Molecular Investigations into Btk and WASP

Lucy MacCarthy-Morrogh

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Molecular Immunology Unit
Institute of Child Health
University College
University of London
Abstract

Bruton's tyrosine kinase (Btk) is a modular non-receptor protein tyrosine kinase which when mutated results in the immunodeficiency X-linked agammaglobulinemia (XLA) in man and xid in mice. The disease is characterised by a block in the development of B cells and as a consequence, levels of serum immunoglobulin of all isotypes are reduced. Btk is therefore crucial for B cell development. It is also implicated in the transduction of signals in both developing and mature B cells.

In humans, Btk has only been reported to be stimulated on ligation of the B cell receptor (BCR), and investigations in this thesis were performed in order to ascertain whether Btk activation is induced upon ligation of the cell surface receptors CD22 and CD38, as might be expected due to the nature of the XLA phenotype.

The modular nature of the Btk protein led to investigations into ligands of these domains in order to determine possible in vitro ligands of Btk. This thesis presents studies of the SH3 domains of Btk, Itk and Tec, expressed as GST fusion proteins. The SH3 domains of all three proteins were seen to bind a similar set of proteins in B cell lysates including the proteins previously identified as Btk SH3 domain in vitro ligands, WASP and c-Cbl. Auto-phosphorylation of the Btk SH3 domain fusion protein led to altered ligand binding. A tyrosine phosphorylated protein observed to bind the phosphorylated but not the unphosphorylated Btk SH3 domain fusion protein was identified as another protein tyrosine kinase, Syk.

The identification of WASP, mutated in patients suffering from another X-linked immunodeficiency, the Wiskott-Aldrich Syndrome (WAS), as an in vitro ligand of Btk, led to investigations into the expression of WASP in WAS patients. Results show that no patients with severe WAS in the study expressed WASP at detectable levels, irrespective of the nature or position of the mutation characterised in these patients.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>αPy</td>
<td>anti-phosphotyrosine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cADPR</td>
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<td>ψL</td>
<td>pseudo/ surrogate light chain</td>
</tr>
</tbody>
</table>
Publications


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Abstracts


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Chapter 1

1 Introduction

The immune system of man has evolved to protect the host from invading pathogens in the form of bacteria, viruses and parasites. It is composed of numerous cell lineages derived from pluripotent haematopoietic stem cells which start to differentiate in the developing embryo and continue to do so throughout adulthood. The resulting distinct cell populations have specific functions, but must interact with each other in a co-ordinated manner to maintain a functioning immune system. The immune system comprises an innate non-specific response, and an adaptive response, comprising specific humoral and cellular responses mediated by lymphoid cells.

Lymphoid cells, such as T and B cells, are the antigen specific cells of the immune system which recognise and respond to potential pathogens via the T cell and B cell antigen receptors (TCR and BCR), respectively. Rearrangement and somatic mutation within the genes coding for the antigen binding domains of these receptors generates highly selective antigen recognition. The signals arising from antigen binding lead to cell activation and development, deletion of cells or anergy, depending the developmental stage of the cell and the nature of the antigen.

The responses to antigen binding of the BCR are mediated by numerous intracellular signalling molecules, many of which are shared with T cells. The interactions that occur between these molecules form a signalling cascade that results in nuclear events.
Insight into the immune system has been greatly enhanced by genetic information from immunodeficiencies caused by single gene defects. Such disorders, along with laboratory created single gene 'knock-out' mice, have helped identify crucial components in the immune system of man and mouse. The defective proteins causing the human primary immunodeficiencies, X-linked agammaglobulinemia (XLA), and the Wiskott-Aldrich syndrome (WAS), were identified in the 1990s following the cloning of the disease genes (Tsukada et al., 1993; Vetrie et al., 1993; Derry et al., 1994a).

XLA is caused by mutations in Bruton’s Tyrosine kinase (Btk), a protein expressed in B and myelomonocytic cell lineages (Tsukada et al., 1993; Vetrie et al., 1993). Mutations in the BTK gene lead to a lack of mature antibody producing B cells in XLA patients, thus implicating Btk as an important molecule essential for B cell development. Btk also plays a role in B cell signalling following recognition and binding of antigen by the BCR (de Weers et al., 1994; Hinshelwood et al., 1995). Studies on the function and activity of Btk should therefore help unravel the sequence of events that lead to the development, and activation of B cells.

WAS results from mutations in another X-linked gene which encodes a protein called WASP (Wiskott-Aldrich syndrome protein). This protein is expressed in all haematopoietic lineages investigated, including the B lineage. WAS patient T, B and megakaryocytic lineages are particularly affected by the disorder. The study of WAS lymphoid cells and the interactions of WASP with other cellular components may provide information on the function of this protein.
1.1 B cell development

Self renewing pluripotential stem cells in bone marrow are believed to develop into both lymphoid and myeloid cell lineages. Commitment to the lymphoid cell lineage is characterised by expression of the Ikaros transcription factor (Georgopoulos et al., 1994). Subsequently a series of committed developmental steps is initiated which finally produce mature B cells capable of foreign antigen recognition and processing, and antibody production (Fig. 1.1).

Throughout life, large numbers of surface immunoglobulin (slg) positive immature B cells are generated from a small number of committed progenitors. Each B cell makes only one type of antibody and excludes the other Ig allele. Functional B cells are selected to enter the long lived mature pools of the periphery. During the development of B cells and on their entry into the periphery, their antigen receptors are screened for recognition of self antigens. Autoreactive B cells are either eliminated by programmed cell death or rendered non-responsive by a mechanism called anergy.

1.1.1 Antigen independent development

The early stages in B cell development from the pluripotent stem cell occur in the bone marrow. These antigen independent stages involve the sequential rearrangement of germline gene segments which code for the variable regions of the BCR to generate receptors with distinct antigen specificities.

The BCR consists of two identical membrane spanning heavy chains (HC), plus two identical extracellular light chains (LC). Both HC and LC contain both variable (V) and constant (C) regions (Fig. 1.2). Antigen is recognised and bound by the variable regions encoded by sets of $V_{H}$, $D_{H}$, $J_{H}$
Figure 1.1 The B lymphocyte development pathway. (Based on Roth et al., 1996). A Cells in transition between pro B and pre B I generate a functional heavy chain, \( \mu \), which is expressed at the pre B I stage, where it complexes with the pseudo light chain proteins Vpre B and \( \lambda 5 \) to form the pre-BCR. Developmental progression to the pre-B II stage requires the prior assembly of the pre-BCR complex, and these pre-B II cells undergo Ig light chain gene rearrangements. Successful completion of the rearrangements allows progression to the immature B cell stage, where \( \kappa \) (or \( \lambda \)) light chains associate with \( \mu \) in place of VpreB and \( \lambda 5 \). This Ig heavy chain and light chain, along with Ig\( \alpha \) and Ig\( \beta \), form the BCR on mature B cells, which also express IgD. An encounter with receptor specific antigen in the periphery results in differentiation into an antibody secreting plasma cell. B human B cell surface markers and Btk expression throughout B cell development are shown.
variable (V), diversity (D), and joining (J) gene segments of the HC), and VL and JL LC gene segments. The constant region of the H chain determines the Ig isotype, and each isotype has a distinct function within the humoral immune system (Melchers et al., 1995; Stavnezer, 1996).

An early event in the development of B cells involves the production of the isotype determining \( \mu \) HC by rearrangement of the HC gene segments which occurs in cells termed pro-B cells (Fig. 1.1). In order for the subsequent stages in development to proceed, successfully rearranged heavy chains must be expressed. Many rearrangements result in non-functional HCs and these cells are selected against. This is accomplished by association of the HC with proteins which assemble to form the surrogate/pseudo light chain (\( \psi L \)) (Sakaguchi and Melchers, 1986; Kudo et al., 1987; Pillai and Baltimore, 1987), and the associated molecules Ig\( \alpha \), and Ig\( \beta \) (Iglesias et al., 1993; Lassoued et al., 1993; Karasuyama et al., 1994). This group of proteins is referred to as the pre-BCR complex (Fig. 1.2). The pre-BCR has a number of properties including associated tyrosine kinase activity (Brouns et al., 1993), suggesting that pre-BCR signalling may be indicative of the presence of a functionally rearranged HC. Tyrosine kinase activity also requires the presence of an intracellular amino acid motif found on the associated proteins Ig\( \alpha \) and Ig\( \beta \) called the immunoreceptor tyrosine based activation motif (ITAM) to signal allelic exclusion and proliferation (Papavasiliou et al., 1995). On passing the pre-BCR expressing checkpoint, B cells are induced to proliferate, and the resulting sizeable population undergoes light chain rearrangements (pre-B I cells, Fig. 1.1). The pre-BCR is also expressed in conjunction with the fully rearranged light chain containing BCR. Developmental stages beyond this point are no longer dependent on the micoreenvironment provided by the stromal cells of the bone marrow, and cells leave for primary lymphoid organs, where antigen dependent development proceeds (Melchers et al., 1995).
Figure 1.2 Structure of the BCR and the pre-BCR complexes. Boxes represent individual immunoglobulin domains. Abbreviations: V=variable, C=constant, H=heavy, L=light, \(\psi L\)= pseudo/surrogate light chain.
1.1.2 Antigen dependent development

Immature B cells are characterised by expression of rearranged light chains associated with heavy chains as sIgM, but they are short-lived cells and do not yet express mature cell determinants (Fig. 1.1). Immature B cells do not yet proliferate or mature into Ig secreting cells upon their exposure to antigen (MacLennan and Chan, 1993). Exposure to self antigen results in the light chain variable genes undergoing further recombination events resulting in their binding specificity being altered so as to no longer recognise self antigens (Rolink et al., 1993). If cells fail to decrease the affinity for self antigens by this process, antigen binding to the BCR results in an arrest in differentiation and the cells are deleted (Melchers et al., 1995).

Mature B cells are found in peripheral lymph nodes and express both IgM and IgD on their cell surfaces. Their differentiation from immature cells is characterised by the expression of certain surface marker molecules, shown in Fig. 1.1. Receptor ligation with self antigen results in anergy or programmed cell death (Melchers et al., 1995), whereas ligation with non-self antigen results in cell proliferation and maturation into antibody producing plasma cells and memory cells (Forster and Rajewsky, 1990; MacLennan and Chan, 1993).

B cell activation results from BCR ligation with both T cell independent and T cell dependent antigens. B cells will develop into antibody secreting plasma cells on ligation with the former without a requirement for the presence of T cells. Somatic mutation of Ig genes is rare in this process and no memory cells are produced (MacLennan and Chan, 1993). T cell dependent antigen activation of B cells however requires the presence of co-stimulatory factors provided by adjacent T cells, to which B cells present antigen via MHC classII:TCR/CD4 interactions (Cooke et al., 1994). Stimulation of the BCR by T dependent antigen results in the production and release of interleukins resulting in B cell proliferation, isotype switching, and the production of
terminally differentiated antibody producing plasma cells and memory cells (Melchers et al., 1995).

Mature B cells express sIgM and sIgD with the same antigen binding specificity on the same cell. Stimulation through these Ig isotypes leads to different phosphorylation and dephosphorylation kinetics in signal transduction pathways (Gaur et al., 1993; Kim and Reth, 1995). The different properties of these signalling cascades are at present unresolved.

1.2 X-Linked Agammaglobulinemia (XLA)

1.2.1 XLA-The disease

XLA is an X-linked recessive disorder occurring in ~1/150,000 live births. The disease was first described as an immune disorder of boys who had no detectable levels of immunoglobulin of any isotypes, and who, as a result of this, suffered from recurrent bacterial infections (Bruton, 1952).

The lack of antibodies of any isotype in affected boys is due to a drastic reduction in the number of circulating B lymphocytes. The bone marrow of XLA patients contains normal or reduced numbers of pre-B cells (Pearl et al., 1978; Conley, 1985; Campana et al., 1990), indicating that the XLA defect does not prevent entry of bone marrow progenitors into the B cell lineage but does seem to result in some block in the differentiation of pro- or pre-B cells into mature B cells and plasma cells. Furthermore, XLA pre-B cells tested have an impaired proliferative response (Campana et al., 1990). The XLA defect was thought to be intrinsic to the B lineage, as these were apparently the only cells affected in XLA patients. Studies on carrier women showed that only mature B cells exhibit an apparent non-random pattern of X chromosome inactivation, presumably due to selection against cells where an X chromosome containing the XLA defective allele is active (Conley et al., 1986; Fearon et al., 1987).
As a consequence of the lack of antibodies and antibody producing cells, boys with XLA suffer from recurrent bacterial infections particularly in the upper and lower respiratory tract. Patients are also susceptible to protozoal infections, chronic enteroviral infections, and in a certain percentage of patients rheumatoid arthritis-like symptoms and neutropenia are observed (Farrar et al., 1996). Without treatment with intravenous immunoglobulin, disease is invariably fatal.

1.2.2 The genetics of XLA

XLA is a single gene disorder that was first mapped to the Xq21.3-Xq22 region on the long arm of the X-chromosome (Kwan et al., 1986) and its localisation was further refined by mapping studies to lie within Xq22 (Lovering et al., 1993). The causative gene for XLA was identified simultaneously by two groups using two quite different methods (Tsukada et al., 1993; Vetrie et al., 1993) discussed in the following section.

1.2.2.1 The Btk gene is defective in XLA

In 1993 Vetrie and colleagues screened a yeast artificial chromosome containing the XLA linked marker DXS178 and surrounding DNA, which led to the isolation of a gene expressed in B cells that was mutated in eight XLA patients, suggesting defects in this gene are the cause of XLA. Sequence analysis of the isolated gene showed it to encode a protein tyrosine kinase (PTK) with similarities to the Src family of PTKs (Vetrie et al., 1993)(section 1.7.1).

Another group interested in isolating genes involved in mouse B cell development (Tsukada et al., 1993), isolated a nearly full length cDNA clone of a gene expressed in murine haematopoietic tissues and cell lines of B but not T lineages, which is tightly linked to a region homologous to the Xq22 region in human. Furthermore, its product was predicted to be a tyrosine kinase, which was found not to be expressed in XLA patient cell lines, and was identical to
the gene identified by positional cloning and reported by Vetrie et al 1993. The gene product, which was found to be mutated in some XLA patients, was named Bruton's tyrosine kinase (Btk) and the gene termed BTK.

The human BTK gene has a length of 37.5 Kb (Tsukada et al., 1993; Vetrie et al., 1993) and contains 19 exons. The encoded Btk protein is a modular protein tyrosine kinase comprising four domains defined by their homology to other proteins including an N-terminal region unique to Btk and several other related PTKs (Fig. 1.3). To date over 175 mutations have been reported from 282 patients characterised for mutations in the BTK gene (Vihinen et al., 1996a). The XLA phenotype can result from mutations affecting all domains of the protein, the mutation frequency being approximately proportional to the length of the domain (Vihinen et al., 1996a). Missense mutations are the most commonly found, however nonsense and splice site mutations, along with insertions, deletions and frameshifts have also been reported (Vihinen et al., 1996a).

No correlations have been observed between the severity of the XLA phenotype and the nature of the mutations. Indeed, varying phenotypes have been observed in different patients that have identical mutations (Vihinen et al., 1996b). These patients may even be members of the same family (Sideras and Smith, 1995) and the phenotypic variation must therefore be explained by other genetic or non-genetic effects, not the specific mutation in the BTK gene. Protein modelling has been used previously to predict changes arising in Btk due to specific mutations which may explain the XLA phenotype (Vihinen et al., 1994b; Sideras and Smith, 1995). Recent studies on XLA patients however, have shown that in the majority of patients the Btk protein is not found expressed at detectable levels irrespective of the nature and position of the mutation (Hashimoto et al., 1996; Gaspar et al., 1998), indicating that the XLA phenotype usually arises due to a lack of Btk expression.
1.2.2.2 The xid mouse has a mutation in the BTK gene

The CBA/N mouse strain or xid mouse, bred in the early 1970s, makes abnormal immune responses to polysaccharide antigens (Amsbaugh et al., 1972; Scher et al., 1973). The pattern of inheritance of the phenotype is consistent with it being caused by an X-linked recessive gene. The gene was termed *xid*, and in 1993 two groups showed that *BTK* maps to the *xid* region of the mouse X chromosome (Rawlings et al., 1993; Thomas et al., 1993). Not only did the *BTK* and *xid* loci co-segregate, but Btk in the xid mouse was found to be mutated at residue number 28 where an arginine was replaced by a cysteine, causing the *xid* phenotype. The xid mouse has therefore be seen as a model to study the function of Btk.

The xid and XLA phenotypes however are not identical; in the xid mouse, although the numbers of mature B cells in the periphery are reduced (Scher et al., 1975) and those found tend to be the IgM<sup>hi</sup>/IgD<sup>lo</sup> immature phenotype (Forrester et al., 1987), B cell numbers are substantial compared to XLA patients who have no B cells in the periphery. The block in B cell development seen in XLA is therefore only partial in the xid mouse. Also, xid mice are capable of responding to most T cell dependent antigens, although the humoral response to a subset of T cell independent antigens is absent. An important difference between XLA patients and xid mice is that Btk mutations in man result in no expression of Btk in haematopoietic cells in the majority of XLA patients studied (Hashimoto et al., 1996; Gaspar et al., 1998), whereas xid B cells express normal amounts of mutated Btk which is still capable of autophosphorylation *in vitro* (Thomas et al., 1993).

Xid mice fail to express the B-1 CD5<sup>+</sup> subset of peritoneal B cells (Hayakawa et al., 1986), which differ from conventional B cells in developmental origin, surface marker expression and antibody repertoire (Hardy et al., 1994). Furthermore, conventional xid B cells do not proliferate when triggered through their surface IgM (Rigley et al., 1989).
Assuming both XLA and xid result from mutations in only one gene, BTK, several explanations were proposed in order to explain the milder xid phenotype. (i) The partial activity of Btk in xid mice may be sufficient to overcome the severe block observed in XLA patients. (ii) Alternatively, mice may have a redundant signalling pathway in early B cell development, or (iii) the severity of the phenotype seen in XLA patients as compared to xid mice may be due to unknown genetic factors. The Btk knock-out mice however have the same phenotypes as the xid mouse (Kerner et al., 1995; Khan et al., 1995), indicating that the presence of active but mutated Btk is not responsible for a phenotype in xid mice that is milder than that of XLA patients where Btk is absent. Explanations (ii) and (iii) are therefore more likely than (i).

