is, it exists predominantly as a linear amino acid chain. However, during immunoprecipitation, the protein is likely to retain much of its native conformation. This may mean that in the case of an anti-peptide antibody such as this one, the peptide may be concealed from the antibody due to the secondary or tertiary structure of the protein, making immunoprecipitation difficult or even impossible. To test the ability of the antisera to precipitate Lyn, antiserum C from Figure 3.2 was bound to protein A-sepharose beads and the successful immunoprecipitation of both isoforms of Lyn is shown in Figure 3.3. Thus, serum C, henceforth referred to as the anti-Lyn antibody, works in both immunoblotting and immunoprecipitation. Convincing evidence that the antisera recognises Lyn is shown in Figure 3.8, which shows that the 56kD band recognised in transfected COS cells is the product of the p56\textsuperscript{lyn} cDNA, and that this band is the same size as the upper band seen in the lysate from WEHI-231 cells.

![Figure 3.3. Immunoblot developed with the anti-Lyn antiserum C. Lane 1, WEHI-231 total cell lysate; lane 2, immunoprecipitation of p53/56\textsuperscript{lyn} from WEHI-231 cells using antiserum C; lane 3, COS-1 cell lysate. The arrow indicates the position of the 53 and 56kD Lyn isoforms.](image)
3.3 Generation of a kinase defective form of p56^lyn^ 

Previous studies have shown that mutation of the lysine residue in the ATP-binding site of the Src-family kinases (corresponding to lysine 275 of p56^lyn^) to methionine, arginine or histidine results in an ablation of both autokinase activity and the ability to phosphorylate an exogenous substrate (Snyder, Bishop et al. 1985; Kamps and Sefton 1986; Kemieicik and Shalloway 1987). However these mutations do not appear to affect these kinases in any other manner. Thus to generate a mutant form of p56^lyn^ lacking kinase activity but retaining functional SH2, SH3 and unique domains that could potentially act as a dominant negative protein when overexpressed, lysine 275 of murine p56^lyn^ was replaced by methionine in the p56^lyn^ cDNA. The codon for this lysine, AAG, was mutated to ATG resulting in a methionine at position 275. This mutation (K275M) was introduced using the single-overlap extension PCR strategy shown in Figure 3.5 and the primers shown in Figure 3.4. The template was the cDNA encoding murine p56^lyn^.

This strategy allowed not only the introduction of the desired mutation, but also the addition of restriction sites at either end of the cDNA. These restriction sites were used to clone the resulting PCR product into the chosen expression vector. To ensure correct initiation of translation, ten bases of the murine lyn cDNA immediately 5' to the initiator methionine were incorporated into the 5' PCR primer (primer no. 1 in Figures 3.4 and 3.5). This sequence includes the Kozak consensus sequence which is critical for efficient initiation of translation (Kozak 1986). Directly upstream of this Kozak sequence an MluI restriction site was added for cloning purposes. A BamHI site was introduced immediately 3' to the
stop codon using primer no.2. As well as generating the mutant p56\textsuperscript{lyn} cDNA carrying the MluI and BamHI sites, this PCR strategy was also employed to generate the wild type p56\textsuperscript{lyn} cDNA carrying the same restriction sites.

Primer no.1:

5'AAACGCGTAGCGAGAAATATGGGATGTATTAAATCA3'  
Primer no.2:

5'ATGGGATCCTACGGTTGCTGCTGATACT3'  
Primer no.3:

5'GTGGCTGTGATGACCCTCAA3'  
Primer no.4:

5'TTGAGGGTCAATCACAGCCAC3'

Figure 3.4. The sequences of the primers used in the PCR protocol shown in Figure 3.4a. Red indicates a restriction site, MluI in the case of primer no.1 and BamHI in the case of primer no.2. The translational start site is coloured blue. Green bases represent the mutation. Ten bases of Kozak consensus sequence were introduced in primer no.1 between the MluI site and the translational start site.

3.4 Vector design

Expression of the mutant and wild type p56\textsuperscript{lyn} cDNAs \textit{in vitro} and \textit{in vivo} requires different expression vectors. For the COS cell transfections described in this chapter, the vector pcDNA3 (Figure 3.7) was used because it contains a strong viral enhancer (the cytomegalovirus (CMV) enhancer) which will drive expression in a variety of cell types, and the SV40 origin of replication allowing episomal replication of the plasmid in cell lines containing the SV40 large T antigen. Both wild type and mutant p56\textsuperscript{lyn} PCR products were digested with MluI and BamHI and the overhangs generated by this
filled in with DNA polymerase, before being ligated into the EcoRV site of pcDNA3, to generate pclyn and pcK275M respectively.

Position of primers on the p56\(^{lyyn}\) cDNA

Figure 3.5. Cartoon showing the PCR strategy used for making the mutant cDNA and adding the MluI and BamHI sites to both the wild type and the mutant Lyn cDNAs. To generate the wild type cDNA carrying the restriction sites, one round of PCR was carried out using only primers 1 and 2. For the mutant cDNA, three reactions were performed, A, B and C, using primers 3 and 4 which span the lysine codon and carry the A\(\rightarrow\)T base change as well as primers 1 and 2. Reactions A and B used the p56\(^{lyyn}\) cDNA as a template; reaction A used primers 1 and 4, reaction B primers 2 and 3. This resulted in two products, one consisting of sequence 5' of, and including, the mutation and also carrying the MluI site; and the other starting with the mutation and containing the 3' sequence including the BamHI site. These two products were then used in PCR reaction C along with primers 1 and 2 to generate the full length mutant cDNA. The asterisk on primers 3 and 4 represents the K275M mutation and the striped box the Kozak consensus sequence.
Figure 3.6. Schematic diagram of the germline and rearranged configurations of the murine heavy-chain locus. The genes are depicted as coloured boxes and the enhancers as green circles. V=variable genes, D=diversity genes, J=joining genes and C=constant genes. For simplicity only the Cμ and Cα constant region heavy chain genes are shown. The 3′Ca enhancer is 16 kb 3′ from the Cα gene.

Whilst these vectors work well in COS cells (see later) they are inappropriate for generating transgenic mice for a number of reasons presented below. I wanted to restrict expression of the transgene to the B cell compartment of the transgenic mice so that the analysis of the phenotype would not be complicated by the possible effects of a mutant p56 lyn in other lineages. pcDNA3 was unsuitable for this because strong viral enhancers such as the CMV enhancer do not confer cell-type specificity upon the gene whose expression they are driving, although they do exhibit optimal enhancer function in their host cells (de Villiers, Olson et al. 1982). To this end, the vector designed to generate the transgenic mice utilised one of the three murine immunoglobulin heavy chain enhancers (Banerjee, Olson et al. 1983; Lieberson, Giannini et al. 1991). These enhancers lie 3′ of the VH promoter in both the germline and rearranged heavy chain loci, as shown above in Figure 3.6. The intronic enhancer (Eμ) was the first
documented cellular enhancer, and one of its most striking properties was its tissue specificity. Eμ is a stronger enhancer than those at the 3' end of the constant chain genes and also activates B cell specific expression at an earlier developmental timepoint; furthermore it has previously been used successfully to facilitate B cell expression in transgenic mice by a number of different groups using a variety of heterologous promoters (Adams, Harris et al. 1985; Reik, Williams et al. 1987). Therefore Eμ was chosen over the 3' enhancers to direct expression in the transgenic mice. The second reason that the pcDNA3 vectors were unsuitable for generating transgenic mice is that these constructs do not contain any introns. There is evidence to suggest that whilst introns may not be necessary when expressing constructs using strong viral promoters such as the CMV promoter, they are necessary for expression in transgenic mice. Splicing is believed to stabilise messenger RNA, help with its export from the nucleus and in some cases to help drive transcription (Hamer and Leder 1979; Palmiter, Sandgren et al. 1991). Therefore the pBISlyn and pBISK275M vectors were built, which are derived from the vector pBIS (Figure 3.7). pPIF2, the parental plasmid of PBIS, lacks Eμ and consists of pBR322 sequence into which the β-globin gene was introduced. pBIS was generated by cloning the 1kb XbaI Eμ fragment into the XbaI site of pPIF2. An engineered MluI site replaces the β-globin ATG and there is a naturally occurring BamHI site at the 3' end of the second exon, into which the Igα cDNA had been cloned, thus replacing the β-globin sequence up to this BamHI site.
Fig. 3.7. pBIS contains the intronic IgH enhancer E\(_\mu\) to direct expression to the B cell compartment, and transcription is driven by the human \(\beta\)-globin promoter (arrow). A splice event is generated by the presence of the 2nd exon, 2nd intron and 3rd exon (yellow boxes) of human \(\beta\)-globin directly downstream of the Lyn stop codon. The polyadenylation signal is also provided by the \(\beta\)-globin sequence. pcDNA3 utilises the cytomegalovirus (CMV) promoter and the bovine growth hormone (bGH) polyA signal. Both plasmids carry the neo gene for selection with G418 in mammalian cells, as well as the ampicillin resistance gene for selection in *E. coli.*
This assembly provides a complete transcription unit, including the promoter and second intron of human β-globin. The rabbit β-globin promoter coupled to the Eμ enhancer has previously been used successfully both in cell lines and transgenic mice and so the BIS vector was expected to give good B cell specific expression. Cloning of the wild type and mutant p56\textsuperscript{lyn} cDNAs into pBIS, replacing the Igα cDNA, generated pBISlyn and pBISK275M respectively.