1.3 Btk

The three C-terminal protein domains of Btk are homologous to the domains first described in the Src family of PTKs (Lin and Justement, 1992; Vetrie et al., 1993). Protein data bank searches (Haslam et al., 1993; Mayer et al., 1993) have found the N-terminus to comprise a pleckstrin homology (PH) domain, and a region unique to Btk and related proteins, termed the Tec homology (TH) domain. Fig.1.3 shows the structure of the Btk protein.

![Figure 1.3 The domain structure of Btk. The approximate number of amino acids in each domain, and the whole protein are indicated. PH=pleckstrin homology, SH=Src homology, TH=Tec homology domains]

1.3.1 The Btk family of PTKs

Btk shares a high degree of homology with several other non-receptor PTKs, and together they form the Btk (also called Tec) family of PTKs.
1.3.1.1 Structure and expression of the Btk family of PTKs

The Btk family consists of at least five different tyrosine kinases with high homology and one, Txk which is more distinct. Itk, Tec, BMX and DrSrc28C all have a structure similar to Btk; they possess SH (Src homology)1, SH2, SH3, TH, and PH domains. All but DrSrc28C, which is a Drosophila protein (Wadsworth et al., 1990), are found in mammalian tissues and are expressed in variable subsets of cell lineages, see Table 1.1.

Btk is found in most haematopoietic cells but not in cells of the T lineage or plasma cells (Genevier et al., 1994; Smith et al., 1994a). Itk was identified in human T cell lines (Tanaka et al., 1993) as an IL-2 induced PTK (Siliciano et al., 1992; Gibson et al., 1993; Tanaka et al., 1993) with its expression restricted to the T and NK lineages (Tanaka et al., 1993). Tec, which was originally cloned from mouse liver (Sato et al., 1994), and BMX, identified as a novel X-linked tyrosine kinase expressed in human bone marrow (Tamagnone et al., 1994), are found in most haematopoietic cells including both B and T cells (Sato et al., 1994; Tamagnone et al., 1994). Txk is expressed primarily in T cells, and although it has homology with Btk over its SH1, SH2 and SH3 domains, it lacks the unique N-terminal region including the PH domain (Haire et al., 1994). Btk, Itk and Tec all have kinase activity and appear to be important in cell signalling, being activated on cell stimulation through various receptors as summarised in Table 1.1, and discussed in the following section.
### Table 1.1 Members of the Btk family of PTKs (Rawlings and Witte, 1995).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol Weight</th>
<th>Cells in which protein expressed (mouse/man unless otherwise stated)</th>
<th>Activation Pathways (mouse unless otherwise stated)</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btk</td>
<td>77kD</td>
<td>B (except plasma) myeloid, erythroid, mast, megakaryocytic cells</td>
<td>IgM (mouse and man), IgD, FcεRI, IL-5R, IL-6R family, G protein βγ subunit, C₄α subunit, CD38</td>
<td>Human XLA Mouse xid</td>
</tr>
<tr>
<td>Itk</td>
<td>72kD</td>
<td>T, NK cells</td>
<td>CD28, CD3, CD2</td>
<td>Unknown</td>
</tr>
<tr>
<td>Tec</td>
<td>62kD</td>
<td>haematopoietic cells</td>
<td>IL-3, G-CSF, IL-6R family, SCF</td>
<td>Hepatocellular carcinoma?</td>
</tr>
<tr>
<td>DrSrc28C</td>
<td>66kD</td>
<td>Drosophila embryonic and oocyte cells</td>
<td>embryogenesis, oogenesis, metamorphosis</td>
<td>Unknown</td>
</tr>
<tr>
<td>BMX</td>
<td>80kD</td>
<td>haematopoietic cells</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Txk</td>
<td>61kD</td>
<td>T, myeloid cells</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

### 1.3.1.2 Function of the Btk family of PTKs

#### 1.3.1.2.1 Btk

In human B cell lines and primary tonsillar B cells, upon ligation of the BCR Btk is tyrosine phosphorylated, correlating with an increase in its kinase activity (de Weers et al., 1994; Hinshelwood et al., 1995) (section 1.8). As the expression of Btk is not restricted to mature BCR expressing B cells (see Table 1.1), it is not surprising that Btk was found to become activated on stimulation through various other cell surface receptors in murine cells, summarised in Table 1.1.

In mouse mast cells, stimulation through the FcεRI receptor results in degranulation and the release of allergic mediators (Stevens and Austen, 1989). Studies showed that on ligation of FcεRI on mouse mast cells Btk was phosphorylated on serine, threonine and tyrosine residues. Btk’s kinase activity was increased and a small proportion of Btk was translocated to the cell membrane (Kawakami et al., 1994). Agents known to activate protein kinase C
(PKC) and to induce Ca++ release from intracellular stores in mast cells, mimicking FceRI ligation, had no effect on Btk phosphorylation, suggesting that Btk plays a role upstream of PKC activation and Ca++ release, or functions on a different pathway (Kawakami et al., 1994).

Murine IL-5 plays an important role in the growth and differentiation of B cells and eosinophils. Btk's activity was increased on stimulation of the IL-5 receptor with IL-5 in a mouse IL-5 dependent early B cell line (Sato et al., 1994). Further evidence for the involvement of Btk in IL-5 signalling was provided by the finding that xid B cells show defective IL-5 signalling (Koike et al., 1995), and an activated mutant of Btk, called Btk*, relieves dependence of these cells on IL-5 (Li et al., 1995).

Xid B cells are also unresponsive to CD38 induced proliferation (Tsukada et al., 1994), and examination of Btk in lysates of CD38 stimulated mouse B cells showed it to be tyrosine phosphorylated (Kikuchi et al., 1995). In the same study it was proposed that the results of CD38 and IL-5 ligation were synergistic. The role of Btk in human CD38 signalling is discussed in Chapter 3.

There is evidence that Btk is important in the activation of phospholipase C (PLC), which results in PKC activation and the mobilisation of intracellular stores of calcium (DeFranco, 1997). In Btk deficient DT40 chicken B cells, the isoform of PLC found in these cells, PLCγ2, fails to become activated following BCR ligation, indicating the requirement for Btk function in the pathway leading to PLCγ activation (Takata and Kurosaki, 1996) (section 1.8.2.4).

As both the XLA and the xid phenotypes result in no, or decreased numbers of B cells past the pre-B cell stage (Hendriks et al., 1996), Btk may play a role in signalling through the pre-BCR, required for development beyond the pre-B cell stage (Melchers et al., 1995). Studies performed in a pre-B cell line showed that Btk may be constitutively active in these cells (Aoki et al., 1994).
Depending on the developmental stage of the B cell, or the specificity of
the antigen receptor, BCR ligation can lead to either cell activation, or
apoptosis. Apoptosis in B cell lymphomas can also be induced by radiation,
however the Btk deficient DT40 chicken B cell lymphoma does not undergo
radiation induced apoptosis, indicating a role for Btk in radiation induced
deletion of B cells. Furthermore, the Btk kinase domain has been shown to be
indispensable for the apoptotic response in this system (Uckun et al., 1996).
Studies in xid mice conversely, implicate a role for Btk in the protection of B
cells from apoptosis, as mutated Btk in xid B cells is correlated with both a
decrease in the levels of the apoptosis suppressing proto-oncogenes Bcl-X
(Anderson et al, 1996), and Bcl-2 (Woodland et al, 1996).

1.3.1.2.2 Btk and Tec

The IL-6 family of receptors share the gp130 subunit through which
signals are generated. Stimulation of gp130 by IL-6 plus soluble IL-6Ra results
in the activation of Btk as well as Tec, both of which were found associated
with gp130 in stimulated and resting cells (Matsuda et al., 1995). In the same
study it was shown that stimulation of a mouse pro-B cell line with IL-3 and
granulocyte colony stimulating factor (G-CSF) results in the activation of Tec
but not Btk. Another group reported contrary results in Mo7e cells (Tang et al.,
1994) where Tec activity was activated by stem cell factor (SCF) binding but not
on ligation of IL-3.

Stimulation of cells through the receptors described above results in the
tyrosine phosphorylation and activation of numerous other signalling
molecules besides Btk. For example, FceRI results in the phosphorylation of the
Src PTKs Lyn, Yes and Src as well as Syk and Btk (Benhamou et al., 1992;
Eiseman and Bolen, 1992; Kawakami et al., 1994), and IL-5R ligation results in
increased tyrosine phosphorylation of PI3-K, Shc, Vav, HS1 and Jak2, and an
increase in the activities of Jak2 and Btk (Sato et al., 1994). Presentation and
binding of antigen to the BCR results in the activation of many signalling
proteins including members of the Src and Syk families (section 1.8). As Btk has not been shown to associate with the receptors through which it is activated, (apart from the gp130 subunit), Btk activation may occur downstream in the signalling process, and is presumed to depend upon other cellular species also regulated by cell stimulation, apart from the ligated receptor itself.

1.3.1.2.3 Btk and Itk

As Itk and Btk are not expressed in the same lymphoid lineage, and as they have 53% amino acid identity, it has been proposed that they may perform analogous functions in B and T cells.

The interaction between Btk and Itk with the $\beta\gamma$ subunits of the heterotrimeric G protein results in the activation of both Btk and Itk due to a direct interaction between the G protein subunits and Btk or Itk, plus the involvement of some unidentified membrane associated factor (Langhans Rajasekaran et al., 1995). Heterotrimeric G proteins exist in all eukaryotic cells and transmit signals to various cellular and membrane effectors (Cantrell, 1994). On activation of G protein coupled receptors, the heterotrimer dissociates into an $\alpha$ subunit and a $\beta\gamma$ heterodimer, both of which have been shown to result in activation of the Btk kinase domain (Langhans Rajasekaran et al., 1995; Bence et al., 1997).

1.3.1.2.4 Itk

Itk has been implicated in CD28 signalling, which results in T cell activation (August et al., 1994; Liao et al., 1997). CD28 and Itk were found to associate in the T cell line Jurkat, and stimulation of CD28 induces tyrosine phosphorylation of Itk (August et al., 1994). Further studies using an Itk deficient cell line showed that CD28 signalling is in fact augmented in the absence of Itk, suggesting a role for Itk as a negative regulator of CD28 signalling. However CD3 mediated proliferation in Itk deficient cells is severely compromised (Liao et al., 1997).
Another T cell surface molecule that is responsible for important co-stimulatory signals is CD2 (Bierer et al., 1989). Stimulation through CD2 results in the tyrosine phosphorylation and activation of Itk, analysed in the T cell line Jurkat (King et al., 1996). Although Lck when activated through CD4 or CD5 does not phosphorylate Itk (King et al., 1996), CD2 mediated tyrosine phosphorylation of Itk is Lck dependent.

No disease has been associated with mutations in Itk to date, however an Itk deficient mouse has been made (Liao and Littman, 1995). These mice exhibit decreased numbers of mature thymocytes that showed defective proliferative responses to TCR crosslinking. Defective T cell proliferation on stimulation through the TCR in these mice is totally rescued by the addition of exogenous IL-2. This indicates that although Itk expression is induced by IL-2 signalling, Itk is not required for signalling through this cytokine receptor (Liao and Littman, 1995).

Itk, like Btk, therefore appears to be involved in more than one cellular response. Although it is not restricted to activation of the cell through any one particular receptor, the activation of Itk is not indiscriminate. The proposal that Btk and Itk play analogous roles in B and T cells, respectively, is strengthened by the fact that both are involved in the analogous signalling processes of BCR (section 1.8) and TCR signalling, that mice lacking Btk or Itk exhibit a block in the development of B or T cells, respectively, and show defective proliferative responses to BCR or TCR ligation, and that both may be involved as effectors of G protein mediated signalling. The exact roles played by Itk and Btk in these signalling processes have as yet to be elucidated.

1.4 Protein domains

Many proteins involved in cell signalling, like Btk, are composed of modular domains. The first to be described were those found in the Rous
sarcoma virus oncogene (Sadowski et al., 1986; Mayer et al., 1988; Koch et al., 1991), v-Src, of which a cellular homologue, c-Src also exists. These domains are referred to as the SH domains, SH1, SH2 and SH3, and they are found in all eukaryotes, except for SH2 domains which are not found in yeast. None have been identified in any prokaryotic proteins. All three SH domains are found in Btk; the SH1 domain comprises the catalytic region of the kinase and the SH2 and SH3 domains are responsible for protein:protein interactions involved in the regulation of Btk. The PH domain is also involved in Btk regulation and it interacts with other protein and lipid components of the signalling machinery.

Regulatory domains (not including SH1) are defined protein or lipid binding molecules which can maintain their tertiary structure in isolation. Structural studies have shown that their N and C-termini are in close apposition to each other on the far side of the domain to the ligand binding surface (Feng et al., 1994; Cohen et al., 1995), so the domain can be plugged into a whole protein without its modular structure being disrupted. The presence of these domains is not restricted to any particular type of signalling molecule and they are found in protein and lipid kinases, phosphatases, transcription factors and adaptor molecules.

The role of these domains as components of signalling proteins is to transduce controlled, and most probably weakly amplified signals to specific targets, as opposed to the generation of second messengers such as Ca²⁺ which flood a cell with information (Berridge, 1997). As many cell signalling proteins contain modular domains they can maintain signals in macromolecular form, rather than relying on the release of small molecules with a broad target. Many signalling proteins contain combinations of modular regulatory domains which may or may not include a catalytic domain. The following sections describe the structure and function of the domains found in Btk and other signalling proteins in greater detail.
1.4.1 The catalytic domain (SH1)

1.4.1.1. SH1: structure

Protein kinases are divided into two subfamilies according to their substrates: protein tyrosine (Tyr or Y) kinases and protein serine (ser)/threonine (thr) kinases. The kinase domains of these two subfamilies show overall sequence and structural similarity. Sequences consist of eleven conserved sub-domains, often separated by variable sequences (Hanks et al., 1988), and the overall structure of the catalytic domain comprises two major lobes. The smaller N-terminal lobe contains the ATP binding site, and the larger C-terminal lobe is important for substrate peptide binding, with the active catalytic site lying in a cleft between the two lobes. Both types of kinases, despite their structural similarity, tend to be highly specific for either tyrosine or ser/thr substrates, and this specificity arises due to particular differences in short amino acid stretches of the kinase domains, each subfamily having its own signature motif (Hanks et al., 1988).

The Btk C-terminus was recognised as a catalytic domain as sequence comparisons with known kinases showed it to contain an ATP binding site, a catalytic site, and a region called the activation loop important for regulation which contains a potential site of tyrosine phosphorylation (Vetrie et al., 1993). The Btk kinase domain contains the non-receptor tyrosine kinase specific motif, DLRAAN, within subdomain VI (appendix A) (Hanks et al., 1988). This motif comprises part of the catalytic loop and differs in sequence from that found in ser/thr kinases, although the conformation formed in both is similar (Hubbard et al., 1994). The catalytic cleft formed in tyrosine kinases such as Btk is designed to position the hydroxyl group of tyrosine to accept phosphate, and will preclude the phosphorylation of ser or thr as their smaller hydroxyl capped side chains do not penetrate deep enough to act as acceptors (Taylor et al., 1995).
1.4.1.2 SH1 function: specificity and regulation

The function of the SH1 domain is to phosphorylate proteins on tyrosine residues. However, in order to send a controlled signal, each catalytic domain must show some ligand specificity so as not to result in indiscriminate protein tyrosine phosphorylation by kinases on cell activation. Individual catalytic domains show substrate specificity which is critical for selective signalling. Nine catalytic domains tested (including members of the Src family of PTKs, but not Btk) (Zhou et al., 1995) have a unique optimal peptide substrate. The substrate specificity of the Btk catalytic domain has not been elucidated, and little is known of physiological Btk substrates. However a major role of the Btk kinase domain is known to be autophosphorylation which is discussed later (sections 1.7.3.1.2, and 1.8.2.3).

A model of how the catalytic domain of Btk is activated is based upon the X-ray crystallographic studies of the kinase domain of the human insulin receptor kinase (IRK) (Hubbard et al., 1994). The IRK structure was seen on crystallisation to resemble the two lobed structure of other catalytic domains investigated (Hubbard et al., 1994), including cyclin-dependent kinase 2 (De Bondt et al., 1993), and cyclic adenosine monophosphate-dependent protein kinase (Knighton et al., 1991). A region called the activation loop which is found in the C-terminal lobe plays an important role in the regulation of the insulin receptor kinase activity and the regulation of Btk’s kinase activity is believed to be controlled in a similar manner.

The inactive conformation of IRK is believed to change to an active conformation upon phosphorylation of a tyrosine residue (Y1162) that lies within the activation loop (Hubbard et al., 1994) (Fig. 1.4). In the inactive state, Y1162 is in such a position that its hydroxyl group lies in the active site of the catalytic domain. Although this tyrosine is in position for phosphotransfer with respect to the catalytic loop residues, the ATP binding site is blocked. Cis autophosphorylation of Y1162 is therefore not believed to take place (Hubbard
et al., 1994), and the position of the Y1162 hydroxyl group effectively inhibits the active site from binding another Tyr residue from a prospective substrate. This is referred to as pseudo-substrate inhibition. (Cobb et al., 1989; Shoelson et al., 1991; Frattali et al., 1992).

The model of activation (Hubbard et al., 1994) proposes that the insulin receptor kinase domain in a resting cell is in an equilibrium between two states, open and closed. The first and highly favoured state is open where Y1162 is unphosphorylated and lying in the active site, which along with the ATP binding site, is blocked (Fig. 1.4 (i)). The second, less favoured, closed state has Y1162 disengaged from the active site, and the ATP binding site and the active sites are both accessible. Insulin binding IRK causes a conformational change in the catalytic domain resulting in a shift to the closed conformation. This allows transphosphorylation of Y1162. Once phosphorylated, Y1162 is disengaged from the active site and the closed, active state is stabilised (Fig. 1.4 (ii)). Thus activation of the receptor on insulin binding results in the observed increase in kinase activity.

The Btk kinase domain contains a site of phosphorylation proposed to be the equivalent of Y1162, which lies at position 551 and is phosphorylated on stimulation of B cells through the BCR (Mahajan et al., 1995; Rawlings et al., 1996). This increase in phosphorylation coincides with an increase in Btk kinase activity, and it is proposed that the low levels of Btk kinase activity in resting cells is dependent on the presence of the unphosphorylated Y551 acting as a pseudo-substrate blocking the active site of Btk. Upon phosphorylation, it has been proposed that Y551 is released from the active site of the Btk catalytic
Figure 1.4 Cartoon representation of pseudo-substrate inhibition. Activation occurs on phosphorylation of Y1162 of IRK (equivalent to Btk Y551) within the activation loop.
domain resulting in the increase in kinase activity of Btk (Rawlings et al., 1996) (section 1.8.2.3).

1.4.2 The SH2 domain

SH2 domains were recognised as components of numerous proteins that bind auto-phosphorylated PTKs. SH2 domains alone were shown to be capable of binding tyrosine phosphorylated, but not unphosphorylated, PTKs (Mayer and Baltimore, 1993). This interaction has been further characterised, showing that although SH2 domains from many different proteins have a similar overall structure, and they all bind phosphotyrosine (pTyr), each SH2 domain (or family of SH2 domains) shows highly specific ligand binding with respect to the residues surrounding the phosphorylated tyrosine.

1.4.2.1 SH2: structure and specificity

Twenty two SH2 domains from various signalling proteins have been examined for their ligand binding specificity (Songyang et al., 1993; Songyang et al., 1994). Peptides from a degenerate library were selected by recombinant SH2 domains and the optimal sequences bound by each ascertained. The SH2 domains in these studies were arranged into groups according to the ligand sequences at positions +1 to +3 C-terminal of pTyr. The Btk SH2 domain is predicted to be in group I (Songyang et al., 1994) which includes the Src family SH2 domains, the ligand binding consensus of which is YEEI.

The Src SH2 domain in complex with a high affinity ligand is described as resembling a two pronged plug (the peptide) engaging a two holed socket (the SH2 domain) (Waksman et al., 1993). The two prongs of the plug are pTyr, and Ile at position +3 relative to pTyr. The pTyr side chain is accommodated in one deep binding pocket of the SH2 domain at the bottom of which lies an Arg residue which is conserved in all SH2 domains. The positively charged side chain of the Arg interacts with the negatively charged phosphate group on the
Tyr residue and the presence of this Arg residue is vital for SH2 domain binding (Mayer and Baltimore, 1993).

The specificity of Src SH2 binding YEEI is due to the accommodation of the Ile residue within the second pocket described in the ‘two holed socket’ model. This Ile binding pocket is lined with hydrophobic residues which practically bury the Ile side chain in the surface of the SH2 domain (Waksman et al., 1993).

1.4.2.2 SH2: function

SH2 domain binding may act as a transducer of cellular signals in two ways. It may be involved in regulation of catalytic activity via intramolecular SH2/ligand binding (section 1.7.3.2). Alternatively, the binding of a ligand to an SH2 domain can result in the subcellular relocalisation of a protein, bringing it closer to its substrate, or to another modifying protein.

For example, relocalisation of the guanine nuclear exchange factor (GEF) Sos to the cell membrane where it activates the GTPase Ras is due to an SH2 domain interaction. The SH2 domain of Grb2, an adaptor to which Sos is constitutively associated (Reif et al., 1994), binds to the epidermal growth factor receptor (EGFR) when it autophosphorylates on cell stimulation, thus bringing Sos to the membrane to which its substrate Ras is associated (Buday and Downward, 1993). As the activity of Sos remains the same in resting and activated cells, the activation of Ras by Sos is the result of the Grb-2 SH2 domain mediated relocation of Sos (Egan and Weinberg, 1993).

The binding specificity of a particular SH2 domain is essential for protein function. This is illustrated by experiments in which a specificity determining residue of the SH2 domain of Src was mutated to that found in the Grb2 SH2 domain involved in the Ras activation pathway. This mutant Src SH2 domain, but not wild type, effectively substituted for the usual Grb2 SH2 domain (Marengere et al., 1994), showing that one residue can modify SH2
selectivity, and that the biological activity of an SH2 domain correlates with its binding specificity.

Studies on the function of the Btk SH2 domain are limited by the fact that so far, no ligands have been identified. Mutations in the SH2 region of the BTK gene of XLA patients have been identified (Vihinen et al., 1996b), suggesting that, unless the mutation results in an unstable and therefore complete absence of protein, the SH2 domain is required for Btk function. Investigation into the expression of Btk in the EBV transformed cell line of a patient with an SH2 domain mutation has shown Btk expression (Genevier et al., 1994) supporting the suggestion that the SH2 domain is critical for Btk function. It has been determined that the SH2 domain plays a role in the regulation of a closely related protein, Itk (Andreotti et al., 1997), discussed in section 1.7.3.1.

1.4.3 The SH3 domain

SH3 domains are found in many proteins, often in combination with SH2 domains, and they too are involved in protein:protein interactions and the regulation of signal transduction. SH3 domains interact with proline rich regions in proteins that form a left handed polyproline type 2 (PPII) helix. Initial studies of two proteins found to bind the SH3 domain of Abl, localised the SH3 binding site within these proteins to a region of 10 amino acids rich in proline residues (Ren et al., 1993). Subsequent analysis of most known SH3 ligands show them to contain a core PXXP binding motif, which forms the PPII helix, essential for SH3 domain binding (Yu et al., 1994). Other residues in the ligand surrounding this motif are important for stabilising the PPII helix and for determining the specificity of SH3 domain binding, as not all ligands containing the PXXP motif will bind all SH3 domains.
1.4.3.1 SH3: structure

The crystal or solution structure of numerous SH3 domains have been reported, including Btk (Chen et al., 1996) and several with bound ligands (for review see Cohen et al., 1995). The solution structure of the Src SH3 domain ligand binding pocket showed it to contain a patch of conserved hydrophobic residues surrounded by two charged and variable loops, called the RT and n-src loops (Yu et al., 1992).

The nuclear magnetic resonance (NMR) determined three dimensional structure of the SH3 domain of phosphatidylinositol 3-kinase (PI3-K) alone, and in complex with a ligand, showed the ligand binding pocket differed little in its structure whether bound to its ligand or not (Yu et al., 1994), indicating that the binding pocket is a preformed template on the surface of the domain. The interaction between the PI3-K SH3 domain and its ligand was stabilised by hydrophobic interactions between the two prolines in the core PXXP motif forming the PPII helix in the ligand, and conserved residues of the SH3 domain. These residues form a hydrophobic patch with two pockets to accommodate the ligand prolines, and include the first Tyr residue of the characteristic ALDYDY motif of SH3 domains (Yu et al., 1994), (see appendix A).

1.4.3.2 SH3: specificity of binding

Detailed studies of the ligands of different SH3 domains have identified residues apart from the critical PXXP core binding motif that are crucial for the specificity of binding. Similarly, variable residues on SH3 domains are also crucial for specificity. X-ray crystallographic and NMR determined structures, plus mutation analysis, have determined interactions between these variable residues on both ligand and SH3 domains, explaining the specificity of binding observed.
Rickles et al (1994) showed by probing a biased peptide library with the SH3 domains of Src, Fyn, Lyn, PI3-K and Abl, that each one selected a peptide with a unique sequence. All sequences were rich in prolines and contained the PXXP motif, but in peptides of 12 amino acids, residues in positions flanking the core motif varied. Both Src and PI3-K SH3 domains selected ligands containing an RXL motif at the C-terminus of the peptide. These three residues interact extensively with residues within the SH3 domain. The conserved Asp-21 of PI3-K (Asp-99 in Src) which lies in the RT loop is critical for ligand binding, forming a salt bridge with the Arg of the RXL motif of the ligand, and is vital for binding ligands in one of two possible orientations. Interestingly, sequence alignments of SH3 domains show that only the Abl SH3 domain has no Asp or Glu residue equivalent to Src Asp-99 or PI3-K Asp-21, but instead it has a threonine. As the Abl SH3 domain specific ligands, 3BP1 and 3BP2, have a hydrophobic residue at the position corresponding to the R of Src and PI3-K ligands, this may explain why Abl binds so poorly to Src ligands and vice versa (Rickles et al., 1994; Yu et al., 1994).

Although the ligands of Src and PI3-K SH3 domains were similar in terms of the RXL motif, other differences in non-proline residues between high affinity peptides were observed. These residues contact more variable regions in the SH3 domain, especially the RT and n-Src loops that surround the hydrophobic binding patch. Mutational analysis of the non- or semi-conserved residues in these loops of Src showed that some residues were involved in SH3 domain interactions (Erpel et al., 1995). Further analysis of the solution structure of the Src SH3 domain showed that a pocket is formed between the n-Src and RT loops which binds sequences flanking the proline rich core motif (Feng et al., 1995). The conformation formed by the RT and n-Src loops differs from one SH3 domain to another, and different ligand binding affinities in SH3 domain:ligand complexes may be accounted for by different interactions involved in this specificity pocket (Yu et al., 1994; Feng et al., 1995).
Work on the interaction between the Nef protein of HIV-1 and the SH3 domain of the Src PTK Hck shows that this interaction, which is the strongest SH3 domain:ligand interaction so far reported, has an affinity determined not only by residues in and around the Nef PXXP motif, but also by interactions involving far regions of Nef (Lee et al., 1995; Saksela et al., 1995; Lee et al., 1996). Nef binds Hck SH3 with high specificity; it does not bind the SH3 domain of closely related proteins such as Fyn (Lee et al., 1995). This specificity is due to an Ile residue within the Hck RT-loop, as mutation of the equivalent residue in the Fyn SH3 domain to Ile (R96I) confers upon the Fyn ‘Hck like’ SH3 domain an ability to bind Nef with the same affinity as Hck SH3. Like the Hck SH3 domain, the high affinity binding of Fyn R96I to Nef required Nef sequences distant from the PXXP motif (Lee et al., 1996).

Thus the PXXP core forming the PPII helix serves as a common anchor used by SH3 binding peptides, while flanking regions allow more extensive contact at the intermolecular interface between ligand and domain providing increased affinity and specificity.

Src SH3 domains can bind ligands in both N to C and C to N orientations (Feng et al., 1994). The two orientations of ligand binding are referred to class I (N to C) or positive, and class II (C to N), or negative. Class I peptides have the consensus motif RXLPPXP. The underlined Ps represent the prolines of the PXXP motif, and the N-terminal Arg (bold) forms a salt bridge with Asp-99 of Src. The P residues are accommodated in two hydrophobic pockets formed by conserved amino acids in the SH3 domain as described above. Class II peptides have the consensus sequence, XPPLPXR. The C-terminal Arg (bold) forms a salt bridge with Src Asp-99, and determines the minus orientation of the bound ligand. The change in direction of binding results in the position of proline residues found in class I ligands being replaced by non-proline residues in the class II ligand, with respect to their position when bound to the SH3 domain. Binding of class II ligands in the minus orientation is possible however, as the crystal structure of a class II
peptide derived from Sos, bound to the SH3 domain of SEM-5 (Lim et al., 1994) shows the proline binding pockets formed by the conserved hydrophobic residues of the SH3 domain are flexible enough to accommodate the ligand, still in the form of the PPII helix, despite the change in the order of the proline and non-proline residues (Feng et al., 1994).

1.4.3.3 SH3: function

Deletion or mutation of the SH3 domain can result in proteins with transforming capabilities (e.g. Src (Hirai and Varmus, 1990; Seidel Dugan et al., 1992), and Abl (Jackson and Baltimore, 1989), and deletion of the SH3 domain from an activated mutant of Btk increases the transforming potential of this protein (Li et al., 1995), implying that the SH3 domain may function to negatively regulate proteins. Deletion of the SH3 domain from the C. elegans SEM-5 protein results in blocked vulval development (Eck et al., 1996), providing evidence for the role of SH3 domains in signalling. Also, studies on the GTPase dynamin, an SH3 domain binding protein, have shown that SH3 domain fusion proteins bound to purified dynamin results in an increase in the GTPase activity of dynamin (Gout et al., 1993).

SH3 domains, like SH2 domains, are also important in determining the cellular localisation of proteins. Many protein functions require their relocation from one compartment of the cell to another. SH3 domains are believed to be important for localising proteins to the cytoskeleton (Mayer and Baltimore, 1993). The SH3 domain of PLCγ is required for its targeting to cytoskeletal microfilaments, and both Grb-2 SH3 domains are required for its localisation to membrane ruffles (Bar Sagi et al., 1993). The constitutive interaction between the Grb-2 SH3 domain and Sos (Reif et al., 1994) is, along with the inducible interaction between the SH2 domain of Grb-2 and the EGFR receptor, required for the activation of Ras (Buday and Downward, 1993). SH3 domain containing proteins are also found abundantly in pathways involving other small GTP binding proteins (Mayer and Baltimore, 1993; Ye and Baltimore, 1994), in the
assembly of the NADPH oxidase system (Finan et al., 1994; Sumimoto et al., 1994), and in the localisation of ion channels in epithelial cells (Rotin et al., 1994).

1.4.4 Ligands of the Btk SH3 domain

Work performed in this laboratory has identified two ligands binding the SH3 domain of Btk. Using a GST fusion protein of the Btk SH3 domain (Btk GST-SH3), the product of the proto-oncogene c-Cbl (Cory et al. 1995), and the Wiskott-Aldrich Syndrome Protein (WASP) (Cory et al., 1996) were precipitated from the lysates of the mature B cell line Daudi.

1.4.4.1 c-Cbl

The cbl gene was first identified in a rearranged form as the oncogene of the Cas-Br-M murine leukemia virus, which causes pre-B cell lymphomas and myeloid leukemias in mice (Langdon et al., 1989). The c-Cbl cellular homologue amino terminal is the same as that of the v-Cbl oncogene product (residues 1-357). c-Cbl also contains protein motifs which include a proline rich region (residues 481-688), a leucine zipper motif (residues 481-C-terminus), a ring finger motif, and multiple SH2 domain binding motifs (Blake et al., 1991) (Fig. 1.5). The proline rich region is notable in containing several consensus SH3 binding motifs. Although c-Cbl contains a nuclear localisation motif, it is only the oncogenic v-Cbl that locates to the nucleus; c-Cbl is found in the cytoplasm (Blake et al., 1991). Fig 1.5 depicts the structure of c-Cbl.
c-Cbl has no known catalytic activity, but is tyrosine phosphorylated upon cross-linking of the BCR (Cory et al., 1995; Tezuka et al., 1996), the TCR (Donovan et al., 1994; Fukazawa et al., 1995) and a number of other receptors on haematopoietic and other cells, including FceRI on mast cells (Ota et al., 1996), GM-CSFR (Oda et al., 1995), FcγRI/II/III (Marcilla et al., 1995; Tanaka et al., 1995), EGFR (Oda et al., 1995) on various haematopoietic cells, and CD38 (Ota et al., 1996) on HL60 B cells. Furthermore, c-Cbl has been reported to be a substrate for several activated tyrosine kinases, including Lyn (Tezuka et al., 1996) in B cells, and Syk (Ota et al., 1996) in mast cells. It has been reported to bind the SH2 and SH3 domains of numerous signalling proteins in both B and T cells including several members of the Src family, (Donovan et al., 1994; Fukazawa et al., 1995; Marcilla et al., 1995; Tanaka et al., 1995; Fournel et al., 1996; Tanaka et al., 1996; Tezuka et al., 1996) the Syk family (Marcilla et al., 1995; Ota et al., 1996), and the Btk family of PTKs (Cory et al., 1995) PI3-K (Hartley et al., 1995; Meisner et al., 1995a), PLCγ (Donovan et al., 1994; Rivero Lezcano et al., 1994), and the adaptor proteins Grb-2 (Donovan et al., 1994; Fukazawa et al., 1995; Meisner and Czech, 1995b; Oda et al., 1995; Smit et al., 1996), Nck (Rivero Lezcano et al., 1994) and Crk (Ribon et al., 1996; Sawasdikosol et al., 1996; Smit et al., 1996). c-Cbl’s role appears to be that of a universal adaptor molecule responsible for the recruitment of signalling proteins.
A regulatory role for c-Cbl has been proposed due to its homology to the SLI-1 protein in *C. elegans*. SLI-1 is a regulator of vulval development and genetic evidence implies that it acts as a negative regulator of Ras activation (Yoon et al., 1995). In the mammalian activation of Ras on BCR crosslinking, c-Cbl may play a negative regulatory role as it competes with Sos PTK for Grb-2 binding (Meisner et al., 1995a) (see section 1.4.2.2).

Another potential regulatory role for c-Cbl in mammalian systems was shown in rat mast cells (Ota et al., 1996; Ota and Samelson, 1997), where c-Cbl co-precipitates with the PTK Syk. The main PTK responsible for c-Cbl tyrosine phosphorylation in mast cells was shown to be Syk. c-Cbl competes with FcεRI for Syk binding, and by doing so inhibits Syk activation as a result of FcεRI binding (Ota et al., 1996; Ota and Samelson, 1997). This results in a situation whereby the cellular phosphorylation of c-Cbl will be decreased as a result of its own action which could subsequently affect its role as a binding partner for other signalling proteins.

Investigations of c-Cbl in DT40 chicken B cells show results that vary from those observed in mast cells. The most prominent kinase resulting in c-Cbl phosphorylation in this system was Lyn; c-Cbl continued to become phosphorylated in the absence of Syk (Tezuka et al., 1996). Furthermore Lyn, but not Syk, co-precipitated with c-Cbl in these cells, the *in vivo* interaction providing more evidence for an enzyme:substrate relationship. In another system, stimulation of mature B cell lines including Daudi (Panchamoorthy et al., 1996) induced a prominent association of c-Cbl with Syk. This is contrary to the reported association in mast cells which was unaffected by cell stimulation through FcεRI. Interestingly, the amount of c-Cbl associated with the SH3 domain of Btk is seen to be decreased on cell stimulation (Cory et al., 1995), showing a reciprocal binding pattern to that seen on Syk binding.

Studies in another system show that c-Cbl is tyrosine phosphorylated in a Src dependent manner in mouse osteoclasts where Src and c-Cbl are also
observed to co-localise and co-immunoprecipitate (Tanaka et al., 1996). This function of Src cannot be compensated for by other members of the Src family, which is consistent with the osteoclast specific dysfunction that is observed in Src deficient mice. Thus c-Cbl appears to play a role in these tissues distinct from T, B and mast cells where other members of the Src family of PTKs are capable of its phosphorylation.

It would appear therefore that c-Cbl is a protein involved in many tyrosine phosphorylation dependent signalling pathways. Within various cell types it is a primary phosphorylated species following appropriate stimulation and it associates with different important signalling proteins. Contradictory evidence on the nature of c-Cbl interactions in different systems studied indicates that the role c-Cbl plays depends on the tissue or cell type under study.