In order to confirm both the fidelity of the PCR, the mutagenesis and the cloning, the wild type and mutant cDNAs were sequenced completely on both strands, from upstream of the MluI site to downstream of the BamHI site (data not shown). The sequences over codon 275 are shown in Figure 3.8. The conclusion from this sequence data was that the PCR had successfully generated both the mutant and wild type cDNAs without incorporating any errors.

Figure 3.8. Autoradiographs showing the sequence over codon 275 of p56\textsuperscript{lyn}, showing the AAG->ATG mutation in pBISK275M. The wild type is shown on the left, the mutant on the right.
3.5 Expression of wild type and mutant p56\textsuperscript{lyn} in COS-1 cells

Before generating transgenic mice and stably transfected cell lines, the expression of the mutant Lyn and of the wild type was compared using transient transfection assays in COS-1 cells. COS-1 is a green monkey kidney cell line widely used for transient expression studies, as it expresses the SV40 large T antigen, allowing plasmids containing the SV40 origin of replication to replicate episomally to high copy number within a transfected cell, thus producing large amounts of protein which can easily be detected by immunoblot. Furthermore, COS-1 cells do not express Lyn, making them a good system for checking the Lyn vectors. Lysates from COS-1 cells transfected with pclyn and pcK275M were analysed alongside lysate from WEHI-231 cells (Figure 3.9). Also included on this immunoblot was a mixture of the two, to ensure that the wild type and mutant Lyn proteins derived from the transfected plasmids exhibited the same electrophoretic mobility as p56\textsuperscript{lyn} from WEHI-231 cells. These results demonstrate that at least in COS-1 cells pclyn and pcK275M express Lyn proteins indistinguishable from p56\textsuperscript{lyn}.

Figure 3.9. Immunoblot of total cell lysates from COS-1 cells or WEHI-231 cells developed with antiserum C. Lane 1, mock transfected COS-1; 2, COS-1 transfected with pcDNA3; 3, COS-1 transfected with pclyn; 4, pclyn lysate+WEHI-231 lysate; 5, WEHI-231 lysate; 6, COS-1 transfected with pcK275M+WEHI-231 lysate; lane 7, COS-1 transfected with pcK275M.
3.6 Analysis of the specific kinase activities of wild type and mutant p56 lyn

For the mutant Lyn to behave as a dominant negative protein, its specific kinase activity must be significantly lower than that of the wild type in order to diminish the phosphorylation (and thus the activation) of target molecules downstream in the signalling pathway. Specific activity was measured by immune complex kinase assays in which Lyn is incubated with $^{32}\text{P}$-ATP. Because Lyn is able to autophosphorylate, the incorporation of $^{32}\text{P}$ can be compared to the amount of protein present to give a measure of the specific activity. The level of $^{32}\text{P}$ incorporation depends on the incubation time, and so in order to determine the time at which the reaction reaches saturation, an in vitro immune complex kinase assay was performed in which the endogenous Lyn from WEHI-231 cells was immunoprecipitated and incubated for varying amounts of time in the presence of $^{32}\text{P}$-ATP. After immunoblotting, the filter was exposed to film before being stained with the anti-Lyn antiserum (Figure 3.10).

After densitometry of the immunoblot and phosphorimager analysis of the autoradiograph, the incorporation at each time point was calculated by dividing the $^{32}\text{P}$ signal by the ECL signal (Figure 3.11). The phosphorimager values for $^{32}\text{P}$ incorporation are linear, but care must be taken when analysing the densitometry data to ensure that the ECL values are taken from a film that is not saturated by the ECL reaction. This was achieved by taking multiple exposures of the
immunoblot, and using the densitometer to check that the exposures were not saturated.

Figure 3.10 Time course (2-20 minutes) of immune complex kinase assay of Lyn immunoprecipitated from WEHI-231. a) $^{32}\text{P}$ incorporation into immunoprecipitated Lyn. b) The same immunoblot stained with anti-Lyn. Each time point was carried out in duplicate. The sizes of p53/56$^{lyn}$ are indicated.

Figure 3.11. Graph depicting $^{32}\text{P}/\text{ECL}$, a measure of specific activity, against time for both isoforms of Lyn. This data was obtained by dividing $^{32}\text{P}$ incorporation values by the amount of protein present from the experiment in Figure 3.10.
From this experiment it was determined that 10 minutes would be the best time point to use, as the reaction is not saturated at this time. Thus Lyn immune complex kinase assays were carried out on COS-1 cells that had been transfected with either pclyn or pcK275M and incubated with $^{32}$P-ATP for 10 minutes (Figure 3.12).

Figure 3.12. *In vitro* kinase assay of immunoprecipitated Lyn. The top panel shows the autoradiograph depicting $^{32}$P incorporation. The bottom panel is the same membrane stained with the anti-Lyn antibody. Lane 1, mock transfected COS-1; lane 2, COS-1 transfected with pcDNA3; lane 3, COS-1 transfected with pclyn; lane 4, COS-1 transfected with pcK275M; lane 5, WEHI-231.

As can be seen in Figure 3.12, the specific activity of the mutant Lyn appears to be much less than that of the wild type. Phosphorimager and densitometer analysis was carried out on these two blots, and the specific activity of the mutant $p_{56}^{lyn}$ was determined to be at least 25-fold less than that of wild type $p_{56}^{lyn}$.

3.8 Summary

The aims of the work presented in this chapter were achieved successfully. The antisera generated recognise both isoforms of Lyn on immunoblots, can immunoprecipitate both and there is very little
background caused by cross reactivity in these experiments (Figures 3.2 and 3.3).

The mutation engineered into p56\textsuperscript{lyn} has reduced its kinase activity to at least 25-fold less than wild type p56\textsuperscript{lyn} without affecting its ability to be expressed, at least in COS-1 cells. Such a reduction in kinase activity should enable the mutant protein to act as a dominant negative when introduced into transgenic mice and B cell lines.
Chapter Four

Generation and analysis of mice transgenic for mutant p56^lyn

B cells are a dynamic population of cells, developing, being selected, becoming activated (or not), dying and being replaced many times during the lifetime of an animal. These responses are critically controlled by signals from the pBCR and BCR. To study the role of Lyn in the signalling of these receptors during these responses, a mutational analysis in the context of the whole animal is required. This chapter describes the generation and analysis of mice transgenic for the mutant p56^lyn developed in the Chapter Three.

4.1 Generation of transgenic mice

The plasmid pBISK275M was digested with \textit{NotI} to remove the prokaryotic sequences, which can impede expression in transgenic mice (Chada, Magram et al. 1985). The resulting 4.9kb fragment (see Figure 4.1) was purified on an Elutip-D column before being injected into the male pronuclei of fertilised eggs. It is common to microinject eggs from an F1xF1 cross, as they give higher egg yields after superovulation, the eggs are hardier, and more likely to survive injection and thus are more efficient for making transgenic mice (Hogan, Constantini et al. 1986). However the genetic background is an important consideration when making transgenic mice as it can affect the resultant phenotype. Transgenic mice from such a cross are necessarily outbred and thus
exhibit more variation due to genetic segregation. To minimise this potential variability in the Lyn mice, inbred mice of the CBA/Ca (CBA) strain were used despite their poorer reproductive capabilities: only 7-10% of re-implanted injected embryos develop to term when using an inbred strain compared to 15-25% when F2 embryos are used.