1.4.4.2 WASP

Microsequencing of a species that precipitated with Btk GST-SH3 from Daudi cell lysates and migrated upon SDS-PAGE analysis with an apparent molecular weight of 65kD identified the protein as WASP (Cory et al., 1996). WASP is the protein encoded by the gene which when mutated causes the primary immunodeficiency WAS (Derry et al., 1994b) (see section 1.5).

WASP contains several interesting motifs illustrated in Fig.1.7 (section 1.5.1). Two regions within WASP, one towards the C-terminus and one towards the N-terminus (see Fig.1.7) are very rich in proline residues and include several PXXP motifs. These regions are believed to be responsible for the association of WASP with SH3 domains of proteins such as Btk, PLCγ, c-Src and Fgr (Banin et al., 1996; Cory et al., 1996; Finan et al., 1996). It is likely that individual SH3 domains interact with specific proline motifs within WASP (Finan et al, 1996) suggesting that WASP may act to recruit and co-localise several SH3 domain containing proteins.
The region of WASP that interacts with Btk GST-SH3 has not been mapped but investigations indicate that motifs within both N and C-terminal proline rich regions (PRR) may be involved in binding Btk GST-SH3. Peptides designed on WASP proline rich sequences within the PRR were used to compete with Btk GST-SH3 for WASP binding. Two peptides were most successful at inhibiting the Btk GST-SH3: WASP interaction: one was designed on a C-terminal PRR and the other on an N-terminal PRR (Cory et al., 1996).

Since the cloning of the WAS gene, WASP has been reported to interact with numerous signalling proteins, and these interactions plus the importance of WASP are discussed in section 1.5.

1.4.5 The TH domain

Between the PH domain and the SH3 domain of Btk lies a stretch of approximately 70 amino acids called the Tec homology (TH) domain. It comprises a region of about 25 amino acids next to the PH domain, followed by a proline rich stretch. The TH domain is conserved amongst Btk and closely related proteins, of which the first to be described was Tec, and the domain was thus named the Tec homology (TH) domain (Smith et al., 1994b; Tamagnone et al., 1994). The 25 amino acids which immediately follow the PH domain are found in two other molecules, a putative Ras GTPase activating protein (Rawlings et al., 1993), and an interferon γ binding protein (Vihinen et al., 1994a). This stretch is named the Btk motif to differentiate it from the region that includes the proline rich stretch (Vihinen et al., 1994a). X-ray crystallography studies of the Btk N-terminus show the Btk motif to form a novel fold held together by a zinc ion bound by three conserved cysteines and a histidine, which packs closely against the PH domain (Hyvonen and Saraste, 1997).

Btk has been found in association with the Src family of PTKs, Fyn, Lyn and Hck in vitro, via an interaction between their SH3 domains and the proline
rich region of the TH domain of Btk (Cheng et al., 1994; Alexandropoulos et al., 1995), although this region does not contain the optimal Src family SH3 binding site. Interestingly, the Src SH3 domain does not bind the Btk TH domain (Alexandropoulos et al., 1995) which indicates a different sequence specificity requirement in the ligands of this SH3 domain. As the Src family of PTKs, especially Lyn, are proposed to be involved in the phosphorylation of Btk, interactions between them and Btk add further evidence to this model of Btk activation (section 1.8.2.3). The TH domain is involved in an intramolecular interaction within Itk (Andreotti et al., 1997), which is proposed to be important for the regulation of Itk and other members of the same family of PTKs, discussed in 1.7.3.1.1.

1.4.6 The PH domain

The PH domain is another regulatory domain involved in interactions with other signalling molecules which was first recognised as a region of approximately one hundred amino acids repeated twice in pleckstrin (Haslam et al., 1993; Mayer et al., 1993), the major substrate of PKC in platelets. Databank searches subsequently identified PH domains in many other proteins including certain PLC isoforms, PI-3 kinase, regulators of monomeric G proteins, cytoskeletal proteins and adaptor proteins (Lemmon et al., 1996; Shaw, 1996). Approximately one hundred PH domains have been identified and it is of note that the classes of proteins containing PH domains closely parallel those containing SH2 and SH3 domains.

PH domains, like SH3 domains are found in all eukaryotes, including yeast, suggesting that primordial eukaryotic signalling molecules may have contained PH and SH3 sequences, with SH2 domains, which are not found in yeast, evolving later.
1.4.6.1 PH: structure

Despite the weak homology in primary sequence, structural studies performed on several PH domains show them to have a well conserved tertiary structure, and that a characteristic arrangement of types of amino acids rather than a primary consensus sequence determines a PH domain (Shaw, 1996).

Solution structures of the PH domains from pleckstrin (Yoon et al., 1994), spectrin (Macias et al., 1994), dynamin (Downing et al., 1994; Fushman et al., 1995), and Btk (Hyvonen and Saraste, 1997) define a conserved polypeptide fold consisting of two anti-parallel β-sheets of three and four strands, with a single C-terminal α-helix positioned at one edge of the interface between the β-sheets. The loop regions connecting the components of the tertiary structure are highly variable and are proposed to form the N-terminal PH domain ligand binding sites.

1.4.6.2 PH: function and ligands

Unlike SH2 and SH3 domains, PH domains have not been reported to bind any particular ligand consensus sequence, and PH domain interactions are complicated by the fact that both the N- and C-termini have been reported to bind ligands.

The membrane associated G protein βγ heterodimer binds the C-terminus of the PH domains of both βARK (Touhara et al., 1994) and Btk (Tsukada et al., 1994). This interaction was detected using PH domain fusion proteins which included sequences C-terminal to the PH domain, and it is therefore not clear whether or not this is a true PH domain interaction. Nonetheless, an interaction between this region of Btk and G proteins provides evidence that a non-receptor tyrosine kinase can serve as a direct effector of G proteins. Furthermore, the binding of specific Gyβ subunit plus a membrane factor results in the activation of Btk (Langhans Rajasekaran et al., 1995).
The PH domains of dynamin, Btk, and PLCδ₁ have been reported to bind to various membrane associated phosphatidylinositol phospholipids (Ferguson et al., 1995; Fukuda et al., 1996; Salim et al., 1996) via the positively charged N-terminus (Hyvonen and Saraste, 1997). The Btk PH domain binds phosphatidylinositol-3,4,5-triphosphate (PI[3,4,5]P₃ or PIP₃), but not other phosphoinositides which are not phosphorylated at the D-3 hydroxyl residue, such as PI[4,5]P₂ and PI[4]P (Salim et al., 1996). Phosphorylation at the D-3 hydroxyl residue of these phosphoinositides is the result of PI3-K action, indicating Btk as a downstream effector of this enzyme (see section 1.8.3.2). The Btk PH domain also binds the inositol-phosphates IP₄, IPs, and IP₆, the soluble head group products of phosphatidyl phosphoinositides hydrolysed by phospholipases (Fukuda et al., 1996).

A transforming mutant of Btk called Btk*, arising from an E41K mutation within the PH domain, transforms NIH 3T3 fibroblasts and is constitutively active and membrane associated (Li et al., 1995). Structural comparison of the Btk PH domain with that of PLCδ indicates that substituting the highly basic residue lysine at position 41 would increase the affinity of the Btk PH domain for an acidic ligand such as PIP₃ (Ferguson et al., 1995). Indeed, a PH domain fusion protein with the mutation E41K introduced does show increased affinity for IP₆ (Fukuda et al., 1996). The Btk* mutation correlates the membrane localisation of Btk with an increase in its phosphorylated state and its activation. Furthermore it provides evidence that the Btk PH domains plays an important part in the regulation of Btk, possibly mediating Btk membrane association, reported to be induced in mast cells on ligation of FcsRI (Kawakami et al., 1994). The importance of the association between the Btk PH domain and these membrane associated phosphoinositide phospholipids is underscored by the finding that PH domains constructed with the xid mutation at R28 incorporated are unable to bind PIP₃ (Salim et al., 1996). This implies that a disruption of the ability of Btk to associate with the membrane leads to a failure in Btk function and the xid phenotype.
PH domains have been suggested to be involved generally in protein membrane association (for review see Shaw, 1996) as (i) a PH domain in one protein is often replaced by a different protein domain, known to mediate membrane association, in another closely related protein. For example, the Src family PTKs have a myristoylation site that is responsible for membrane association instead of Btk's PH domain (see section 1.7.1.1, and Fig.1.9). (ii) Many substrates for PH domain containing proteins are lipids or membrane anchored proteins, such as PIP\(_2\) which is the membrane associated substrate of PH domain containing PLC\(_{\gamma}\), and (iii) many PH domain containing proteins including dynamin, PKC\(_\mu\), \(\beta\)ARK and Btk are found as both soluble and membrane associated forms (Shaw, 1996).

Another ligand of the PH domain of Btk is PKC. Several isoforms of PKC interact with the Btk PH domain \textit{in vitro}, but only one, PKC\(\beta\)I, a calcium dependent isoform, was found associated with the whole Btk molecule in stimulated and unstimulated mouse mast cells (Yao et al., 1994). The implications of this Btk: PKC interaction in cell signalling are unknown, however Btk was found to be a substrate of PKC \textit{in vitro}, and this ser/thr phosphorylation of Btk correlated with a decrease in the activity and tyrosine phosphorylation of Btk (Yao et al., 1994) (see section 1.8.3.1, and Fig.1.15). It is also of interest to note that the PKC\(\beta\) deficient mouse exhibits a range of immunological defects very similar to the xid phenotype, suggesting that Btk and PKC\(\beta\) function in the same pathway (Leitges et al., 1996). A summary of the ligands of Btk indicating the domains to which they bind is shown in Fig.1.6.
Chapter 1

N

Regulatory Region

C

Catalytic domain

Unique Region

| PH | TH | SH3 | SH2 | SH1 |

Btk Ligands:

- G protein βγ subunits
- Blk c-Cbl
- Btk Y223 (section 1.7.3.1.2)
- PIP3, IP4, IP6 Fyn WASP
- PKC* Lyn Vav (see section 1.8.3.4)
- Hck Sam-68 (see section 1.8.3.4)

Figure 1.6 Btk Ligands. The characteristic domain structure of Btk is shown with reported ligands listed. * indicates the protein has been found to co-precipitate with Btk. All others are in vitro associations.

1.5 The Wiskott-Aldrich Syndrome

Of immediate interest on the identification of WASP as a ligand for the Btk SH3 domain was the fact that when mutated, WASP results in another primary immunodeficiency, the Wiskott-Aldrich syndrome, that effects several haematopoietic lineages, including B cells.

WAS is a rare X-linked disorder which is characterised by thrombocytopenia, eczema, combined immunodeficiencies, and in severe cases susceptibility to lymphoreticular malignancy and autoimmune phenomena. The disease affects many haematopoietic cell lineages including platelets, and affects both the humoral and cell mediated responses (Remold O’Donnell et al.,...
1996). It is characterised by a failure of B cells to respond to polysaccharide antigens and deficiencies in chemotaxis in the myeloid cell lineage have also been reported (Zicha et al., 1998). The cellular defects in WAS patients also include cytoskeletal abnormalities of T cells and platelets (Molina et al., 1992), with T cells showing an unusual smooth surface due to reduced density and size of surface microvilli (Molina et al., 1992), suggesting that a defect in the organisation of the cytoskeleton may lie at the basis of the syndrome.

Linkage studies placed the gene to a >1 Mb region in Xp11.22-p11.23 (Greer et al., 1992) and investigations using a positional cloning strategy involved construction of a clone contig in this interval. The subsequent isolation of cDNAs led to the identification of a sequence, expressed in lymphocytic and megakaryocytic lineages, which is altered in individuals with WAS (Derry et al., 1994a).

The WAS gene spans ~9 Kb of genomic DNA and comprises 12 exons encoding a cDNA of about 1800 bp (Derry et al., 1994a). WASP expression has been observed in all haematopoietic cells examined (Stewart et al., 1997). Apparent non-random X chromosome inactivation is observed in T and B lymphocytes, granulocytes, monocytes, and platelets of carrier mothers of the mutant WAS allele (Gealy et al., 1980; Fearon et al., 1988; Greer et al., 1989; Notarangelo et al., 1991).

WAS patients are classified according to their phenotype depending on the occurrence and the severity of the symptoms described above. An immunodeficiency known as X-linked thrombocytopenia (XLT), has subsequently been shown to be due to mutations in the same WAS gene (Villa et al., 1995), and patients previously diagnosed with XLT are now recognised as WAS patients with a mild phenotype. Attempts to correlate the WAS phenotype with specific mutations within the WAS gene have not proved successful and are discussed in Chapter 6.
1.5.1 The Wiskott-Aldrich Syndrome Protein (WASP)

The primary structure of the WAS gene predicts it to encode a proline rich protein (Derry et al., 1994a). PXXP motifs are found in WASP and on its identification, WASP was proposed a likely SH3 domain binding protein.

Further examination of WASP revealed that it contains regions with homology to other proteins (Fig. 1.7). Along with the two regions rich in proline residues, WASP contains an N-terminal PH domain (Miki et al, 1996) (section 1.4.6) and a GTPase-binding site similar to those found in Rac/Cdc42 effector proteins, e.g. PAK (Symons et al., 1996). In addition, two distinct sequences are found in the WASP N and C-terminal regions that are conserved in several proline rich proteins which are known to be involved in the organisation of the actin cytoskeleton (Symons et al., 1996). As these regions were originally identified in WASP they have been termed the WH (WASP homology) 1 and 2 domains.

1.5.2 WASP interactions: WASP and PTK mediated signalling

The proline rich regions of WASP are responsible for the association of WASP in vitro with the SH3 domains of several proteins, including the Btk family PTKs (Cory et al., 1996) as discussed in section 1.4.4.2. The first WASP:SH3 domain interaction reported was between the three SH3 domains of the adaptor protein Nck and WASP (Rivero Lezcano et al., 1995). A unique clone isolated from a cDNA library using the three SH3 domains of Nck GST

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**Figure 1.7 The Structure of WASP.** WH = WASP homology, GBD = GTPase binding domain, PRR = proline rich region, NLS = nuclear localisation site, Apm = actin polymerisation site
fusion protein as a probe was found to have homology to the WAS gene. The same fusion protein was also found to precipitate WASP from HL60 cells. Although only the C-terminal SH3 domain could bind WASP directly, the presence of all three SH3 domains resulted in a precipitated WASP band of higher intensity than that seen bound to the C-terminal SH3 domain alone. This idea that the three domains may co-operate to enhance Nck's binding of WASP may explain the fact that WASP could be detected in Nck immunoprecipitates from COS-7 cells transfected with WASP (Rivero Lezcano et al., 1995), whereas the co-precipitation of WASP with Btk, which contains only one SH3 domain has not been successfully performed (Cory et al., 1996).

WASP was subsequently reported to associate with a number of SH3 domain containing proteins which suggest that it plays a role in tyrosine kinase mediated signal transduction, and may be involved in linking these signalling pathways with the cytoskeleton. As well as binding the SH3 domains of the Btk family of PTKs and Nck, WASP has also been reported to bind the SH3 domains of PLCγ (Cory et al., 1996), c-Src, c-Fgr, Fyn (Banin et al., 1996), the p85 sub-unit of PI3-K (Finan et al., 1996), and the N-terminal SH3 domain of Grb-2, (Cory et al., 1996). Of these, apart from the Nck:WASP interaction in COS cells, only Fyn has been found in a WASP immunoprecipitate, from myeloid cells (Banin et al., 1996).

1.5.3 WASP interactions: WASP and the cytoskeleton

WASP also interacts with Cdc 42, a member of the Ras related Rho family of GTPases. This interaction involves the GTPase binding site (GBD) of WASP, similar to that found in other Rac/ Cdc 42 effector proteins. This site in WASP interacts specifically with activated (GTP bound) Cdc 42, and very weakly with Rac, but not with another member of the same family, Rho (Symons et al., 1996).

The Rho family of small GTPases function as molecular switches that cycle between the active GTP-bound state and the inactive GDP-bound state,
and are key elements in the dynamic organisation of the actin cytoskeleton, including lamellipodia formation (Ridley et al., 1992b), stress fibres regulation (Ridley and Hall, 1992a) bud formation in yeast (Johnson and Pringle, 1990), and the induction of microspikes and filopodia formation in mammalian cells (Nobes and Hall, 1995). All three family members are involved in the formation of specialised adhesion complexes and are believed to be organised in a linear cascade to control the actin cytoskeleton (Nobes and Hall, 1995).

WASP transfected into endothelial cells (which do not express endogenous WASP) induced actin polymerisation, and in both these endothelial cells and Jurkat T cells WASP was found co-localised and clustered with some cellular filamentous actin (f-actin), an interaction that is dependent on WASP containing an intact GBD (Symons et al., 1996). This clustering of WASP and f-actin was inhibited by the addition of dominant negative Cdc 42. Furthermore, the formation of lamellipodia and bundling of stress fibres seen in endothelial cells transfected with constitutively active Cdc 42 and Rac was inhibited on co-transfection with WASP. The phenotype associated with transfection of constitutively active Rho was unaffected by co-transfection with WASP, consistent with the lack of binding observed in vitro between WASP and this member of the Rho family (Symons et al., 1996). WASP is therefore proposed to function as a regulator of the actin cytoskeleton, and is regulated itself by Cdc 42 (Symons et al., 1996).

WASP may therefore act to co-ordinate the actions of PTKs such as Btk and Fyn, and Cdc42 in controlling the actin cytoskeleton.

1.6 B cell signalling

Lineage commitment and B cell development (Fig. 1.1) are determined, at least in part, by extracellular signals. Signalling through cell surface markers expressed at various stages of development will determine the progression of
the cell throughout its determined lineage. Some downstream targets of signals are known, such as the lymphoid lineage determining Ikaros gene (Georgopoulos et al., 1994).

Cell surface receptors on lymphocytes trigger many physiological processes including differentiation and activation. Most immune reactions require the integration of distinct signalling pathways, connecting cell surface receptors to the nucleus. Determining the molecular interactions within these distinct pathways, as well as understanding how these signals are integrated is required in order to understand complex immune phenomena.