Five hundred eggs were microinjected (with the help of A. Mellor at NIMR) with the 4.9kb fragment from pBISK275M containing p56lynK275M and those that had reached the two-cell stage after incubation overnight were transferred into (B6 x CBA) F1 psuedopregnant foster mothers. The resulting forty pups were screened for the presence of the transgene by Southern blot analysis of tail DNAs digested with BamHI, using the 0.9kb BamHI-EcoRI fragment of human β-globin derived from pBISK275M and shown in Figure 4.1. This probe was chosen for a number of reasons. As it consists of human sequence, it will only be present in the DNA of transgenic pups, allowing them to be distinguished from nontransgenics. Also, since most transgenes integrate into the host genome in multiple copies arranged in a tandem array (Hogan, Constantini et al. 1986), this probe allowed differentiation between mice carrying single copies of the transgene and those with multiple copies. Furthermore it shows the orientation of multiple copies with respect to one another. Mice containing a single copy of the transgene will be identifiable as there is only one BamHI site within the construct, so the adjacent BamHI sites will depend upon where in the genome the transgene integrates; since integration occurs at seemingly
random positions, the size of the band will be unpredictable and unique for each single copy integration. Multiple copies of the transgene in a tandem array can be in a head-to-tail, head-to-head or tail-to-tail orientation. A BamHI digest will thus generate a 4.9kb band if the copies are integrated head-to-tail, whereas an head-to-head orientation will give a 3.2kb band, and a tail-to-tail 6.6kb.

Figure 4.1. Schematic diagram showing the 4.9kb transgene excised from pBISK275M by digestion with NotI and the 0.9kb probe used for its detection on Southern blots. The green box represents the 1kb Xbal Eμ enhancer; the blue box the mutant Lyn cDNA and the asterisk the mutation. The yellow boxes represent the human β-globin 2nd and 3rd exons. The black lines represent the β-globin promoter (arrow), 2nd intron and polyadenylation sequences. For simplicity the EcoRI site in the Lyn cDNA is not shown. N, NotI; X, Xbal; M, MluI; B, BamHI; and E, EcoRI.

Figure 4.2. Southern blot showing tail DNAs from the seven transgenic mice digested with BamHI and hybridized to the 0.9kb BamHI-EcoRI human β-globin probe. The positive control (+) is tail DNA from a mouse transgenic for the whole human β-globin locus (a gift from F. Grosveld's lab.) also digested with BamHI and predicted to give a band of 4.2kb with this probe. Also included is DNA from nontransgenic littermates as a negative control (-).

Of the forty pups born, seven were transgenic as shown by tail DNAs on the Southern blot in Figure 4.2. All seven transgenic mice show a band of 4.9kb, indicative of multiple copies of the transgene in a head-
to-tail array. This result does not exclude the possibility of more than one integration event in any of the transgenic mice. Such a multiple integration event would only become apparent on breeding the transgenic mice, when the two (or more) integrations would segregate.

The seven transgenic mice were named Lyn Dominant Negative (LDN) followed by a number corresponding to their identification number assigned at the time of weaning, so that the seven founders were called LDN14, LDN21, LDN31, LDN32, LDN33, LDN36 and LDN37 respectively. These founder mice were then mated to nontransgenic CBA mice to establish transgenic lines. Table 4.1 shows a table documenting this breeding.

<table>
<thead>
<tr>
<th>Founder Mouse</th>
<th>No. transgenic/No. total progeny</th>
<th>% transgenic progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDN14</td>
<td>22/56</td>
<td>40</td>
</tr>
<tr>
<td>LDN21</td>
<td>0/49</td>
<td>0</td>
</tr>
<tr>
<td>LDN31</td>
<td>0/27</td>
<td>0</td>
</tr>
<tr>
<td>LDN32</td>
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<tr>
<td>LDN36</td>
<td>4/6</td>
<td>66</td>
</tr>
<tr>
<td>LDN37</td>
<td>0/38</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.1. Table showing the first six months breeding of the founders. The total number of progeny for each founder is shown, plus the percentage of these progeny who had inherited the transgene.

As can be seen from Table 4.1, only three of the seven transgenic mice transmitted the transgene to their progeny: LDN14, LDN33 and LDN36.

The expected frequency of transmission of the transgene by a founder transgenic mouse containing a single integrant is 50%. LDN14 and LDN36 transmitted to 48% and 67% of their progeny, whereas LDN33
only passed on the transgene to 12% of its offspring, suggesting that this founder was a mosaic.

Once the three lines of transgenic mice had been established, the number of copies of the transgene carried by each line was determined. This was done so that the magnitude of any phenotype of the mice could be correlated to the copy number. A similar approach was used with transgenic mice expressing a dominant negative mutant of Lck, where mice expressing the highest levels of the mutant Lck had a more severe phenotype (Levin, Anderson et al. 1993). Since the Lyn transgene included the murine Eμ enhancer, the number of copies of the transgene could be derived by comparing its hybridisation to the single copy endogenous Eμ by Southern blotting.

![Diagram of transgene structure](image)

Figure 4.3. The 4.9kb transgene showing the 0.6kb PvuII-Xbal probe and the predicted size of the Eμ containing fragment that the probe would hybridise to on a Southern blot after digestion with PvuII and SacI. Colouring is the same as in Figure 4.1. N, NotI; X, Xbal; P, PvuII; M, MluI; E, EcoRI; S, SacI.

To evaluate copy number, DNA from wild type CBA mice as well as each of the three transgenic lines was digested with PvuII and SacI and probed with a 0.6kb PvuII-Xbal fragment from Eμ. This probe was
predicted to hybridise with a 2.8kb PvuII-SacI fragment from the transgene (Figure 4.3) and hybridised to an endogenous 1.0kb PvuII-PvuII fragment from the Eμ locus (Figure 4.4). The Southern blot of the transgenic DNA is shown in Figure 4.5.

Figure 4.4. Southern blot of wild type CBA genomic DNA with a variety of enzymes and probed with the 0.6kb Eμ probe. Lane 1, PvuII; lane 2, PvuII/EcoRI; lane 3, PvuII/HindIII; lane 4, PvuII/MluI; lane 5, PvuII/SacI.

Figure 4.5. Southern blot showing genomic DNAs from transgenic (+) and nontransgenic (-) mice. Tail DNAs from three mice of each line plus three nontransgenic littermate controls were digested with PvuII and MluI. The blot was probed with the Eμ probe.
To determine the copy number, the intensities of the bands due to the transgene were measured using a phosphorimager and the value divided by the intensity of the endogenous $\text{E}_{\mu}$ band for each transgenic mouse. Table 4.2 shows the average copy number for each line of transgenic mice.

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDN14</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>LDN33</td>
<td>62 ± 18</td>
</tr>
<tr>
<td>LDN36</td>
<td>90 ± 48</td>
</tr>
</tbody>
</table>

Table 4.2. Table showing the copies of the transgene in each line of transgenic mice as calculated by phosphorimager analysis of the Southern blot in Figure 4.5. The mean of three mice from each line is shown, plus the standard deviation.

4.2 Analysis of B cell development in p56$^{\text{lyn}}$ transgenic mice

Since signals through the pre-B cell receptor (pre-BCR) and BCR are crucial for B cell development, if Lyn is involved in these signalling processes B cell development may be disrupted in the transgenic mice. Therefore the analysis of the transgenic mice began by studying B cell development in the bone marrow.

Pro-B, pre-B and IgM$^+$ B cells were analysed by flow cytometry using the markers B220 (the isoform of CD45 expressed on B cells), CD25, the $\alpha$ chain of the IL2 receptor, IgM and IgD. The expression patterns of these proteins during B cell development are shown in Figure 4.6 and were used to assign the cells to the pro-B, pre-B and IgM$^+$ compartments.
Bone marrow from each of the three transgenic lines was compared to bone marrow from sex and age matched control mice of 6-8 weeks old. The total bone marrow cellularities of each transgenic line were not significantly different from wild type CBA mice (Figure 4.7).

Figure 4.6. Cartoon of B cell development showing the expression patterns of the markers used to analyse the transgenic mice. See text for further details.

Figure 4.7. Graph showing the mean number of cells (of all lineages) present in the bone marrow from each of the three lines of transgenic mice compared to wild type control mice. Twelve mice were analysed, three for each group, taking both femurs from each mouse. Columns represent the mean cellularity of the three mice in each group, and the error bars represent the standard deviation from the mean.
Figure 4.8. Dot plots showing bone marrow staining for IgM and B220. The polygon region enclosing cells that express both IgM and B220 contains both immature and mature B cells. The square is drawn around cells that express B220 but not IgM, i.e. pro- and pre-B cells. These gated B220+ IgM- cells have their CD25 profile displayed as a histogram on the right. Vertical bars on the histograms separate CD25- cells (pro-B) from CD25+ cells (pre-B).
Figure 4.9. Graphs showing the proportions of pro-, pre- and IgM⁺ B cells present in bone marrow as a percentage of the total number of lymphocytes. Cells were gated as in Figure 4.8. Columns and error bars represent the mean and standard deviation for three mice of each transgenic line.

Flow cytometric analysis showed that the transgenic mice contained similar proportions of B cells as demonstrated by the presence of cells expressing both IgM and B220 (Figure 4.8). This fraction contains both
immature and mature B cells. To enumerate pro-B and pre-B cells, the CD25 staining of B220⁺, IgM⁺ cells was displayed as a histogram (Figure 4.8, right-hand side). Pro-B cells are CD25⁻ and pre-B cells CD25⁺.

The numbers of pro-B, pre-B, and IgM⁺ cells as percentages of the total number of lymphocytes were calculated for each of the twelve mice analysed (Figure 4.9). There does not appear to be any significant variation between genotypes, suggesting that B cell development in the transgenic mice is grossly normal.

4.3. Analysis of mature B cell compartments in p56 lyn transgenic mice

Mature B cells in the spleen were analysed by flow cytometry using IgM and IgD as markers. Mature cells express both isotypes, with IgD typically more highly expressed than IgM. A dot plot showing staining typical of wild type CBA mice and the three transgenic lines is presented in Figure 4.10. All three transgenic lines showed a similar proportion of B cells to CBA mice. Furthermore they all showed a high ratio of IgD/IgM typical of mature B cells. These results suggest that there is no effect on the development and survival in the periphery of mature B cells in the LDN mice.

The B1 population of B cells, which predominate in the peritoneal cavity, can be distinguished from conventional cells (B2) by virtue of a high ratio of IgM:IgD. Therefore to determine whether or not this subpopulation was affected in the LDN mice, peritoneal cells were harvested from transgenic and control mice and stained with anti-IgD
and anti-IgM antibodies. Flow cytometric analysis of these cells showed that the B1 population in the transgenic mice is still present in apparently normal proportions (Figure 4.11).

Figure 4.10. Dot plot of IgM against IgD staining of splenocytes from a sex and age matched CBA control mouse, and one of each of the three transgenic lines LDN14, LDN33 and LDN36.

Thus the Lyn transgene does not appear to affect development or maintenance in the periphery of any B cell lineage. In contrast the Lyn knockout mice have normal numbers of B1 cells but lower levels of mature recirculating B2 cells (Hibbs, Tarlinton et al. 1995; Nishizumi, Taniuchi et al. 1995).
4.4 Analysis of T cells in p56lyn transgenic mice

The ability of the transgenic mice to generate T cells was studied because whilst Lyn is not expressed in normal T cells, the Eμ enhancer can occasionally direct the expression of transgenes under its control to this lineage. Since T cells express other members of the Src family of tyrosine kinases which are important for T cell development, it is possible that the mutant Lyn could interfere with T cell development in a manner analogous to that of a dominant negative Lck mutant (Levin, Anderson et al. 1993). Therefore splenocytes from the three
lines of transgenic mice were stained with antibodies against CD4 and CD8 and analysed by flow cytometry to ensure that T cells were able to develop and populate the secondary lymphoid organs (Figure 4.12). There does not appear to be any significant difference in the ability of any of the transgenic lines to develop T cells, as Figure 4.12 shows.

Figure 4.12. Typical dot plot showing flow cytometric analysis of splenocytes with antibodies to CD4 and CD8.

4.5 Summary

Three lines of mice have been generated that carry a transgene encoding the mutant form of p56^lyn^. The number of copies integrated into the genomes of these three lines varies from about five to one hundred and twenty.

Analysis of B cell development in the transgenic mice suggests that it is not perturbed when the transgene is present. Furthermore, mature B cells in the spleen, plus the B1 subset residing in the peritoneal cavity,
are present in normal proportions. Likewise T cell development does not seem to be affected.
Chapter Five

Expression of mutant Lyn in transgenic mice

Before any further conclusions could be drawn from the lines of transgenic mice it was necessary to ensure that the transgene was being expressed. The presence of the Εμ enhancer in the transgenic constructs should direct the mutant Lyn expression to the lymphoid compartments of the animals, specifically B cell compartments, but possibly also to T cells (Strasser, Harris et al. 1990). Therefore the bone marrow, spleen, lymph node and thymus were studied for evidence of expression of the transgene by immunoblotting using the anti-Lyn antibody. The rationale being that in the transgenic mice, the amount of p56lyn would increase with respect to p53lyn. In this way, the p53lyn acts as an in-built control. However, the possibility that the levels of expression of the two isoforms are tightly linked and regulated such that they are always expressed the same relative ratio cannot be formally excluded. Overexpression of one isoform may lead to the downregulation of expression of the endogenous protein, or upregulation of the expression of the other isoform. Since this would negate the use of p53lyn as an internal control, the blots were also restained with an antibody against Vav.

4.1 Transgene expression in lymphoid compartments

The Εμ enhancer is active early in B cell development (at the pro-B cell stage), and so bone marrow was included in the expression analysis. Protein extracts made from single cell suspensions of femoral bone marrow from each line of mouse, plus age- and sex-matched controls, were analysed by SDS-PAGE and immunoblotting with the anti-Lyn and anti-Vav antibodies. This showed that the ratio of p56lyn to p53lyn
was unchanged in the transgenic mice (Figure 5.1). Furthermore the ratio of p56\textsuperscript{lyn} to Vav is unchanged in the three transgenic lines.

![Immunoblot showing bone marrow cell lysates stained with anti-Lyn (top panel) and anti-Vav (bottom panel). Lysates from the transgenics are marked either LDN14, 33 or 36; - indicates sex and age matched control mice. The sizes of the molecular weight markers are also shown.](image)

Figure 5.1 also shows that there appears to be greater amounts of both isoforms of Lyn in some of the mice; however by virtue of the Vav staining, variation between lanes can be seen to be due to unequal loading rather than any increase in expression of Lyn in the transgenics.

Whilst it may be easy to conclude from this result that there is no expression of the mutant Lyn in the bone marrow, where it is expected, it may be that the overexpression of p56\textsuperscript{lyn} is very slight; if so, because it will only be expressed in B cells, it may be masked in this blot by the normal expression of Lyn in other haematopoietic cells of the bone marrow. Therefore the spleen and lymph node were also analysed for expression. Spleens provide a good source of mature and recirculating
B cells; the spleen of a four month old mouse yields around $10^8$ cells of which approximately 40% are B cells, the remainder being T cells and myeloid cells. Lymph nodes provide probably the cleanest population of lymphocytes in terms of the absence of other cell types. Figure 5.2 shows an immunoblot carried out using lysates made from single cell suspensions of splenocytes, again from each line of mice plus nontransgenic controls.

![Immunoblot](image.jpg)

**Figure 5.2.** Typical immunoblot showing splenocyte cell lysates stained with, top panel, the anti-Lyn antibody; bottom panel, the same blot stained with the anti-Vav antibody. Lysates from the transgenics are marked either LDN14, 33 or 36; - indicates sex and age matched control mice. The sizes of the molecular weight markers nearest the bands of interest are also shown.

As Figure 5.2 shows, there also does not appear to be any overexpression of p56\(^\text{lyn}\) in the spleens of any of the transgenic lines, because neither the p56/p53\(^\text{lyn}\) ratio nor the p56\(^\text{lyn}\)/Vav ratio is altered in any of the LDN mice.

Figure 5.3 shows an immunoblot of total cell lysates made from lymph nodes stained as before first with anti-Lyn and then with anti-Vav.
antibodies. As in both bone marrow and spleen, there is no difference in the amount of protein in the p56^lyn^ band compared to the p53^lyn^ band for any of the transgenics, nor a change in the p56^lyn^/Vav ratio. Any difference in the amount of both isoforms present is probably due to unequal loading between lanes.