Antigen recognition and ligation by the BCR is the major event in initiating cell signalling cascades, resulting in various cellular responses that will vary depending on the developmental stage of the ligated cell. The early molecular consequences of BCR ligation are discussed in section 1.8. Antigen receptors such as the BCR also rely on information from accessory cell surface signalling molecules, and integrating signals from these molecules may determine the occurrence of an appropriate immune response. Amongst the membrane associated proteins on B cells which play roles in the final outcome of antigen recognition by the BCR are CD19, CD22 and FcγRIIb1, (Fig. 1.8). These receptors are discussed briefly prior to a discription of cytoplasmic signalling PTK families which are essential for BCR mediated signalling in which Btk function is implicated.

1.6.1 CD19

CD19 is expressed throughout B cell development until the plasma cell stage (Fig. 1.1). CD19 ligation can prolong the expression of RAG during pro-B cell development, which may extend the opportunity for gene diversification (Billips et al., 1995). CD19 deficient mice show normal development of conventional B-2 peripheral B cells, but the peritoneal B-1 population is diminished, suggesting a role for CD19 in the development or maintenance of
this cell population (Rickert et al., 1995), probably in conjunction with sIgM. CD19 deficient mice have major reductions in the antibody responses to T cell-dependent antigens (Rickert et al., 1995), and in the absence of CD19 it would appear trafficking of B cells to T-cell rich areas is altered (Behr and Schriever, 1995), contributing to an impairment of interactions between T and B cells. CD19 amplifies antigen receptor signalling when they are co-ligated and it is required for a normal T cell-dependent response. As it associates with CD21, a complement receptor, CD19 is also involved in coupling the B cell to the innate immune system (Croix et al., 1996; Doody et al., 1996).

1.6.2 CD22

CD22 is a B cell specific marker and its surface expression correlates with that of sIgM and sIgD (Dorken et al., 1986). CD22 associates with the BCR (Peaker and Neuberger, 1993), and on crosslinking of both the BCR and CD22 the intracellular region of CD22 becomes phosphorylated on tyrosines (Schulte et al., 1992; Peaker and Neuberger, 1993). Tyrosine phosphorylated CD22 interacts with other signalling proteins including Syk (Wienands et al., 1995; Law et al., 1996b), PLCγ (Law et al., 1996b), and the protein tyrosine phosphatase SHP-1 (Doody et al., 1995). CD22 co-ligated with the BCR lowers the quantity of anti-IgM antibody required to elicit BCR mediated cell signalling (Pezzutto et al., 1987; 1988), perhaps due to its ability to sequester increased quantities of Syk and PLCγ to the BCR complex. The B cells of CD22 deficient mice however, are hyper-responsive on antigen binding the BCR, indicating CD22 plays a negative role in BCR signalling (O'Keefe et al., 1996). CD22 presumably exerts its negative effects due to its association with SHP-1, as SHP-1 deficient B cells from the viable motheaten (me°) mouse show a similar hyper-responsiveness on ligation of the BCR (Cyster and Goodnow, 1995). CD22 signalling is discussed further in Chapter 3.
1.7.3 FcγRII

Signalling through Fc receptors for IgG suppresses signalling through mIgM when co-ligated with the BCR (Chan and Sinclair, 1971). The cytoplasmic domain of both FcγRIIb1 and FcγRIIb2 isoforms contain a site of tyrosine phosphorylation lying in a consensus region called the immunoreceptor tyrosine based inhibitory motif (ITIM). Phosphorylation at this site within FcγRII on cell activation leads to the recruitment of the phosphotyrosine phosphatase SHP-1 (D'Ambrosio et al., 1995), and FcγRIIb1 and FcγRIIb2 exert their negative regulatory effects by inhibiting the extracellular flux of calcium ions across the cell membrane (Choquet et al., 1993). The effects these accessory molecules exert in modulating signalling through the BCR are summarised in Fig.1.8.
Figure 1.8 Possible roles for B cell co-receptor molecules in BCR signalling. (Adapted from Doody et al., 1996). A: CD22 ligation by glycoconjugates on the surfaces of other cells may recruit SHP-1 or Syk to the signalling complex. B: Co-ligation of CD19/CD21 complex recruits positive signal transduction effectors. C: FcyRII co-ligation with the BCR by antigen in complex with IgG exerts an inhibitory effect on IgM signals through its association with SHP-1.
1.7 Non-receptor protein tyrosine kinases

A group of proteins that are crucial for mediating mitogenic signals are the non-receptor PTKs. These proteins, along with many others, contain modular SH1, SH2, SH3 and PH domains together in various combinations. The non-receptor tyrosine kinases are grouped into families according to their domain composition. The similarity between members of these families indicates the likelihood of them having similar or redundant functions. Three families involved in the early events of signalling through the BCR, are the Btk (section 1.3), the Src, and the Syk families of non-receptor tyrosine kinases. Members of both Syk and Src families of PTKs have been implicated in the regulation of Btk in B cell signalling, and the characteristics of these PTK families are discussed in this section.

1.7.1 The Src Family of PTKs

The Src family of PTKs consists of nine proteins so far identified, summarised in Table 1.2, and they are characterised by their structural homology depicted in Fig.1.9.

1.7.1.1 Structure of the Src family of PTKs

Src PTKs, like members of the Btk family contain SH1, SH2 and SH3 domains. Unlike the Btk family they do not possess the long N-terminal sequence containing the PH and TH domains, and they have an important regulatory tyrosine residue in their C-terminal tail (Chow and Veillette, 1995).

The Src PTKs contain a short unique sequence N-terminal to the SH3 domain that varies between members of the family. It is proposed that this region helps determine the unique roles of different Src PTKs, for example, Lck has been shown to interact with the CD4 and CD8 T cell antigens via this region (Shaw et al., 1989; Turner et al., 1990), and it may be involved in the
association between subunits of the BCR and Src PTKs in resting cells (section 1.8.2.1).

Table 1.2 The Src family of PTKs and their expression patterns (Chow and Veillette, 1995).

<table>
<thead>
<tr>
<th>Name</th>
<th>Mol. Weight</th>
<th>Expression Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src</td>
<td>60kD</td>
<td>ubiquitous, high in platelets</td>
</tr>
<tr>
<td>Yes</td>
<td>62kD</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Fgr</td>
<td>58kD</td>
<td>granulocytes, monocytes, macrophages</td>
</tr>
<tr>
<td>Fyn</td>
<td>59kD</td>
<td>ubiquitous, high in T cells</td>
</tr>
<tr>
<td>Lck</td>
<td>56kD</td>
<td>T cells, NK cells, peripheral B cells</td>
</tr>
<tr>
<td>Hck</td>
<td>56/59kD</td>
<td>granulocytes, monocytes, macrophages, platelets</td>
</tr>
<tr>
<td>Lyn</td>
<td>53/56kD</td>
<td>B cells, mast cells, monocytes, macrophages, platelets</td>
</tr>
<tr>
<td>Blk</td>
<td>55kD</td>
<td>B cells</td>
</tr>
<tr>
<td>Yrk</td>
<td>60kD</td>
<td>ubiquitous</td>
</tr>
</tbody>
</table>

The N-termini of Src PTKs, sometimes referred to as the SH4 domain, contain several membrane association signals. This includes a consensus sequence for the attachment of the membrane fatty acid myristate and often a sequence subject to palmitoylation. Furthermore, N terminal lysine residues also form interactions with membrane phospholipids (Resh, 1994).

The C-termini of the Src PTKs contains a major site of Src tyrosine phosphorylation at Y527 in Src, not found in Btk or Btk related proteins. This tyrosine residue is vital for Src regulation and its mutation is sufficient to generate a transforming protein (Hunter, 1987) (section 1.7.3.2).

1.7.1.2 Function of the Src family of PTKs

While the expression of most of the members of the Src family of PTKs is restricted, especially to haematopoietic cells, at least three, Src, Fyn and Yes, are more generally expressed (Table 1.2). All members show a striking similarity in the sequence of their catalytic domains which implies that they might share common substrates and exhibit a degree of redundancy. The functions of the family members are not necessarily redundant however. For example as
discussed in section 1.4.4.1, phosphorylation of c-Cbl in mouse osteoclasts is dependent on the presence of Src whose function (in its absence) cannot be replaced by any other Src PTKs present (Tanaka et al., 1996).

Src family members have been implicated in cell signalling through the T cell receptor and the T cell antigen CD4 (Xu and Littman, 1993; Lorenz et al., 1996), through surface IgD and IgM on B cells (Burkhardt et al., 1991), through growth factor receptors (Roche et al., 1995), and through some cytokine receptors (Seckinger and Fougereau, 1994). Src PTKs have also been implicated in cell cycle arrest (Scheuermann et al., 1994), cytoskeletal organisation (Thomas et al., 1995) and cell cycle progression (Roche et al., 1995). The role of Src PTKs in the latter is an example where they exhibit functional redundancy. DNA synthesis in response to PDGF, EGF and CSF-1 in fibroblasts is repressed when all three Src PTKs present in these cells, Fyn, Src and Yes, are inhibited (Roche et al., 1995), however inhibition of any one of these Src family members has no effect (Twamley Stein et al., 1993).

Further evidence for the importance of Src PTKs comes from the effects on ‘knock out’ mice. The Src-deficient mouse suffers from osteopetrosis, a deficiency in the bone resorption by osteoclasts (Soriano et al., 1991). This indicates that despite the ubiquitous nature of the expression of Src, its lack of expression elsewhere in the Src deficient mouse, for instance in the haematopoietic lineage, may be compensated for by other members of the family.

Fyn and Lck deficient mice show defects in signalling via TCR/CD4/CD8, and in the development of T cells (Appleby et al., 1992; Grant et al., 1992; Molina et al., 1992; Stein et al., 1992), in accordance with the high levels of expression of Fyn and Lck in this lineage (Table 1.2) The Lyn deficient mouse has reduced numbers of mature B cells and defective B cell signalling in response to BCR and CD40 cross linking. It has circulating autoreactive antibodies and fails to mediate an allergic response to IgE crosslinking in mast
Chapter 1

cells (Hibbs et al., 1995; Nishizumi et al., 1995). These results indicate that the
Src PTKs Fyn and Lyn play critical roles in T and B cell development
respectively, and that Lyn is indispensable in immunoglobulin mediated
signalling for clonal expansion and terminal differentiation of peripheral B
cells, particularly in establishing B cell tolerance.

1.7.2 The Syk family of PTKs

The Syk family of protein tyrosine kinases comprises just two identified
members, ZAP-70 and Syk. ZAP-70 was isolated as a TCR ζ chain associated
protein, found to become tyrosine phosphorylated and activated upon
stimulation of T cells through the T cell receptor (Chan et al., 1991). Its
expression is limited to T cell lineages (Chan et al., 1992; 1994) and shares high
homology (73%) with Syk (for review see van Oers and Weiss, 1995). Syk is
found expressed in multiple haematopoietic lineages including T cells, but its
expression is greatest in B cells (van Oers and Weiss, 1995). Syk associates with
the BCR, and like ZAP-70, becomes phosphorylated on tyrosines and activated
on ligation of the BCR (Hutchcroft et al., 1992) (section 1.8.2.2).

1.7.2.1 Structure of the Syk family of PTKs

The Syk family of PTKs is characterised by two N-terminal SH2 domains
and a C-terminal catalytic domain with a short linker region between the C-
terminal SH2 domain and the SH1 domain, (see Fig.1.9). Syk contains a unique
sequence of unknown function within this linker region. Neither Syk nor ZAP-
70 contain any regions that may result in translocation to the membrane, nor do
they have a C-terminal regulatory tyrosine (for review see van Oers and Weiss,
1995).
Figure 1.9 Comparative structures of the Src, Syk and Btk PTK families. Modular domains are shown as blocks of different colours. Sites of tyrosine phosphorylation with respect to catalytic domain activation are indicated. Abbreviations: My = myristylation site, SH = Src Homology, CTT = C terminal tail, Y = Tyrosine, PH = Pleckstrin Homology, PRR = Proline rich region.
Syk was first identified as a 40kD protein from porcine spleen (Kobayashi et al., 1990), which was found to possess the ability to autophosphorylate by an intramolecular process (Kobayashi et al., 1990). An antibody raised against the C-terminal tail of this protein recognised two proteins, one of 72 and one of 40kD. The 40kD species was subsequently found to be a proteolytic product of the full length 72kD protein which was missing the entire N-terminus including both SH2 domains and the linker region (Taniguchi et al., 1991) (Fig. 5.15 (B)).

1.7.2.2 Function of the Syk family of PTKs

Syk and ZAP-70 have both been implicated in antigen receptor mediated signalling and the role of Syk in BCR signalling is discussed in section 1.8.2.2. Syk is also seen to be tyrosine phosphorylated and activated in cells stimulated with IL-2 (Minami et al., 1995), G-CSF (Corey et al., 1994), various platelet agonists (Clark et al., 1994), and through Fc receptors including FcγRI, FcγRII (Agarwal et al., 1993), and FcεRI (Costello et al., 1996).

Syk knock-out mice produced by two groups provide more evidence for the function of Syk (Cheng et al., 1995; Turner et al., 1995). Syk⁻/⁻ foetuses haemorrhage in utero, indicating that Syk may play a role in vascular integrity or wound healing during embryogenesis. Interestingly the pattern of haemorrhaging was similar to that seen in mice deficient in platelet derived growth factor-B or its receptor, of which Syk has been reported to be downstream effector (Clark et al., 1994).

Syk deficient mice die perinatally, but the development of cells derived from the foetus could be examined by their introduction into RAG-2 mutant or irradiated recipients (Cheng et al., 1995; Turner et al., 1995). Syk⁻/⁻ haematopoietic lineages, with the exception of the B lineage, developed normally. B cell development was blocked at the pro- to pre-B I cell stage in differentiation, indicating a possible role for Syk in signalling through the pre-BCR. Since the very small number of immature B cells found did not further
develop into mature B cells, it appears that Syk deficiency results in a second later block in differentiation. Similar to PKCβ1 deficient (Leitges et al., 1996), and xid mice (Hayakawa et al., 1986), Syk deficient mice appear to have no B-1 peritoneal B cells (Cheng et al., 1995; Turner et al., 1995).

ZAP-70 deficiency results in a form of severe combined immunodeficiency (SCID) in humans, characterised by a deficit of peripheral blood CD8+ and dysfunctional CD4+ T cells (Perlmutter, 1994). Due to their homology and tissue distribution it has been proposed that Syk and ZAP-70 play homologous roles in signalling from the TCR and the BCR. Indeed, the non-functional BCR that results from loss of Syk PTK can be reconstituted by the expression of ZAP-70 in these Syk- B cells (Kong et al., 1995), and the defective development and function of thymocytes, blocked at the CD4+CD8+ stage in ZAP-70 deficient mice is corrected by Syk PTK (Goug, 1997). However, two cell lines, one with a mutated ZAP-70 and one with CD45 deficiency, provide interesting evidence concerning both the similarities and differences in the functions of Syk and ZAP-70. In a ZAP-70 deficient patient, Syk function can restore TCR signalling (van Oers and Weiss, 1995), and in a CD45 deficient cell line, defective TCR signalling was restored when Syk expression was high (Chu et al., 1996). The non-redundant functions of Syk and ZAP-70 may be explained by the finding that ZAP-70 requires tyrosine phosphorylation by Src kinases to become fully active, whereas Syk activation appears to be Src independent (Chu et al., 1996) (section 1.7.3.3).

It would appear therefore that the homology observed between Syk and ZAP-70 results in a certain degree of functional redundancy.

Syk activity, like that of Btk (section 1.3.1.2), has also been implicated in BCR mediated activation of PLCγ2 (Takata and Kurosaki, 1996) (section 1.8.2.4). Downstream signalling effects that result in actin assembly and α-tubulin phosphorylation have also been shown to require or involve the presence of Syk (Cox et al., 1996; Peters et al., 1996).
1.7.3. Regulation of non-receptor tyrosine kinases

Members of the Btk, Src and Syk families of non-receptor PTKs all become activated in B cells on BCR ligation. The regulation of this activation, vital for PTK function, is discussed in the following section. Two phenomena are critical for PTK regulation: tyrosine phosphorylation, and the formation of intra- and inter-molecular associations.

1.7.3.1 Regulation of Btk and Itk PTKs

1.7.3.1.1 Itk intramolecular associations

NMR studies of Itk show an intramolecular interaction between the SH3 domain and the adjacent proline rich sequence of the TH domain which contains an Itk SH3 domain binding consensus (Andreotti et al., 1997), (depicted in Fig.1.10). The structure of this intramolecular complex and investigations into cellular ligand binding suggests that the intramolecular interaction between the proline rich region and the SH3 domain, stabilised by the SH2 domain and the Btk motif of the TH domain (Fig. 1.10), regulate the binding of cellular ligands to both the SH3 domain and the proline rich region of Itk.

A fusion protein consisting of the Itk SH3 domain plus the proline rich region bound a decreased amount of SH3 ligands, but the high affinity ligand Sam-68 remained bound, consistent with a relatively weak affinity between Itk SH3 and the proline rich region as determined by the NMR studies. However fusion proteins consisting of the SH3 domain, the proline rich region plus the SH2 domain and Btk motif no longer bound Sam-68, nor did it bind Grb-2, which associates with the proline rich region of Itk via its SH3 domain. Itk was thus proposed to form one of two conformations, open, or closed (see Fig.1.10). In the open state (Fig. 1.10(B)) the intramolecular interaction is disrupted and both SH3 domain and the proline rich region are accessible to cellular ligands. In the closed conformation, the intramolecular association between the SH3
domain and the proline rich region, stabilised by the SH2 domain and the Btk motif, results in an Itk molecule where neither SH3 nor proline rich ligands can gain access to their respective binding sites (Fig. 1.10(A)).