Finally, to look for potential expression of p56^lyn^ in the T cell lineage, thymic cell extracts were analysed by immunoblotting with the Lyn and Vav antibodies as before (Figure 5.4). The conclusion from Figure 5.4 is definitive: there is no detectable expression of either isoform of Lyn in the thymus of the transgenic or wild type mice. Any expression of the transgene in the thymus would be readily visible, since there is no endogenous expression of Lyn in this tissue.
In order to confirm that the mice studied were indeed transgenic, tail DNAs from the mice used to make the protein samples were taken at the time of sacrifice and analysed by Southern blotting. A typical result is shown in Figure 5.5, which clearly shows that the mice used to analyse expression had the expected genotypes.

Figure 5.4. Immunoblot showing total cell lysates from thymocytes of transgenic and control mice. The upper panel depicts the blot stained with anti-Lyn, the lower panel with anti-Vav.

Figure 5.5. Southern blot showing tail DNAs from the mice used to analyse expression. The DNAs were digested with BamHI and run on a 1% agarose gel before being transferred to a nitrocellulose membrane. Hybridisation was then performed using the 0.9kb human β-globin probe.
5.2 Lyn kinase activity in spleen

In light of the results presented above, one would conclude that there is no detectable expression of the transgene in any of the lines of transgenic mice. However it may be possible that the level of expression is too low to be detectable by immunoblotting in the presence of the endogenous p56\textsuperscript{lyn}. As the kinase activity of the mutant is undetectable above background, expression could possibly be seen by a decrease in the specific kinase activity of p56\textsuperscript{lyn} in the transgenic mice. To test this, \textit{in vitro} kinase assays were performed on splenocytes isolated from each of the three lines of mice, plus the appropriate controls. The kinase assays shown in Chapter Three were modified to account for the smaller size of primary cells compared to cell lines and the higher levels of degrading proteases that appeared to be present in these cells. Figure 5.6 shows a typical kinase assay performed on one of each of the three lines of LDN mice. Each transgenic sample was run alongside a sex- and age-matched control and WEHI-231 cells were used as a positive control for the immunoprecipitation, as primary cells were more difficult to assay. After autoradiography, the immunoblot was stained with the anti-Lyn antibody to enable the specific kinase activity to be calculated. As with the immunobLOTS on different tissues, the genotypes of the mice used in the assay were confirmed by Southern blotting (data not shown).

The kinase assay shown in Figure 5.6 suggests that there is no less kinase activity in the splenocytes of the transgenic mice than the control mice. Several similar assays were performed and the phosphorimager and densitometer analysis is presented in Figure 5.7.
Figure 5.6. In vitro kinase assay performed on splenocytes taken from transgenic mice plus control animals. Upper panel, autoradiograph; lower panel, the same blot stained with anti-Lyn.

Figure 5.7. Graph showing kinase activities of p53/56\textsuperscript{lyn} from splenocytes of control, wild type mice, and mice from each of the transgenic lines. Each column represents the mean taken from two mice for each group and the error bars denote standard deviation. \textsuperscript{32}P/ECL gives a measure of the specific kinase activity.

The specific activities of p53\textsuperscript{lyn} and p56\textsuperscript{lyn} alone were also calculated and did not differ appreciably between wild type and control splenocytes (data not shown). From this it can be concluded that there
is no significant decrease in the kinase activity of p56\textsuperscript{lyn} any of the three transgenic lines.

5.3 Summary

Taken together, the immunoblot and kinase assay data suggest that there is no detectable expression of mutant p56\textsuperscript{lyn} in any of the tissues studied in the transgenic mice.
Chapter Six

Expression of wild type and mutant Lyn in WEHI-231 cells

Whilst transgenic technology allows the effects of targeted mutations to be studied in the context of the immune system as a whole, an in depth biochemical analysis of signal transduction through the BCR can be carried out more conveniently using transformed cell lines in vitro. This chapter presents experiments using the B cell lymphoma line WEHI-231, which undergoes apoptosis in response to BCR ligation. This line was chosen so that potential alterations in the threshold necessary for apoptosis after BCR ligation in WEHI-231 cells overexpressing wild type or mutant p56\textsuperscript{lyn} could be studied. Also such transfectants would enable the study of downstream targets of p56\textsuperscript{lyn} to be identified by immunoblotting lysates of BCR-stimulated cells with antibodies against phosphotyrosine. Likewise potential changes in calcium fluxing after BCR ligation in the presence of exogenous p56\textsuperscript{lyn} could be analysed.

6.1 Apoptosis in WEHI-231

The antibody b.7.6 is a rat monoclonal antibody directed against the C\textsubscript{H}2 region of murine IgM (Julius, Heusser et al. 1984). Whilst it is mitogenic for resting splenic B cells, it induces apoptosis in WEHI-231 cells (Hasbold and Klaus 1990). In order to determine the optimal concentration of b.7.6 needed to obtain maximal apoptosis in a culture of WEHI-231 cells a titration was carried out. $10^4$ cells per well were
plated out in triplicate in 96-well tissue culture plates and cultured with varying dilutions of the antibody for 48 hours after which the metabolic activity of the cells was measured using the MTT assay. Proliferation was measured by the uptake of tritiated thymidine (Figure 6.1). The metabolic activity of the cells decreased rapidly on addition of the antibody, as did the incorporation of $^3$H-thymidine, with almost no live cells left at an antibody concentration of 10μgml$^{-1}$.

Figure 6.1. (a) Graph showing the metabolic activity of cells cultured with varying dilutions of b.7.6 antibody plus MTT. After lysis, wells containing live cells that have metabolised the MTT appear blue and thus have a higher optical density at a wavelength of 560nm. (b) Graph of tritiated thymidine uptake in WEHI-231 treated with b.7.6.
To confirm that this result was due to apoptosis, electron microscopy of cells cultured for 48hr either with b.7.6 (10μg/ml) or without were analysed by electron microscopy. Figure 6.2 shows the results, and clearly demonstrates that the addition of b.7.6 leads to apoptosis. The untreated control cells look healthy and normal, whilst the treated cells are mostly dead or dying via an apoptotic pathway as evidenced by their appearance. Apoptosis can be broken down into stages, each of which are represented in this micrograph. In early apoptosis, the nucleus begins to condense. This is followed by even further condensation, an example of which can be seen in the top right-hand corner of the photograph. At this point the cell begins to disintegrate via the formation of vesicles which are shed at the cell surface.

6.2 Transfection of WEHI-231 with pBISlyn and pBISK275M
WEHI-231 cells were transfected in triplicate with pBISlyn, pBISK275M and pSV2neo (as an empty vector control) by electroporation. All of the constructs were linearised with *XmnI* prior to electroporation. After three weeks of selection clones were clearly visible. Transfectants generated with pBISlyn (containing the wild type p56 lyn cDNA) were named WLW, for WEHI-231 Lyn Wild type, those made with pBISK275M (the mutant Lyn cDNA) WLD, and the control transfectants containing pSV2neo named WSV. 13 WLW clones, 21 WLD clones and 16 WSV were picked, expanded and their genomic DNA analysed by Southern blotting. The DNA was digested with *EcoRI*, which liberates an internal fragment of 1.1kb from the
Figure 6.2. Electron micrographs of upper panel, untreated cells, and lower panel, cells treated with B.7.6 for 48 hours. Magnification x 2400.
constructs, and NotI, which generates a 4.9kb band (Figure 6.3). Blots were hybridised with the human β-globin probe to confirm the integration of the constructs and a typical Southern blot is shown in Figure 6.4.

As can be seen, only approximately 50% of the clones show a band of the correct size. All of the transfectants harbouring the exogenous DNA were then analysed for the overexpression of p56lyn by immunoblotting using the Lyn antibody and parental WEHI-231 cells as well as the WSV transfectants, which were used as controls. A typical result is shown in Figure 6.5.

There does not appear to be any overexpression of p56lyn, as the ratio of p56lyn:p53lyn in the WLW and WLD transfectants is similar to the ratio of the two isoforms in the control parental cells and the WSV transfectants.
Figure 6.4. Southern blots of WLW and WLD transfectants probed with the human β-globin probe. Top panel, DNA digested with *NotI*; bottom panel, DNA digested with *EcoRI*. The sizes of the molecular weight markers are shown.

Figure 6.5. Typical immunoblot showing five WLW transfectants, five WLD transfectants and five control cell lines. Top panel, stained with anti-Lyn; bottom panel, stained with anti-α-tubulin as a loading control.