In the closed conformation, the pTyr binding pocket of the SH2 domain however is still accessible. A fusion protein containing both SH3 and SH2 domain binds pTyr containing ligands synergistically from cell lysates. A fusion protein comprising the entire TH domain, and the SH3 and SH2 domains was incubated with lysates containing varying levels of tyrosine phosphorylated proteins. The accessibility of the Itk proline rich region to Grb-2 binding gives an indication of the degree of ‘openness’ of the fusion protein, and it was observed to increase with increasing tyrosine phosphorylation of cellular proteins added. The intramolecular interaction within Itk may therefore be disrupted by ligands which bind both SH2 and SH3. Initially they may bind Itk SH2 even in the closed conformation, and with increasing quantities may out compete the intramolecular SH3:proline rich interaction, and result in cellular ligand binding by both Itk SH3 and Itk proline rich binding sites. Alternatively, the closed conformation may be disrupted by direct phosphorylation of the binding regions of the SH3 domain or proline rich regions. Y180 of Itk is believed to be involved in this intramolecular interaction, and it is equivalent to the Btk site of autophosphorylation at Y223. The proline rich region contains consensus sites for phosphorylation by several kinases (Andreotti et al., 1997) including PKC (Woodgett et al., 1986).
Figure 1.10 *Itk regulation by intramolecular interactions* (Adapted from Andreotti et al., 1997). A: in the closed conformation, Itk SH3 bends around and binds the adjacent proline rich (PR) region. The conformation resulting from this interaction is stabilised by the presence of the SH2 domain and the Btk motif, that may be in close proximity. Neither the SH3 domain nor the PR region can interact with cellular ligands, but the SH2 domain binding site has access to pTyr ligands. B: The open conformation may be induced by the binding of a bi-dentate ligand to the SH2 domain of the closed conformation. Subsequent binding of the same ligand to the SH3 domain competes with the PR region. Itk in the open conformation can bind ligands via its PR region, and its SH3 and SH2 domains.
Protein domains function as mediators of protein:protein interactions which, as discussed in section 1.5, are often crucial for the normal action of the protein in which they are found, be they involved in relocalisation of the protein or its activation. The work of Andreotti et al. on the structure of Itk provides evidence that a complex set of intra- and intermolecular interactions involving regulatory domains of a member of the Btk family of PTKs with high homology to Btk are involved in the regulation of protein:protein interactions and hence, presumably, the action of the PTK.

1.7.3.1.2 Btk tyrosine phosphorylation

The correlation between tyrosine phosphorylation and the activation of Btk was noted in early studies on Btk after BCR and FcεRI cross-linking (de Weers et al., 1994; Kawakami et al., 1994; Hinshelwood et al., 1995). Investigations into the pattern of phosphorylation of Btk have mapped two crucial sites of tyrosine phosphorylation. One is a major site of trans-phosphorylation and lies at position 551 within the activation loop of the catalytic domain (Mahajan et al., 1995; Rawlings et al., 1996). Phosphorylation at this site is believed to result in activation of the catalytic activity of the Btk kinase domain as discussed in section 1.5.1. The second residue, which lies at position 223 within the SH3 domain, is a primary site of Btk autophosphorylation (Park et al., 1996; Rawlings et al., 1996).

Residue Y223 of Btk, as mentioned above, is equivalent to a tyrosine at position 180 of Itk, which from NMR structural studies was shown to lie on the binding surface of the SH3 domain (Andreotti et al., 1997). Phosphorylation at this position is likely to affect SH3 binding and determine Btk function upon auto-phosphorylation (see section 1.8.2.3 and Chapter 5).

Phosphorylation of Btk regulates its activity therefore by resulting in the activation of the protein's catalytic domain, and by potentially altering the binding capacity and specificity of the SH3 domain. The regulation of Btk in the context of BCR signalling is discussed in section 1.8.2.3.
Btk has also been reported as a substrate of the ser/thr kinase PKC, and phosphorylation at these non-tyrosine residues may act to negatively regulate Btk (Yao et al., 1994). How this may be achieved is not known.

1.7.3.2 Regulation of Src family PTKs

The regulation of the catalytic activity Src family kinases has been studied extensively, and accumulated genetic and biochemical evidence has led to the proposal of a model of enzyme inhibition. This proposes that a C-terminal tyrosine residue (Y527 in Src) unique to Src family kinases forms an intramolecular interaction with the SH2 domain when phosphorylated, thus inhibiting enzymatic activity (Superti-Furga and Courtneidge, 1995) (Fig. 1.11). Src activity was therefore thought to be dependent upon the rival activities of phosphatases, e.g. CD45 (Shiroo et al., 1992) and the kinase Csk (Bergman et al., 1992) which act to determine the phosphorylated state of Y527. The determination of the crystal structures of Src and Hck and of the activation of Hck by Nef binding (Moarefi et al., 1997), have confirmed the importance of Y527 and also of the SH3 domain (Superti-Furga et al., 1993) in the regulation of the Src family.

The crystal structures of Src and Hck (Sicheri et al., 1997; Xu et al., 1997) in an inactive conformation have confirmed the presence of an intramolecular interaction that could result in catalytic inhibition. The SH2 domain is seen to be bound to the C-terminal tail and this interaction occurs to the side of the catalytic domain opposite the catalytic face. This interaction would not therefore appear to be directly responsible for the inhibition of Src kinase activity. The SH3 domain in this conformation was surprisingly seen to interact with the linker region between the SH2 domain and the catalytic domain, (see Fig.1.11). Although this region in Src only contains one proline residue, its interface with the SH3 domain showed it to be in the form of a PPII helix. Kinase inhibition is explained by interactions between the SH3 domain bound linker, and the catalytic domain, which results in displacement of a region...
called helix C within the N-terminal lobe of the catalytic domain to a position that results in disordering of the activation loop containing Y416, and an inactive kinase conformation.

It is proposed that the activation of the kinase domain is accomplished by disruption of the SH3 and SH2 domain interactions and by phosphorylation of Y416. As Csk rescue of *S. pombe* from cell death induced by Src expression was dependent on Src containing an intact SH3 domain, the inhibitory intramolecular association was proposed to involve the SH3 domain (Superti Furga et al., 1993). Further evidence for the involvement of the SH3 domain in inhibition of Src PTK activity came from studies showing that binding of the high affinity ligand Nef to the Hck SH3 domain results in activation of the protein (Moarefi et al., 1997). Y416 in an unphosphorylated state is believed to act as a pseudosubstrate catalytic inhibitor as described in section 1.5.1.2; on relaxation of the intramolecular interaction of kinase inhibited Srcs, phosphorylation of Y416 in the ordered activation loop is proposed to lead to activation of the kinase domain.

The interactions between the SH2 domain and the C-terminal tail, and between the SH3 domain and the linker region showed that both interactions were not of high affinity (Moarefi et al., 1997; Sicheri et al., 1997), as would be expected from the evidence that Src activation may be accomplished by displacement of intramolecular interactions by higher affinity ligands of the SH2 and SH3 domains (Courtneidge et al., 1991; Songyang et al., 1993; Moarefi et al., 1997). Interestingly mutations within Src PTKs that had been reported as activating were all found to lie in regions that were implicated in the intramolecular associations formed by the inactive crystallised Hck and Src (Xu et al., 1997; Sicheri et al., 1997).
Figure 1.11 A cartoon representation of kinase inactive Src. Y527 at the C terminus is phosphorylated by Csk and interacts with the SH2 domain. The SH3 domain interacts with the PPII like helix formed by the linker between the kinase domain and the SH2 domain, rendering the activation loop of the kinase domain disordered, Y416 is unavailable for phosphorylation, and the kinase catalytic site is inactive.

Figure 1.12 Syk Activation on domain binding. On binding a peptide designed to contain a doubly phosphorylated ITAM, Syk activity was reported to increase (Shiue 1996), or not (Johnson 1996). The same activation was observed when the C terminal tail (CTT) bound an antibody raised against it (Kimura 1996). However, Syk immunoprecipitated from cells using this same CTT antibody, was not activated by the addition of the ITAM like peptide (Shiue 1995).
1.7.3.3 Regulation of Syk family PTKs

The activity of the Syk family of PTKs is regulated by tyrosine phosphorylation. There is also \textit{in vitro} evidence that direct interactions of peptides or antibodies with the SH2 domains and the C-terminal tail of Syk may also regulate its activation.

Within ZAP-70, six sites of tyrosine phosphorylation have been mapped: two are sites of autophosphorylation and four are transphosphorylated by the Src PTK Lck (Watts et al., 1994). One of these four tyrosine residues lies within the catalytic domain in a sequence identical to the Src catalytic domain trans-autophosphorylation site, and is equivalent to Y1162 of IRK. In Syk, due to differences in the primary amino acid sequence, the equivalent tyrosine residue does not lie in a Src autophosphorylation consensus sequence (El Hillal et al., 1997).

The activation of Syk independent of other PTKs was illustrated by studies where anti-CD16 antibody mediated clustering of a chimeric molecule with a CD16 extracellular domain and a Syk intracellular domain transfected into T cells was observed to be sufficient to initiate certain T cell activation responses. A chimera containing a ZAP-70 intracellular region has to be co-clustered with a Fyn:CD16 chimera in order to induce the same responses (Kolanus et al., 1993). Syk is therefore capable of initiating these cellular responses independent of Src PTKs, but ZAP-70 function is entirely dependent on the actions of a Src PTK such as Fyn (Zoller et al., 1997). This would explain the requirement of CD45 for ZAP-70 activation (Chu et al., 1996), and also the requirement of ZAP-70 mediated signalling for Src PTK activity in thymocytes which may be overcome by elevated expression of Syk (van Oers and Weiss, 1995). It could possibly be an explanation for the relative differences in the activity of the Syk and ZAP-70 catalytic domains (Latour et al., 1996), as clustering of Syk may result in increased tyrosine trans-phosphorylation and kinase activity in \textit{in vitro} kinase assays, whereas ZAP-70 clustering under these
conditions will result in no increased tyrosine phosphorylation without the presence of a Src PTK such as Fyn or Lck. It also explains the high levels of Syk activity observed on crosslinking of even relatively weak Fc receptor stimuli (El Hillal et al., 1997).

Syk activation results from a series of events initiated by BCR ligation (section 1.8). Activation of Syk catalytic activity occurs due to tyrosine phosphorylation at Y518/519 in the activation loop (Kurosaki et al., 1995), believed to result in the release of the unphosphorylated tyrosine pseudosubstrate from the active site of the kinase. This phosphorylation has been proposed to be initiated by the action of the Src PTK Lyn on Syk (Kurosaki et al., 1994), producing an activated Syk molecule. However Syk is capable of trans-autophosphorylation at Y518/519 in a Src PTK independent manner (Kurosaki et al., 1995), resulting in an exponential rise in Syk activity. This is discussed in section 1.8.2.2.

Structural regions within the Syk molecule are also involved in its regulation. The SH2 domains of Syk are implicated in the regulation of its activity, as the 40kD proteolytic fragment of Syk containing only the kinase domain exhibits elevated catalytic activity relative to the wild type (Paolini et al., 1992), indicating a negative regulatory role for the SH2 domains in the 72kD full length protein. Furthermore, addition of phosphopeptides with high affinity for the Syk SH2 domains results in an increase in the activity of wild type, (Fig. 1.12), but not the 40kD Syk. These phosphopeptides were designed to contain phosphorylated ITAMs from FcεRI which were shown to be phosphorylated upon FcεRI ligation in mast cells and to be critical for Syk activation (Paolini et al., 1992; Shiue et al., 1995). However conflicting results concerning the binding of Syk SH2 domains which indicate that it does not activate Syk have also been reported (Johnson et al., 1995) and are discussed in section 1.8.2.2.
The Syk C-terminus may also be involved in the regulation of the protein. Syk adopts more than one conformation, as detected by immunoprecipitating Syk using antibodies raised against different parts of the protein (Kimura et al., 1996). An antibody raised against the C-terminal 16 residues of Syk, called αSykC, precipitates Syk from activated cells, but in the lysate of resting cells αSykC does not recognise Syk. As αSykC recognises denatured Syk from resting cells on immunoblotting, these results imply that its inability to precipitate Syk from resting cell lysate is due to a conformational change in native Syk that masks the C-terminal tail from the antibody.

Syk adopts a conformation where the C-terminal tail is free to bind αSykC upon its tyrosine phosphorylation, or upon binding to phosphopeptides designed to contain a phosphorylated ITAM. Both events are correlated with an increase in Syk kinase activity, indicating that the conformational state of Syk in resting cells, apparently involving the C-terminal tail, may somehow be involved in repressing Syk catalytic activity. Furthermore, αSykC added to a Syk immunoprecipitate was reported to induce an increase in Syk autophosphorylation (Kimura et al., 1996), (Fig. 1.12), perhaps by sequestering the C-terminal tail away from an inhibitory association with the Syk kinase domain.

However, a contradictory report showed that Syk could be immunoprecipitated from resting cells using an antibody raised against the same C-terminal residues as αSykC was, but that this population of Syk was not activated on the addition of an ITAM containing phosphopeptide, shown to activate Syk in resting cell lysates (Shiue et al., 1995). This discrepancy has not been addressed. Both studies (Shiue et al., 1995; Kimura et al., 1996) were performed using the same RBL-2H3 cells, and antibodies raised against the same C-terminal sequence of Syk.
1.8 BCR signalling

Recognition and binding of antigen by the BCR leads to the activation of many signalling cascades, resulting in cell activation or deletion depending on the developmental stage of the cell. Understanding the molecular interactions that are induced by BCR crosslinking will provide critical information for our understanding of how antigen recognition results in immune responses which protect the organism from invading pathogens.

1.8.1 Tyrosine phosphorylation at the BCR

Cross-linking of the BCR results in the activation of several PTKs, leading to the tyrosine phosphorylation of protein substrates and activation of multiple biochemical pathways, including the mobilisation of calcium (Gold et al., 1990; Carter et al., 1991; Kurosaki, 1997). The μ heavy chain of the BCR is a transmembrane protein but its intracellular domain consists of only 3 amino acids, KVK. Two IgM associated molecules, Igα and Igβ, are essential for the surface expression of IgM (Hombach et al., 1990). Although they contain no kinase activity of their own, Igα and Igβ have long cytoplasmic domains which encode a region containing six conserved amino acids including two tyrosine residues. This region was first identified (Reth, 1989) as an amino acid motif found in the cytoplasmic tails of several antigen receptor sub-units including those associated with the BCR, the TCR and FcεRI, all of which were known to have similar structural and functional properties, including the ability to result in cell activation in a phosphotyrosine mediated manner upon cross-linking of the receptor (Weiss and Littman, 1994; Cambier, 1995a). This region is referred to as the ITAM, (Fig. 1.13) (Cambier, 1995b), and its presence in receptor sub-units is crucial for receptor mediated tyrosine phosphorylation and cell activation, but not for antigen presentation.
Figure 1.13 The consensus sequence of the ITAM. X=any amino acid, Y=sites of tyrosine phosphorylation.

Examination of the components of the BCR in mouse B cell lines show that the Igα and Igβ subunits are inducibly phosphorylated at tyrosines on BCR ligation (Gold et al., 1991). The sites of phosphorylation were subsequently mapped to include the two tyrosines of the ITAM (Cambier et al., 1994).

Evidence that Igα and Igβ are critical for signalling through the BCR comes from several reports. Mutation of polar residues in the transmembrane region of IgM that disrupt its association with Igα and Igβ, but leave it membrane anchored, results in a non-functional BCR unable to elicit signalling responses. Ligation of a chimeric molecule containing the extracellular and transmembrane domains of the mutated IgM fused to the intracellular domains of Igα or Igβ (IgM:Igα and IgM:Igβ), restored signalling responses (Sanchez et al., 1993). The Igα and Igβ subunits are therefore essential and sufficient for BCR induced signalling, but results indicate that their functions may not be exactly the same. Interestingly CD8: Igα and Igβ chimeras used to signal in B cells could also induce signal transduction and IL-2 production in T cells (Burkhardt et al., 1994) without the involvement of TCR components, indicating the functional homology of the ITAM motif.

Furthermore, for the development of pre-B cells from pro-B cells, the association between IgM and the Igα/Igβ heterodimer was shown to be critical, and these early antigen independent developmental events could be induced by the presence of the phosphorylated cytoplasmic domain of Igβ (Papavisiliou et al., 1995).
Signalling through a CD8:Igα chimera in B cells with mutations introduced at the tyrosine residues showed that the two Y residues within the ITAM are critical for signal transduction. Mutation of only one of these, Y23 (the first in the ITAM sequence), was sufficient to abolish signal transduction. In vitro, this tyrosine 23 was a major substrate of the Src PTK Fyn (Flaswinkel and Reth, 1994). Similarly the sites of tyrosine phosphorylation within Igβ required for B cell development from the pro-B cell stage were mapped to lie with the ITAM motif (Papavasiliou et al., 1995).

Tyrosine phosphorylation at the ITAMs has been proposed to be a mechanism for recruiting SH2 domain containing proteins which could result in tyrosine kinase activity being associated with the receptor upon its stimulation. Mutation of tyrosine and non-tyrosine residues within the ITAM indicates that a disruption in SH2 domain binding consensus within the ITAM could be the cause of abrogated signalling.

1.8.2 The roles of Src family, Syk and Btk non-receptor PTKs in BCR signalling

1.8.2.1 Src PTKs

Suppression of BCR signalling observed in cells deficient in the B cell Src-like PTKs, Blk and Lyn indicate that these PTKs are involved in BCR mediated signalling (Yao and Scott, 1993; Takata and Kurosaki, 1996). Physical and functional associations between the BCR and Src PTKs have been reported substantiating this proposal.

Ligation of the BCR on resting splenic B cells leads to the tyrosine phosphorylation of numerous cellular proteins. This increase in phosphorylation was seen to correlate with an increase in the kinase activity of the PTKs, Blk, Lyn and Fyn. LPS activation of B cells, which does not result in tyrosine phosphorylation of cellular proteins, also did not result in an increase in the activity of these PTKs, indicating that their activity is a requirement of
antigen receptor mediated (the effect was also seen on ligation of IgD), and not general, B cell activation (Burkhardt et al., 1991). IgM precipitations from digitonin, but not NP40, lysates of stimulated B cells contained the Src PTKs, Blk, Lyn and Fyn (Burkhardt et al., 1991; Yamanashi et al., 1991; Lin and Justement, 1992), indicating both a physical and functional relationship between these PTKs and the BCR.

The increase of Lyn and Blk activity upon ligation of the BCR in mouse WEHI-231 B cells was correlated with an increase in the tyrosine phosphorylation of the Igα and Igβ BCR sub-units (Saouaf et al., 1994). This, and the observations that (i) Fyn was seen to phosphorylate the Igα ITAM in vitro (Flaswinkel and Reth, 1994), (ii) Lck and Fyn kinase activity is required for the phosphorylation of the tyrosine residues within the ITAM of the TCR ζ chain (Goldman et al., 1994), and (iii) on reconstitution of BCR signalling in COS cells (Saouaf et al., 1995), Igα and Igβ tyrosine phosphorylation was only observed upon their co-expression with Blk, give a strong indication that Src PTKs are responsible for the phosphorylation of ITAMs in vivo (see Fig. 1.14).