There is a difference in the amount of total Lyn between lanes, however the p56 lyn/α–tubulin ratio is not altered in the WLW and WLD lines. Given this result, and the fact that none of the lines of transgenic mice seem to express the mutant p56 lyn, one possible conclusion is that the BIS vectors are not capable of expressing the Lyn
cDNA. Therefore a further set of transfectants were generated using different expression vectors and are described below.

### 6.3 Transfectants generated with pclyn and pcK275M

The pcDNA3 based vectors have been shown to express appreciable levels of p56^lyn^ when transfected into COS-1 cells, as demonstrated in Chapter Three, and although not specifically designed for expression in lymphocytes, unlike the BIS vectors, the viral enhancer and promoter present in pcDNA3 can drive expression of DNAs in a broad range of cell types. The constructs were linearised by digestion with *Pvu*I, introduced into the cells by electroporation and clones picked after three weeks of selection in media containing G418. 22 transfectants generated with pclyn were picked and named WPCL1-22; 18 clones made with pcK275M were picked and named WPCLKM1-18, whilst two clones were picked from the control experiment with pcDNA3 and named WPCL and 2. Genomic DNA was prepared and analysed by Southern blotting, using the 1.1kb bovine growth hormone (bGH) fragment generated by digestion of pcDNA3 with *Xba*I and *Xma*I as a probe. The genomic DNAs were digested with *Xma*I, which generates a 1.9kb fragment containing the bGH polyA sequence plus 0.8kb of the 3' half of the Lyn cDNA (Figure 6.6). WEHI-231 genomic DNA spiked with 100pg of pcDNA3 and digested with *Xma*I was run alongside the transfectant DNAs as a positive control, and WEHI-231 DNA alone was used as a negative control.
A typical Southern blot, showing 10 pclyn transfectants and 10 pcK275M transfectants, is shown in Figure 6.7. Again the expected band is seen in approximately 50% of the transfectants. Note that a second, unexpected, band is present in all of the lanes. Since this is seen in the WEHI-231 and WPC controls, this may correspond to murine growth hormone that has hybridised to the bGH probe. Also some lanes appear to show aberrant integration patterns. In these cases the constructs may have been partially degraded by endogenous nucleases or rearranged some of their DNA prior to integration. The clones were analysed by immunoblotting, the result of which is shown in Figure 6.8.

As with the transfectants made with the BIS vectors, there does not appear to be any overexpression of p56lyn in the second set of clones (Figure 6.8). Possible reasons for this lack of expression are discussed in Chapter Seven although some of the possibilities were investigated by further experiments described below.
Figure 6.7. Southern blots showing the clones from, upper panel, the pclyn, or lower panel, the pcK275M transfection experiment, probed with the bGH probe. About 50% of the clones show the expected 1.9kb transfected DNA. All show hybridisation to endogenous murine growth hormone sequence.

Figure 6.8. Immunoblots of the pclyn and pcK275M transfectants stained with anti-Lyn.
It is possible that the constructs used so far were expressing exogenous p56\textsuperscript{lyn}, but at a level that was undetectable above the background of endogenous p56\textsuperscript{lyn}. To address this, the mutant and wild-type p56\textsuperscript{lyn} cDNAs were epitope tagged so that protein derived from the constructs could be distinguished from endogenous p56\textsuperscript{lyn} using an antibody specific for the epitope tag. The peptide sequence of the epitope used was YPYDVPDYAS, which is derived from influenza haemagglutinin (HA), and which is the minimal peptide sequence recognised by the well-documented monoclonal antibody 12CA5 (Niman, Houghten et al. 1983). If placed at the amino terminal end of Lyn, the tag may interfere with myristoylation and the localisation of the protein, and so it was engineered at the carboxy terminal end, although this may affect the function of the protein due to the proximity of the regulatory tyrosine at position 507. The tagging was achieved using the PCR strategy outlined in Figure 6.9, which takes advantage of the unique SacI site in the Lyn cDNA so that only a fraction, rather than the entire tagged cDNA, is generated by PCR, as the integrity of the PCR product must be confirmed by sequencing after cloning. Thus this strategy is less labour intensive than using the full-length cDNA as a template. A 161bp SacI-BamHI consisting of the 3' end of the Lyn cDNA plus the HA tag was generated and ligated into SacI-BamHI digested pBISlyn and pBISK257M. The integrity of the new constructs was confirmed by sequencing from immediately 5' of the SacI site until past the BamHI site. The new vectors were named pBLF (pBISLynFlu), and pBLKMF. Furthermore, the plasmids pCLF (pCDNA3LynFlu) and pCLKMF were
built by cutting out the tagged wild type and mutant cDNAs from pBLF and pBLKMF, and ligating them into pcDNA3 that had been digested with EcoRV.

![Diagram of PCR strategy](image)

Figure 6.9. Schematic diagram depicting the PCR strategy used to generate the epitope-tagged pSé275 cDNA. The two primers give a fragment which after digestion with Sac I and Bam HI can replace the Sac I-Bam HI fragments of pBLSlyn and pBISK275M, giving constructs identical to the parental ones except for the addition of the HA epitope immediately 3' of the Lyn stop codon. Primer 1: 5' AGCGCACTGTCACAGGG3'. Primer 2: 5' TTTTGGATCCTATGAGGCGTAGTCGGG-CAGTGTAGGGGTAGGCCGTGCTGCTGATACTG3'. The nucleotides in bold represent the Bam HI site.

The next step was confirmation that the tagged proteins retained the same kinase properties as the untagged. Therefore COS-1 cells were transfected with either pclyn, pCK275M, pCLF or pCLKMF and an *in vitro* kinase assay performed (Figure 6.10). This suggested that the epitope tagging of wild type p56lyn to generate p56lynHA has not interfered with its ability to autophosphorylate, although any effects on the regulation of this protein *in vivo* due to the presence of the epitope tag near the negative regulatory tyrosine at position 507 cannot be determined from this assay. As expected the mutant p56lynK275MHA remains unable to autophosphorylate. Figure 6.10 also shows that the
tagged proteins are larger than the untagged p56\textsuperscript{lyn} proteins and the same size as each other, confirming that the cloning was successful.

![Image](image_url)

Figure 6.10. \textit{In vitro} kinase assay performed on transfected COS-1 cells. The upper panel is the autoradiograph; the bottom panel, the same membrane stained with anti-Lyn. Lane 1, no DNA; lane 2, pcDNA3; lane 3, pclyn; lane 4, pCLF; lane 5, pcK275M; lane 6, pCLKMF.

6.5 Transfection of WEHI-231 with pBLF and pBLKMF

A third set of WEHI-231 transfectants was then generated, using the pBLF and pBLKMF constructs. 24 clones for each construct were picked and named WBLF1-24 (for p56\textsuperscript{lynHA}), WBLKMF (for mutant p56\textsuperscript{lynHA}) and WSVF (for the pSV2neo controls). These cells were analysed by immunoblotting for the overexpression of p56\textsuperscript{lyn} by staining with the anti-Lyn antibody and for the expression of the HA epitope by staining with the 12CA5 antibody (Figures 6.11 and 6.12).

There does not appear to be any overexpression of p56\textsuperscript{lyn} in the transfectants as the p53/p56\textsuperscript{lyn} ratio is unchanged. Furthermore, there is no staining of a band with 12CA5 that could correspond to either p56lynHA or p56\textsuperscript{lyn}K275MHA. 12CA5 does stain a protein of around
Figure 6.11. Immunoblot of 9 of the WBLF transfectants with, upper panel, anti-Lyn; lower panel, 12CA5. Lysates from 4 WSVF clones, WEHI-231 cells and COS-1 cells were used as negative controls. Immunoprecipitated HA-tagged ERK1 was used as a positive control for the 12CA5 staining.

60kD, but this appears in the control samples as well as in the transfectants. Since this antibody has successfully recognised the HA-
tagged ERK-1 present on the blot as a positive control, it can be concluded from these two blots that if the tagged Lyn is being expressed, it is below the level of detection in this experiment.

One possibility that could influence the results seen on the immunoblots presented so far is the fact that Lyn is anchored to the inside of the plasma membrane via its myristoylation. If the lysis buffer used to make the cell lysates (which uses NP40 as a detergent) is insufficient to fully solubilise the membrane-bound proteins, then only a fraction of the cell’s total Lyn may be present on the blots, and in this manner, the expression of p56lyn in the transfectants may be missed. To rule this out, further lysates were made in which the cells were lysed in Laemmli sample buffer, which contains SDS, a stronger detergent than NP40. These samples were then analysed by SDS-PAGE and immunoblotting, as before, and are shown in Figure 6.13.

As can be seen, there is no expression of a 56kD protein recognised by 12CA5, suggesting that incomplete solubilisation of cell membranes is not the reason for the inability to detect Lyn overexpression. The only difference between the samples made using sample buffer and those made with NP40 lysis buffer is that some of the Lyn has migrated further through the gel, probably due to the excess SDS present in the samples. Again this effect is seen in the control samples as well as in the transfectants.
Figure 6.13. Immunoblot of the lysates made with Laemmli sample buffer, stained with, top, anti-Lyn, and bottom, 12CA5. Again the same controls as for the previous 2 blots were used.

One further attempt to demonstrate the expression of the transfected Lyn was carried out by immunoprecipitating both isoforms of Lyn from the transfectants using the anti-Lyn antibody and then performing an immunoblot with 12CA5. This is in general a more sensitive approach, as it reduces background and concentrates the protein of interest in the sample. Clones were analysed by Southern blotting for incorporation of the constructs (data not shown) and representative clones were used. A typical example is shown in Figure 6.14. However there is no detectable epitope-tagged Lyn in any of the samples.
Figure 6.14. Immunoblot showing Lyn immunoprecipitated from representative clones made with the epitope tagged constructs. Top panel, stained with anti-Lyn; bottom panel, stained with 12CA5.

6.6 Summary

This chapter shows that in three different sets of WEHI-231 transfectants, generated with different constructs, overexpression of p56^{ lyn } cannot be detected by immunoblotting. The possible reasons for this are discussed in detail in Chapter Seven. It also demonstrates that it is possible to epitope tag a member of the Src family of kinases and maintain kinase activity, although the regulation of this activity could not be assessed in these experiments.
Chapter Seven

Discussion

The aim of this project has been to study the role of Lyn in signal transduction through the BCR. The initial strategy was to generate mutant mice lacking functional Lyn alleles. However, after the discovery that the Lyn locus was duplicated, an alternative dominant negative mutation strategy was employed. I have successfully generated an antibody that recognises both isoforms of murine Lyn, both in immunoblotting and immunoprecipitation. I have also generated a kinase defective mutant of p56\textsuperscript{lyn}, the kinase activity of which is undetectable above background. After building expression vectors I then attempted to express this mutant both in transgenic mice and the B cell line WEHI-231. However expression of the mutant p56\textsuperscript{lyn} was undetectable in all three lines of transgenic mice, by both immunoblotting and \textit{in vitro} kinase assays using the Lyn antibody. Similarly no detectable expression of either wild type or mutant p56\textsuperscript{lyn} was found in any of the three sets of WEHI-231 transfectants generated. The possible reasons for this are discussed in this chapter.

7.1 Lyn knockout mice

During the course of this project, two groups published the successful generation of Lyn\textsuperscript{-/-} mutant mice (Hibbs, Tarlinton et al. 1995;
Nishizumi, Taniuchi et al. 1995). The two groups overcame the problems associated with the duplication of the locus by different means. Hibbs et al. targeted the Lyn promoter, which is not present in the psuedogene, replacing it with the neomycin resistance gene used for the positive selection of transfected ES cells, which successfully ablated expression from the locus. Nishizumi et al. used PCR to distinguish between the Lyn gene and the psuedogene, as there are a number of nucleotide differences between the two (Hibbs, Stanley et al. 1995). After transfecting embryonic stem cells they then used this PCR assay to determine which clones had integrated the targeting vector into the correct locus.

Similar experiments conducted on the Lyn−/− mice by both groups show the same phenotype, confirming that both have correctly targeted the Lyn locus. Young mice are grossly normal, but show defects in both B cell and mast cell function. Common findings between the two laboratories include the reduction in the number of mature, peripheral B cells, impaired proliferative responses of these B cells, elevated levels of serum IgM, and the production of autoantibodies. The overproduction of serum Ig results in glomerulonephritis in both sets of Lyn−/− mice. The reduction in the number of mature cells is not caused by a developmental block, as pro-B, pre-B and immature cells are present in normal numbers. Rather, the authors suggest that this aspect of the phenotype is due to a failure of the cells to proliferate or persist in the periphery. Thus it would appear that whilst Lyn is not necessary for B cell development, it is required for negative selection
events, as demonstrated by the autoantibody production and autoimmune disease.

The tyrosine phosphorylation patterns of Lyn⁻/⁻ B cells after BCR ligation differ significantly from wild type B cells. Phosphorylation of Vav, Cbl and PLCγ2 is absent or delayed in the mutant cells, suggesting that they are all involved in a signalling pathway that includes Lyn (Nishizumi, Taniuchi et al. 1995). Since the expression of both isoforms of Lyn is ablated in these animals the specific functions of each one cannot be evaluated from these experiments.

7.2 LDN transgenic mice

It is possible that the LDN mice may have exhibited a phenotype similar to the Lyn⁻/⁻ mice, had the transgene been expressed. The possibility that the transgene was transcribed but failed to be translated in the three lines of LDN transgenic mice cannot be excluded. This could have been tested by analysing mRNA from the lymphoid tissues of the mice by Northern blot. However the aims of the project were thought to be better served by accelerating the efforts to achieve expression of both the mutant and wild type p56lyn cDNAs in WEHI-231 cells, and so this was the course of action taken.

7.2.1 Factors affecting expression in transgenic mice

Many factors affect the expression of exogenous DNA introduced into the mouse germline. The first of these is the design of the constructs used to drive expression. One possibility for the lack of expression seen
in the three lines of transgenic mice is that the constructs are simply not capable of driving expression. This seems unlikely, however, since both the E\(_\mu\) enhancer and the \(\beta\)-globin promoter have been used previously in transgenic systems with success (Reik, Williams et al. 1987; Brinster, Allen et al. 1988).

The integrity of the constructs was rigorously tested both before and after the generation of the mice. After building the transgenic vector, its structure was confirmed by restriction mapping, and the p56\(^{K275M}\) Lyn cDNA sequenced in its entirety from upstream of the MluI site to 3' of the BamHI site (data not shown). After integration into the mouse genome, the Southern blot analysis shown in Chapter Four demonstrates that the construct has integrated in all three LDN lines without rearrangement or deletion. Furthermore, the mutant and wild type Lyn cDNAs, when subcloned into pcDNA3 and expressed in COS-1 cells, gave rise to proteins of the correct size, demonstrating their integrity. These results demonstrate that the transgene should be capable of expression.

Another factor, and a possible reason for the lack of expression demonstrated here, is the effect of the position in the genome at which the transgene integrates. If the transgene integrates into a heterochromatic region, for example, it is unlikely to be expressed. Likewise position effects can be caused by regulatory elements such as enhancers or silencers in the vicinity of transgene exerting their influence on its expression, and explain why transgenes are not usually
expressed in a copy-number dependent manner. Since the integration of transgenes appears to occur randomly throughout the genome and cannot be controlled by the experimenter, it is possible to generate high-copy number transgenic mice that lack expression. It is possible that position effects could account for the lack of expression in all three lines of LDN mice.

As an illustration of this argument, Strasser et al. have conducted an analysis of the efficacy of two different constructs to overexpress Bcl-2 in lymphoid cells of transgenic mice (Strasser, Harris et al. 1990). The two constructs both contained the E\(\mu\) enhancer to direct expression to the B cell lineage. The first construct, designated E\(\mu\)SV, uses the SV40 promoter, splice site and polyadenylation signal and has been shown to work previously (Hariharan, Harris et al. 1989; Rosenbaum, Webb et al. 1989). The second vector consisted of a functionally rearranged Ig \(\mu\) gene with the Bcl-2 cDNA inserted between the \(V_H\) promoter and the downstream coding sequence. Of the 28 founder mice generated, 18 were bred to wild type animals and of these, 17 transmitted the transgene: 7 were deemed to be mosaic on the basis that significantly less than 50% of their progeny inherited the transgene, 8 transmitted the transgene with a frequency of around 50%, and two gave rise to two sublines each, demonstrating integration of the transgene at two unlinked positions in the genome in each case. The mice were then scored for expression: 8 harbouring the E\(\mu\)SV construct and 16 the E\(\mu\)lg construct. Of the eight E\(\mu\)SV mice, three did not express the transgene,
three expressed it exclusively in the B lineage, one exclusively in the T lineage and one in both B and T cells. Four of the EjIg mice did not express the transgene. Thus for two different constructs, 25-37% of the mice did not express the transgene, presumably because of position effects. This is comparable to my data, in which 3 out of 7 founder mice gave rise to stable lines, with one of these founders, LDN33, probably being mosaic. It also suggests that the lack of expression seen in the three lines of LDN mice could be the result of position effects.