Consistent with the idea that Igα and Igβ play partially distinct roles in BCR signalling (Sanchez et al., 1993), investigations into the Igα and Igβ associated molecules showed that fusion proteins based on the Igα and Igβ cytoplasmic tails bound overlapping but distinct groups of proteins (Clark et al., 1992). The proteins bound by Igα in the B cell lymphoma K46 included the Src PTKs Lyn and Fyn, and the site of binding was mapped to lie within the ITAM of Igα.

Although Lyn, Fyn and Igα associate constitutively, the association in BCR stimulated cells is stronger than in resting cells. Moreover, the sites of interaction on Fyn and Igα are distinct in resting and stimulated cells . In resting cells the site of the Fyn-Igα interaction has been shown to occur between a motif, DCSM, within the Igα ITAM (Clark et al., 1994), and the N-terminal 10 amino acids of Fyn (Pleiman et al., 1994). On BCR ligation, the DCSM motif is
no longer required for binding, as the interaction between Fyn and Igα in activated cells occurs exclusively between the SH2 domain of Fyn and tyrosine phosphorylated ITAM of Igα (Clark et al., 1994; Pleiman et al., 1994).

This re-orientation of the binding of Fyn at the BCR upon its ligation correlates with an increase in Fyn kinase activity (Pleiman et al., 1994). This could be due to the Fyn SH2 domain binding a cellular ligand resulting in de-repression of kinase activity (section 1.7.3.2). Lyn has been reported to become activated due to a direct interaction with a peptide designed on the doubly phosphorylated ITAM (\((p)₂\text{ITAM}\) of the CD3ε subunit of the TCR (Johnson et al., 1995) an interaction which requires CD45 presumably to dephosphorylate the C-terminal tail (see Fig. 1.11).

1.8.2.2 Syk

Several lines of evidence support an essential role for Syk in BCR signalling, (i) In chicken B cells where the Syk gene has been disrupted by homologous recombination, the tyrosine phosphorylation and calcium mobilisation seen on BCR cross-linking in wild type cells is ablated (Kurosaki et al., 1994), (ii) Analysis of Syk deficient mice has shown that Syk is required for development of mature B cells, apparently by generating the signal required for the maturation of pro/pre-B cells (Cheng et al., 1995; Turner et al., 1995) (iii) Cross-linking of the BCR of mouse WEHI-231 B cells results in a rapid increase in the tyrosine phosphorylation and activation of Syk (Hutchcroft et al., 1992). Furthermore, in vitro kinase assays performed on Syk immunoprecipitates show the presence of tyrosine phosphorylated Igα subunits. Although the amount of Syk associated with Igα increases on BCR ligation of transfected COS cells (Saouaf et al., 1994), depletion of Igα from WEHI-231 cell lysates under conditions where Syk is co-precipitated showed that only a small fraction of the total cellular Syk pool was associated with the BCR subunit (Hutchcroft et al., 1992).
The Syk interaction with Igα (and Igβ (Saouaf et al., 1994; Kurosaki et al., 1995)), has subsequently been shown to occur between both Syk SH2 domains and the \((p)_2\)ITAM motif of Igα and Igβ. The crystal structure of the two SH2 domains of ZAP-70 in complex with the TCR \(\zeta\) subunit (Hatada et al., 1995) confirms the likelihood of this interaction. In the latter case the interaction involves both ZAP-70 SH2 domains binding the bidentate \((p)_2\)ITAM, with the linker region between the two SH2 domains also being involved in the interaction.

The association of Syk with the BCR on cell stimulation may cause Syk activation. The interaction between peptides designed to contain a \((p)_2\)ITAM and Syk has been shown in contradictory reports to either result (Rowley et al., 1995; Shiue et al., 1995), or not result (Johnson et al., 1995) in the activation of Syk. This discrepancy has yet to be resolved, however precipitation of Syk molecules from cell systems (Rowley et al., 1995; Shiue et al., 1995) would result in Syk aggregation, and Syk would also have had contacts with Src PTKs in mast cells. As both Syk aggregation and Src PTKs are implicated in the activation of Syk (discussed below), results may be affected by experimental artefacts.

Nevertheless, the association of Syk with \((p)_2\)ITAMs is essential for Syk function and is the first of two steps required for Syk mediated BCR signalling. This first step involves the recruitment of Syk to the BCR upon its ligation, via an interaction between the \((p)_2\)ITAM of the Igα and Igβ subunits of the BCR, and the two SH2 domains of Syk. Mutation of either or both of these SH2 domains results in the ablation of Syk mediated BCR signalling, as illustrated by the signalling defects observed on the introduction of Syk SH2 mutant constructs into the Syk deficient DT40 chicken B cell line (Kurosaki et al., 1995).

The second requirement for Syk mediated BCR signalling is Syk phosphorylation, which results in its activation. Although mutation of Y518/519 within the activation loop of Syk to phenylalanine does not interfere
with the association of Syk and the BCR, cellular protein tyrosine phosphorylation is compromised on ligation of the BCR in cells expressing this Syk mutant. Unless mutation of this Y to F results in changes in the tertiary structure of the kinase domain rendering Syk catalytic function inactive, these results indicate that phosphorylation at Y518/519 is the second step required for BCR signalling (Kurosaki et al., 1995).

Syk tyrosine phosphorylation results from the kinase activities of both Src PTKs, such as Lyn (Kurosaki et al., 1994), and autophosphorylation (Kolanus et al., 1993; Kurosaki et al., 1995; Rowley et al., 1995; Zoller et al., 1997). A Syk kinase inactive mutant transfected into Syk deficient DT40s is not tyrosine phosphorylated at Y-518/519 on BCR cross-linking, indicating that this is a site of autophosphorylation (Kurosaki et al., 1995). As lack of phosphorylation at this site results in a decrease in BCR induced protein tyrosine phosphorylation, despite phosphorylation at other sites in Syk, Syk autophosphorylation would appear crucial for BCR mediated signalling (Kurosaki et al., 1995). Initiation of Syk activation required for this autophosphorylation, which itself results in a rapid increase in Syk activation, may be induced by phosphorylation of Syk at other sites besides Y518/519 by Src PTKs (Kurosaki et al., 1995). Alternatively, the role of Src PTKs in Syk activation may be the phosphorylation of Igα and Igβ, which allows binding of Syk, and which may provide the initial event required for Syk activation (Kurosaki et al., 1995). Syk has been reported to be associated with Lyn in activated B (Sidorenko et al., 1995) and mast cells, (Amoui et al., 1997), and synergistic protein phosphorylation was seen in cells co-transfected with Lyn and Syk compared to cells transfected with one or the other (Kurosaki et al., 1994). A representation of early events involving Syk at the BCR in resting and stimulated cells is depicted in Fig.1.14.
1.8.2.3 Btk

The role of Btk in early events of BCR signalling is less well understood. However, impaired B cell development observed in Btk deficient and xid mice (Rawlings et al., 1993; Thomas et al., 1993; Kerner et al., 1995; Khan et al., 1995), and XLA patients (Lovering et al., 1997), and the fact that Btk is tyrosine phosphorylated and activated on ligation of sIgM in B cells (de Weers et al., 1994; Hinshelwood et al., 1995), indicate the importance of Btk in BCR mediated B cell signalling.

Despite in vitro evidence (section 1.4.5), no in vivo interactions have been reported between Btk and any Src PTKs. Nor has Btk been reported to associate with the BCR. Nevertheless, activation of Btk on BCR ligation has been shown to be dependent on activated Src PTKs (Mahajan et al., 1995; Afar et al., 1996; Rawlings et al., 1996). Phosphorylation at Y551 within the activation loop of the Btk catalytic domain is proposed to result in the activation of the catalytic activity as discussed in section 1.4.1.2. Trans-phosphorylation at Y551 within Btk has been shown to be the result of the action of mainly Src PTKs (Rawlings et al., 1996), with one report indicating the critical involvement of Syk (Kurosaki and Kurosaki, 1997).

Co-expression of Btk with active Src PTKs in COS cells causes Btk, but not the Src PTKs, to become increasingly tyrosine phosphorylated and activated. The site of tyrosine phosphorylation was mapped to Y551 (Mahajan et al., 1995). The same result was found on co-expression of Btk with Lyn or Fyn, but not Syk, in EBV transformed B cells, and the increase in Btk tyrosine phosphorylation was the same as that observed in BCR stimulated B cells (Rawlings et al., 1996). Co-expression of a Lyn kinase inactive mutant and Btk in the non-haematopoietic NIH-3T3 fibroblast cell line resulted in no tyrosine phosphorylation of Btk. This indicates that in the absence of any other haematopoietic PTKs, Lyn is required for transphosphorylation of Btk, and that this process cannot be initiated by Btk autophosphorylation (Rawlings et al.,
Co-transfection of active Lyn with a Y551F Btk mutant into NIH-3T3 fibroblasts resulted in a 90% decrease in Btk tyrosine phosphorylation compared to wild type Btk. This confirms that Lyn is required for trans-phosphorylation at Y551 in this system, which lies within a Src like phosphorylation consensus sequence, and that maximal Lyn dependent phosphorylation of Btk requires both an intact Btk Y551, and Lyn kinase activity (Rawlings et al., 1996). Lyn induced tyrosine phosphorylation of Btk was also impaired when Lyn was transfected with a Btk kinase negative mutant, indicating that maximal Btk tyrosine phosphorylation is also dependent on auto-phosphorylation, presumably induced by transphosphorylation at Y551 (Rawlings et al., 1996). In CD45 deficient cells and fibroblasts co-expressing Btk and Csk, Btk phosphorylation is considerably impaired, providing further evidence for the requirement of Btk activation for Src PTKs (Afar et al., 1996; Pao et al., 1997). However due to the fact that Btk phosphorylation is not totally abrogated in CD45 deficient cells, Btk may be a substrate for other kinases, such as Syk (see later).

Studies on Btk*, an activated mutant with an E41K mutation in the PH domain, show that its transforming activity in NIH-3T3 cells is associated with increased membrane association and tyrosine phosphorylation of Btk*. The sites of phosphorylation of Btk* are the same as those mapped to wild type Btk in BCR activated B cells, and Btk co-expressed with Lyn in COS cells (Afar et al., 1996; Rawlings et al., 1996). Btk*’s transforming potential is upregulated on co-expression with Lyn, and downregulated in the presence of Csk (Afar et al., 1996). These studies into Btk* are consistent with, and provide further evidence for a close relationship between Src mediated Btk phosphorylation, Btk activation and Btk membrane association.
Figure 1.14 Early PTK mediated events on BCR ligation. (i) The Src PTK, Lyn is regulated by the opposing functions of CD45 and Csk acting at the C-terminal tail regulatory Tyr. On BCR ligation Lyn is activated (Lyn+), and phosphorylates the Tyr residues within the ITAMs of Igα and Igβ. (ii) Activated Lyn also phosphorylates Btk, initiating its autophosphorylation and activation. It may also play a role in Syk activation. (iii) Syk is recruited to the phosphorylated ITAM, a proportion of Btk locates to the membrane, and their concerted actions result in the activation of PLCγ. Dotted lines indicate phosphorylation events leading to activation, slashed lines indicate phosphorylation leading to repression, solid lines indicate activation events, + indicates activated proteins.
Although co-transfection of Btk and Syk did not result in Btk activation (Mahajan et al., 1995), Btk is tyrosine phosphorylated in Lyn deficient B cells when Syk activity is upregulated by transphosphorylation at Y518/519 (Kurosaki and Kurosaki, 1997). This Syk induced tyrosine phosphorylation of Btk is not observed until ~10 minutes after BCR ligation, and is not observed in Syk deficient DT40s. The timing of Syk induced tyrosine phosphorylation of Btk correlates with studies on the temporal activation of PTKs which reported Src PTK activation to occur within seconds of BCR ligation, followed by Btk activation peaking at ~3 mins, and finally Syk activation reaching a maximum at ~10 mins post BCR stimulation (Saouaf et al., 1994). As Btk and Syk activity decrease at a similar rate between 10-60 mins after stimulation, Syk induced activation of Btk could be masked by the initial Src induced Btk activation. The physiological relevance of Syk induced activation of Btk is not known, however there is evidence that Btk and Syk are involved in the same pathway that leads to PLCγ activation, IP₃ generation and the mobilisation of calcium (section 1.8.2.2/3 and 1.8.3.1).

Whichever tyrosine kinase is responsible, transphosphorylation at Y551 is essential for BCR signalling. Transfection of Btk deficient DT40s with Btk Y551F results in no Btk tyrosine phosphorylation and abrogates BCR induced calcium mobilisation (Kurosaki and Kurosaki, 1997). A direct consequence of transphosphorylation at Y551 is Btk autophosphorylation, phosphopeptide analysis of tyrosine phosphorylated Btk, purified from NIH 3T3 cells where it was overexpressed with Lyn, plus peptide sequencing of an auto-phosphorylated Btk GST-SH3 domain fusion protein mapped the site of Btk autophosphorylation to lie within the SH3 domain, at position Y223 (Park et al., 1996). The importance of Y223 and phosphorylation at this site is less clear. Transfection of Btk Y223F into Btk− DT40s merely results in a slight delay in the observed increase in Btk tyrosine phosphorylation, and Y223 does not appear to be essential for BCR signalling (Kurosaki and Kurosaki, 1997).
Y223 has however been shown to be involved in Btk regulation. Deletion of the SH3 domain, or mutation of Y223 to F in Btk* results in a further increase in its transforming activity (Park et al., 1996). Unless a Y-F substitution at this point interferes with SH3 domain function, as Btk* is phosphorylated constitutively at Y223, the increase in the transforming activity of Btk* on mutation of Y223 to F indicates a negative regulatory role for the SH3 domain, and possibly for phosphorylation at Y223. From structural comparisons with the SH3 domains of Itk and Fyn, Btk Y223 is predicted to lie at the surface of the SH3 domain binding groove (Andreotti et al., 1997; Noble et al., 1993), and its mutation to F may disrupt ligand binding, as may phosphorylation at this site in wild type Btk (Park et al., 1996).

1.8.3 Effectors of Btk mediated BCR signalling

The overall cellular protein tyrosine phosphorylation observed following BCR stimulation of Btk deficient DT40 cells was not significantly different to that seen in wild type cells (Takata and Kurosaki, 1996). Further investigations showed that the activities of neither Syk nor Lyn were affected by the absence of Btk. Lyn/ Syk deficient cells on the other hand showed an almost complete defect in tyrosine phosphorylation induction, indicating that these PTKs function upstream of Btk (Takata and Kurosaki, 1996), despite the report that Syk activation follows that of Btk (Saouaf et al., 1994). As Syk activation is dependent upon ITAM tyrosine phosphorylation, the possibility that Btk is responsible for Igα/Igγ phosphorylation is excluded. Btk deficient cells also showed no defects in the BCR mediated activation of MAPK, indicating that Btk is not required for coupling the BCR to the Ras pathway.

1.8.3.1 PLCγ and PKC

Activation of PLCγ2 (the only PLCγ isoform expressed in DT40 cells) was not observed on BCR ligation of Btk deficient cells (Takata and Kurosaki, 1996),
nor was it observed on the stimulation of Syk deficient cells (Takata et al., 1994).

Phosphorylated and activated PLC\(\gamma\) is responsible for the hydrolysis of PIP\(_2\), releasing diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP\(_3\)), which result in the activation of PKC and the mobilisation of intracellular stores of calcium, respectively (DeFranco, 1997) (see Fig.1.15). Ca\(^{++}\) release and signalling results in cellular effects such as B cell proliferation, and regulation of apoptosis. It also regulates the activation of several transcription factors, and ser/thr and tyrosine kinases (for review see Tsien and Tsien, 1990; Clapham, 1995). Btk and Syk deficient cells were incapable of IP\(_3\) release and Ca\(^{++}\) mobilisation on BCR ligation, indicating that these PTKs do function on a similar pathway in BCR induced cell activation (Takata et al., 1994; Takata and Kurosaki, 1996).

PLC\(\gamma\)2 activation is almost totally lost in Syk\(^{-/-}\) cells (Takata et al., 1994), whereas in Btk\(^{-/-}\) cells its activation is reduced about 3 fold (Takata and Kurosaki, 1996). Ca\(^{++}\) mobilisation was restored to normal levels in Btk\(^{-/-}\) cells transfected with wild type Btk, but transfectants expressing Btk with mutations in the PH, SH2 or kinase domains showed a much smaller Ca\(^{++}\) and IP\(_3\) response (Takata and Kurosaki, 1996). As Syk appears to be unaffected by the absence of Btk in these DT40 cells, it is possible that Syk regulates Btk in BCR mediated PLC\(\gamma\)2 activation.

PLC\(\gamma\)1 has three distinct sites of tyrosine phosphorylation required for its activation on cell stimulation through growth factor receptors (Lee and Rhee, 1995), and one key regulatory site at Y783 is a target of Syk (Law et al., 1996a). Indeed, PLC\(\gamma\) tyrosine phosphorylation is dependent upon both maximal Syk activation and an interaction between the C-terminal SH2 domain of PLC\(\gamma\) and the tyrosine phosphorylated linker region of Syk (Law et al., 1996a).
PLCy activation results in the production of DAG which activates PKC (Bijsterbosch et al., 1985). The PKCβ1 isoform has been reported to bind and phosphorylate Btk resulting in a decrease in Btk activity (Yao et al., 1994). As another PKC isoform, PKCμ has been reported to similarly bind Syk and decrease its ability to phosphorylate PLCγ (Sidorenko et al., 1996), the activation of PKC may act as feedback inhibitory role for PIP2 hydrolysis, (see Fig.1.15).