7.2.2 Locus Control Regions

Regulatory elements capable of controlling the temporal and tissue-specific expression of a gene, and that also confer copy-number dependent, position-independent levels of transgene expression, have recently been defined. These sequences, termed locus control regions (LCRs), act as long-range, cis-acting mediators of transcription by controlling chromatin structure and thus the availability of enhancers and promoters to transcription factors and RNA polymerase (Dillon and Grosveld 1994). It is thought that each gene has its own LCR controlling its expression, in much the same way as enhancers act on individual genes. When used in transgenic constructs, the pattern of transgene expression directly mirrors that of the gene from which the LCR was isolated (Grosveld, van Assendelft et al. 1987). Apart from allowing expression only at the correct time and in the correct tissue, when used in transgenic constructs, LCRs are thought to buffer the transgene from any regulatory elements that it may find itself near to on integration. When the Lyn transgenic constructs were built, there
were no reports of a B cell specific LCR in the literature, and so the design of the Lyn transgene incorporated the best available reagents at that time. Subsequently an LCR that operates in B cells has been documented, and if the experiments presented here were to be repeated, this LCR would be included in the constructs to give rise to position-independent, copy-number dependent transcription of the transgene (Madison and Groudine 1994).

7.3 WEHI-231 Transfectants

Three different sets of transfectants have been generated, each with either the wild type or mutant p56 lyn. The first, the WLW/WLD transfectants, used the pBIS vectors, the second, the pcDNA3-based vectors, and the third set the epitope-tagged p56 lyn cDNAs, again in the pBIS vectors. Chapter Six shows that none of the clones tested showed detectable expression. Like transgenic mice, stable transfectants are also subject to position effects, although it seems unlikely that position effects are the cause of the lack of expression in this case, given that 120 different clones were analysed.

Again, as in the transgenic experiments, the entire wild type and mutant p56 lyn cDNA sequences in the expression vectors were confirmed, as were the restriction maps of the plasmids in all three sets of transfection experiments. Likewise after integration, the clones were analysed by Southern blot analysis and the majority of DNA positive transfectants were shown in Chapter Six to have integrated the
constructs in an unrearranged fashion and without deleting any segments of the expression cassette.

The possibility that the lack of apparent expression is that overexpression of p56\textsuperscript{lyn} causes either downregulation of the endogenous p56\textsuperscript{lyn} or upregulation of p53\textsuperscript{lyn}, such that both isoforms are always expressed in approximately equal amounts, was investigated using the epitope-tag strategy. Immunoblotting with the 12CA5 antibody specific for the epitope showed no detectable expression. This finding does not show whether or not the expression of the two Lyn isoforms is regulated as suggested above, but it does confirm the previous conclusion that there was no expression in the WLW/D clones.

There are a variety of reasons to explain why all three sets of transfectants failed to overexpress p56\textsuperscript{lyn}. The first, and perhaps most obvious, is that the vectors are not capable of expression. This seems unlikely, since the pcDNA3 vector gives good p56\textsuperscript{lyn} expression in COS-1 cells and is capable of driving expression in lymphoid cell lines (Salmeron, Ahmad et al. 1996). It has also been used to express proteins transiently in WEHI-231 cells (M. Choi, personal communication).

7.3.1 Potential toxicity of overexpressed p56\textsuperscript{lyn}

A second explanation is that overexpression of p56\textsuperscript{lyn} is toxic to the cells, meaning that only those clones not expressing the exogenous Lyn survive, whilst the expressing cells die before they can clonally expand.
Since Lyn is important for signalling through the BCR as demonstrated by the Lyn\textsuperscript{-/-} mutant mice, it may be that its overexpression causes the cells to become exquisitely sensitive to apoptosis, the response of these cells to BCR ligation, thereby removing them from the pool of transfectants. If toxicity is the case, it would necessarily point to a kinase independent function of p56\textsuperscript{lyn}, since transfectants harbouring both the wild type and the mutant p56\textsuperscript{lyn} failed to express. This potential kinase-independent activity, mediated via the unique, SH3 or SH2 domains, could enable exogenous p56\textsuperscript{lyn} to act as a dominant activator of a signalling pathway that leads to growth arrest or apoptosis. Since both the wild type and the mutant p56\textsuperscript{lyn} contain functional unique, SH3 and SH2 domains, this would explain the same phenotype in both the wild type and mutant transfectants. Such putative kinase independence for at least some functions would not be unprecedented, since some workers have defined a kinase independent function for Lck (Xu and Littman 1993).

Toxicity is a plausible explanation as it has been observed in the overexpression of another tyrosine kinase, Abl. Attempts to generate stably transfected fibroblasts that overexpress this protein have proved fruitless, as after selection, the expanding clones all consist of cells that have downregulated expression of Abl. Further experimentation demonstrated that this is because Abl is a negative regulator of cell growth (Sawyers, McLaughlin et al. 1994). Interestingly, mutations in either the Abl SH2 domain or the kinase domain allow stably
expressing transfectants to develop, suggesting that both domains are necessary for the growth inhibitory effect in this case.

7.4 Further experiments

The potential toxicity of p56\textsuperscript{lyn} could be assessed by carrying out a toxicity assay. One way of doing this would be to transfect WEHI-231 cells with pBIS\textsuperscript{lyn}, pBISK275M and pBIS, select the stable transfectants and compare the numbers of clones established with each construct. This can be expanded to include transfections with the plasmids digested both at a site irrelevant for p56\textsuperscript{lyn} expression, such as the ampicillin resistance gene, or at a unique restriction site in the cDNA itself. Any difference in the number of colonies generated by cells transfected with vectors capable of p56\textsuperscript{lyn} expression and those without (such as pBIS, as well as pBIS\textsuperscript{lyn} and pBISK275M digested within the cDNA) would be indicative of counterselection against p56\textsuperscript{lyn} expressing clones. In support of this strategy, one of the factors in determining the cell-growth related function of Abl was that in transfection experiments, 10-fold fewer colonies developed in cells transfected with wild type Abl compared to those transfected with Abl mutants (Sawyers, McLaughlin et al. 1994). Since in the transfection experiments shown in this thesis I routinely carried out fewer empty vector control than p56\textsuperscript{lyn} transfections, I am unable to compare their relative transfection efficiencies and thus to come to any conclusions about the potential toxicity of p56\textsuperscript{lyn}. 

152
Apart from a toxicity assay, transient expression of p56\(^{\text{lyn}}\) in WEHI-231 could provide a method by which to bypass the inability to clone overexpressing stable transfectants. Transient transfection would appear, from the data presented in this thesis, to be perhaps the only way to overexpress p56\(^{\text{lyn}}\) in WEHI-231 cells. Whilst only a fraction of cells in a given population will take up the exogenous DNA in a transfection experiment, the transient transfectants could be enriched for by cotransfecting with a protein whose expression enabled the cells to be distinguished from non-transfectants. I began experiments of this nature, cotransfecting WEHI-231 cells with the p56\(^{\text{lyn}}\) constructs and either CD4 or Green Fluorescent Protein (GFP) expression vectors. CD4 or GFP expression would allow transfected cells to be sorted by flow cytometry. However time constraints prevented these experiments from being completed.

A further option would be to use a different cell line to make stable transfectants, as the problem with expression may be specific to WEHI-231 cells. The lymphoma line Bal-17, for example, has characteristics of mature B cells and does not apoptose in response to BCR ligation. Again, time constraints prevented this from being attempted for the work presented here.

7.5 Summary

After generating a kinase defective mutant of p56\(^{\text{lyn}}\) I attempted to study the role of p56\(^{\text{lyn}}\) in BCR signalling, both in the context of the whole immune system by creating lines of transgenic mice, and by
conducting biochemical analyses in stably transfected B cells. However both of these strategies proved unfruitful due to the lack of p56\textsuperscript{lyn} overexpression. In this chapter I have proposed some possible explanations for this, as well presented some possible further experiments to explain my data and to pursue the study of Lyn in B cell signalling.
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