Further evidence that Syk and Btk may function in a similar BCR mediated pathway which leads to PLCγ activation comes from comparisons of knock-out mice. Syk (Cheng et al., 1995; Turner et al., 1995), Btk (Kerner et al., 1995; Khan et al., 1995), PKCβ (Leitges et al., 1996), Igα (Torres et al., 1996), and Igβ deficient (Gong and Nussenzweig, 1996), and xid (Rawlings et al., 1993; Thomas et al., 1993) mice all have similar phenotypes. Similarities include (i) a total or partial block in antigen independent B cell development, and in Btk and Syk deficient B cells, a secondary block in development of the reduced numbers of B cells to reach the antigen dependent stages of B cell development occurs, (Cheng et al., 1995; Hendriks et al., 1996), (ii) a failure to develop the peritoneal CD5+ B-1 population of B cells, (iii) reduced levels of circulating immunoglobulins, and (iv) a defect in T independent type II antigen induced responses. As mice deficient in these proteins share so many phenotypic similarities, it implies that the same signalling pathway in all these animals may be disrupted, and that the proteins defective in these mice may all function on that same pathway. Further evidence for a role for Btk in the BCR mediated signalling pathway in humans comes from studies of agammaglobulinemia patients found to have μ chain mutations (Yel et al., 1996).
Figure 1.15 Phosphoinositides in Btk mediated BCR signalling
1.8.3.2 PI3-Kinase

PI3-K, named after its ability to catalyse the phosphorylation of the D-3 hydroxyl residues in the inositol headgroups of PI, PI4 monophosphate or PI 4,5-bisphosphate, was first described as a lipid kinase activity associated with several activated PTKs including v-Src (for review see Abraham, 1996; DeFranco, 1997). The PIP₃ products of the PI3-K reaction have been implicated as second messengers involved in a host of cellular responses (Panayotou and Waterfield, 1997).

PI3-K is recruited to the plasma membrane on cell activation, activating multiple signal transducing pathways (Klippel et al., 1996), and bringing its enzymatic activity into contact with its membrane associated substrates. PI3-K is also tyrosine phosphorylated and activated on BCR and CD19 crosslinking (Gold et al., 1992; Tuveson et al., 1993), and when bound by Lyn and Fyn SH3 domains in vitro (Pleiman et al., 1994). PI3-K inhibition with wortmannin blocks Ig production in human B cells (Beckwith et al., 1996). The interaction reported between the PI3-K product PIP₃ and the PH domain of Btk (Salim et al., 1996) implies a functional relationship between the two proteins, however the consequence of this interaction is not known. PIP₃ interacting with Btk may be important for Btk dependent activation of PLCγ (Fig. 1.15), as a reduction in cellular PIP₃, due to enhanced actions of the tyrosine phosphorylated inositol polyphosphate 5-phosphatase, SHIP, inhibits PLCγ activation (Sarkar et al., 1996), (Fig. 1.15). Thus PI3-K activity may be an upstream effector of Btk activity.
1.8.3.3 Heterotrimeric G proteins

Heterotrimeric guanine nuclear binding proteins (G proteins) consist of an \( \alpha \beta \) and a \( \gamma \) subunit. Stimulation of a G protein coupled receptor leads to dissociation of the \( \beta \gamma \) and \( \alpha \) subunits, which can both initiate downstream events. The intrinsic GTPase activity of the \( \alpha \) subunit is involved in the downregulation of G protein activity (Cantrell 1994). G proteins are involved in the regulation of key cellular events including transmembrane signalling, proliferation and differentiation (Harnett and Rigley, 1992). Signalling via \( \mathrm{slg} \) receptors can be blocked by the introduction of the G protein blocker GDP\( \beta \mathrm{S} \) (Gold et al., 1987), suggesting a role for these proteins in BCR mediated signal transduction. G protein mediated signals from muscarinic acetylcholine (mACh) receptors have also been reported to require Lyn and Syk in B cells (Wan et al., 1996). On receptor stimulation, heterotrimeric G proteins dissociate into the \( \beta \gamma \) and \( \alpha \) subunits both of which have been reported to cause Btk activation (Langhans Rajasekaran et al., 1995; Bence et al., 1997). This G protein-tyrosine kinase association may provide a link to extend G protein signalling to a broad range of physiological processes.

1.8.3.4 Other proteins

Other proteins that may be upstream or downstream effectors of Btk have also been reported. A protein of 135 kD has been reported associated with Btk via the Btk PH domain and is a substrate of Btk phosphorylation. This protein, called BAP-135, has no homology with any other known protein and its function is unknown (Yang and Desiderio, 1997).

The SH3 domain has been reported to bind to c-Cbl and WASP as discussed (section 1.4.4). Further recent Btk SH3 \textit{in vitro} associations have been reported with Vav and Sam-68 (Guinamard et al., 1997). Sam-68 is a mitotic target of Src PTKs (Taylor et al., 1995), and it binds activated Src in mitotic cells via both its SH2 and SH3 domains. The product of the \textit{Vav} proto-oncogene is a
Chapter 1

haematopoietically restricted protein containing an SH2 and two SH3 domains as well as a region with homology to the Rho family GEF, Dbl (Hu et al., 1993; Khosravi Far et al., 1994). Vav tyrosine phosphorylation is induced on stimulation through numerous haematopoietic receptors, including the BCR (Bustelo and Barbacid, 1992), and Vav deficient B and T cells have defective antigen receptor responses (Bonnefoy Berard et al., 1996). Vav phosphorylation appears to be dependent on its association with Syk (Deckert et al., 1996), and it is also found associated with CD19. However Vav has been reported to function as a nucleotide exchange factor involved in activation of members of the Ras family (DeFranco, 1997). Although it has been proposed to be a potential link between the Ras-like GTPases and tyrosine kinases, its involvement, and that of Sam-68, in Btk mediated BCR signalling is unknown.

1.9 Summary, aims and approaches

B cell development is controlled via surface receptors resulting in the regulation of transcriptional activity in the nucleus. Signals must be passed through the cytoplasm and this is performed by small second messengers such as Ca++, or more selectively by molecule to molecule cascades of reactions which mediate the activation of limited and specified targets. Important to this latter type of signalling are protein domains, which are capable of mediating highly specific interactions between proteins acting in the signalling cascade, and the phenomena of phosphorylation and dephosphorylation.

Many cellular proteins are involved in B cell signalling through numerous cell surface receptors. Investigations into individual components of signalling cascades are crucial for the understanding of the mechanisms of B cell differentiation and activation which results in the production of antibodies by plasma cells, crucial for the functioning of the humoral immune system. One protein known to be essential for B cell development and activation is Btk,
because a mutation in the gene encoding Btk can cause the immunodeficiency XLA in humans, and in xid mice Btk is mutated at R28.

The aim of the studies presented in this thesis was to attempt to determine some aspects of the role of Btk in B cell signalling. Two approaches were undertaken.

- Btk activity was investigated on stimulation of human B cell receptors CD22 and CD38 in order to try and establish potential *in vivo* signalling pathways that require Btk. Defects in such signalling pathways may explain the block in B cell development in XLA patients and xid mice, and the impaired B cell signalling observed in xid mature B cells.

- An *in vitro* approach using SH3 domain GST fusion proteins of Btk and related PTKs was used in order to examine *in vitro* SH3 domain ligands, providing evidence for what may be *in vivo* interactions. The Btk SH3 domain fusion protein was phosphorylated *in vitro* in order to determine whether Btk autophosphorylation may alter the specificity of ligand binding of the SH3 domain fusion protein.

  In addition, the identification of WASP as a ligand of the SH3 domain of the Btk family of PTKs led to investigations into the expression of WASP in WAS patients.
Chapter 2

2 Materials and Methods
2.1 Reagents and buffers

2.1.1 Antibodies

Antibodies used are shown in Table 2.1

Table 2.1 Antibodies used, source and type

<table>
<thead>
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<th>Specificity</th>
<th>Name/ Cat#</th>
<th>Source</th>
<th>Raised in</th>
<th>Type</th>
</tr>
</thead>
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<tr>
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<td>Katz et al., 1994</td>
<td>rabbit</td>
<td>serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Santa Cruz Biotechnology, SCB</td>
<td>rabbit</td>
<td>serum</td>
</tr>
<tr>
<td>WASP</td>
<td>SK3/ JD1</td>
<td>Banin et al., 1996</td>
<td>rabbit</td>
<td>serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Symons et al., 1996</td>
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<td>serum</td>
</tr>
<tr>
<td>Syk</td>
<td>sc-573/ 4G10</td>
<td>SCB/ Upstate Biotechnology Inc.</td>
<td>rabbit</td>
<td>serum</td>
</tr>
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<td></td>
<td>mouse</td>
<td>monoclonal IgG (mAb)</td>
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<tr>
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<td>T. Lester, this lab</td>
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<td>serum</td>
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<tr>
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<td>SCB/ Dako</td>
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<td>serum</td>
</tr>
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<td>Funaro et al., 1990</td>
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<td>mAb</td>
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<td>D-8406</td>
<td>Sigma</td>
<td>mouse</td>
<td>mIgE-αDNP</td>
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<td>goat</td>
<td>F(ab′)2 fragment</td>
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<tr>
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<td>goat</td>
<td>HRP conjugate</td>
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<td>Sigma</td>
<td>goat</td>
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<td>Sigma</td>
<td>rabbit</td>
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<tr>
<td>Mouse IgG</td>
<td>FO479</td>
<td>Dako</td>
<td>goat</td>
<td>F(ab′)2</td>
</tr>
</tbody>
</table>

2.1.2 General reagents

Unless otherwise stated, all reagents were obtained from Sigma.

Radioactive isotopes [γ32P]-ATP, and [35S] trans label were obtained from ICN Biomedical.
2.1.3 Cell lines

Daudi is a human Burkitt's lymphoma B cell line, Molt-4 is a T cell leukaemia cell line. Wiskott-Aldrich cell lines were human B cells from two Wiskott-Aldrich patients transformed by EBV (Lau et al., 1989).

Syk negative and positive control primary fetal liver mouse mast cells and the WEHI 3B mouse B cell lymphoma were a kind gift of P. Costello, NIMR (Costello et al., 1996).

2.1.4 Buffers

PBS: PBS tablets (Oxoid) were dissolved 1/100ml water
PBS-T: PBS, 0.05% (v/v) Tween-20
STE: 10mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1mM EDTA
NP40 lysis buffer: 1% (v/v) NP40 (Calbiochem), 20mM Tris-HCl pH 8.0, 130mM NaCl, 10mM NaF, 1mM DTT, 20μM leupeptin, 1% aprotinin, 100μM NaVO₄, 1mM PMSF
Transfer buffer: 48mM Tris-HCl, 39mM glycine (Calbiochem), 20% (v/v) methanol (BDH), pH 9.2
2X SDS loading buffer: 125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 2% 2-mercaptoethanol, 0.001% (w/v) bromophenol blue (BDH)
SDS-PAGE running buffer: 25mM Tris-HCl, pH 8.3, 192 mM glycine, 1% (w/v) SDS
TBE: 90mM Tris-HCl, 90mM boric acid, 1mM EDTA, pH 8.0
Kinase buffer: 10mM MgCl₂, 10mM MnCl₂
Phosphorylation buffer: 20mM Pipes, pH 7.0, 20mM MnCl₂, 20mM MgCl₂, 100μM ATP
Glutathione elution buffer: 100mM Tris-HCl, pH 8.5, 100mM NaCl, 10mM glutathione, 5mM DTT
Binding buffer: 20mM Tris-HCl, pH 7.4, 10mM EDTA, 100mM NaCl, 1% (v/v) NP40, 0.2mM Na₃VO₄
**Phosphotyrosine elution buffer:** 100mM phenolphosphate in binding buffer

**Column regeneration buffer:** 1.2 g NaCl dissolved in 10ml water

**Glycine buffer:** 50mM glycine, pH 2.3

**Neutralisation buffer:** 24.2g Tris, 8.7g NaCl, 200μl 0.5M EDTA, 10ml 5% sodium azide, in 100ml H2O, pH 8.0

**L-broth:** 10g bactotryptone (Difco), 5g yeast extract (Difco), 5g NaCl dissolved per litre of water. 15g/L bacto-agar (Difco) added for LB agar plates. 100μg/ml ampicillin was used in L-broth and agar plates for selection of antibiotic resistance

**SOB medium:** 20g bactotryptone (Difco), 5g yeast extract (Difco) and 0.5g NaCl dissolved in 1 litre of water/25mM KCl/10mM MgCl2, pH 7.0

**SOC medium:** 1 litre of SOB medium plus 20ml 1M glucose

**6X DNA loading buffer:** 1.5g Ficoll per 100ml water, bromophenol blue, xylene cyanol

**TE:** 10mM Tris-HCl, pH 8.0, 1mM EDTA

**Immunoblot buffer (H360B):** 200mM Tris-HCl, pH 7.5, 10% (v/v) FCS, 0.05% (v/v) Tween-20, 5% (w/v) non-fat milk (Marvel)

**Immunoblot buffer (4G10):** 0.05% (w/v) non-fat milk in PBS

**Immunoblot buffer (all antisera except above):** 5% non fat milk (w/v) in PBS-T

**FP storage buffer:** 20mM Tris-HCl, pH 8.0, 130 mM NaCl, 1mM DTT, 10% (v/v) glycerol

**PCR reaction buffer:** Per 50 μl reaction- 1ng Btk cDNA, 200μM dATP, dCTP, dTTP and dGTP (Promega), 1.5 mM MgCl2 (Bioline), 1X NH4Cl buffer (Bioline), 1pM forward and reverse primers, 0.5μl Taq polymerase (Bioline)

**2.1.5 PCR primers**

PCR primers were designed to contain restriction digest sites for *Bam HI*, (forward primers, F) and *Eco RI* (reverse primers, R) so that digested products
would ligate in the correct orientation in similarly digested pGEX-2T. For primers, see Table 2.2.

Table 2.2. **PCR primers used for the amplification of the Itk and Tec SH3 domain coding regions.** The annealing temperature for both Itk and Tec forward and reverse primers was 60°C.

<table>
<thead>
<tr>
<th>GST fusion protein:</th>
<th>Itk-SH3</th>
<th>Tec-SH3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Primer</strong></td>
<td>CTGAGGATCCGTGGTCAATT</td>
<td>CTGAGGATCCGTTTGTAGCC</td>
</tr>
<tr>
<td></td>
<td>GCCTTATATGAC</td>
<td>ATGTATGATTTC</td>
</tr>
<tr>
<td><strong>Reverse Primer</strong></td>
<td>TATCAGAATTCTTTTCCA</td>
<td>TATCAGAATTCTTTCCGTT</td>
</tr>
<tr>
<td></td>
<td>CCAGATAACTGCTTG</td>
<td>ACGTAATTACTTG</td>
</tr>
<tr>
<td><strong>Size of Product:</strong></td>
<td>156nt</td>
<td>153nt</td>
</tr>
<tr>
<td><strong>Size of Product: Amino acids</strong></td>
<td>52 aa</td>
<td>51 aa</td>
</tr>
<tr>
<td><strong>Predicted Molecular weight of product, including GST moiety</strong></td>
<td>~31kD</td>
<td>~31kD</td>
</tr>
</tbody>
</table>

2.2 Cell staining

Approximately $10^6$ cells were used for staining with first step antibodies at a concentration of $\sim 1\mu g/ml$ in PBS, with 0.05% BSA, 0.02% sodium azide, for 30 mins at 4°C. Cells were washed twice and incubated with fluorescent isothiocynate (FITC) conjugated goat anti-mouse F(ab')$_2$ fragments. Samples were analysed by flow cytometry using a FACSCalibur.

2.3 Cell stimulation and immunoprecipitation

2.3.1. Cell culture

The Daudi lymphoma B cell line, Molt-4 leukemic T cell line and EBV transformed Wiskott-Aldrich patient B cells were grown in RPMI media
containing 10% (v/v) FCS, 5mM L-glutamine and 10μg/ml ciprofloxin (Bayer) at 37°C in 5% CO₂ to a density of 0.5-1X10⁶/ml.

Mouse primary mast cells were grown in RPMI complete medium containing, 10% (v/v) FCS, 10% sterile filtered WEHI 3B conditioned media, 5mM L-glutamine, 5% non essential amino acids (Gibco BRL), 5% pyruvate (Gibco BRL), 1 unit penicillin/ml, 0.5μg streptomycin/ml.

The WEHI 3B mouse B cell line was grown in DMEM containing 10% FCS, 5mM L-glutamine, 5X10⁻³M 2-mercaptoethanol and 1 unit penicillin/ml, 0.5μg streptomycin/ml. Conditioned media was collected through a 2 micron filter when the cell density was several million per ml and the media had turned yellow.

2.3.2. Cell stimulation

Daudi cells were washed in RPMI with no additives and resuspended in a minimal volume of the same. Stimulating antibodies against surface IgM and CD22 were added to a final concentration of 100μg/ml. Antibodies against CD38 were added to a final concentration of 10μg/ml. Stimulating antibodies were incubated with cells in RPMI at 37°C for times indicated before lysis.

Mouse mast cells were primed overnight with 0.5μg/ml of mIgE-anti-DNP at 10⁶ cells/ml in complete RPMI medium. Cells were washed in RPMI with no additives and resuspended at 10⁷/ml in RPMI 0.1% bovine serum albumin (BSA). Cells were stimulated by the addition of 100ng/ml DNP-HSA at 37°C for the length of time indicated prior to lysis.

2.3.3. Cell lysis

Cells were lysed for 10 mins on ice with ice cold NP40 lysis buffer at a concentration of 3X10⁷/ml. Mast cells were lysed in NP40 lysis buffer
containing 10X normal quantities of added protease inhibitors. Lysates were centrifuged at 12,000g at 4°C for 15 mins to remove any insoluble material.

2.3.4. Immunoprecipitation

All steps were performed at 4°C. Lysates of cells were pre-cleared by incubation with Gammabind Sepharose beads (Pharmacia Biotech) previously incubated with normal rabbit serum and washed thoroughly with NP40 lysis buffer before being added to the lysates. Lysate from 4X10^6 cells was immunoprecipitated with ~1μg of polyclonal rabbit antiserum attached to 10μl Gammabind Sepharose beads (Pharmacia Biotech) by rolling for 2 hr. Beads were washed 5 times with NP40 lysis buffer. Samples to be analysed at this point were re-suspended and boiled for 3mins in 2X sample buffer and resolved by SDS-PAGE.

2.3.5 SDS-PAGE analysis

Resolving gels contained 8-10% (w/v) acrylamide, (containing 0.8% bisacrylamide) (Protogel, National Diagnostics) in 375mM Tris-HCL, pH 8.8, 0.1% (w/v) SDS. Stacking gels were poured on top of set resolving gels and contained 4% acrylamide in 125mM Tris-HCl, pH 6.8. Gels were polymerised using 0.1% (w/v) ammonium persulphate and 0.1% (v/v) Temed.

Samples loaded on minigels (8x5cm) were electrophoresed across 70-150V. Samples loaded onto large gels (12x17cm) were electrophoresed across 40-50V.

2.3.6 Coomassie brilliant blue staining

250 mg/L Coomassie brilliant blue was dissolved in an aqueous solution containing 40% (v/v) methanol, and 10% (v/v) acetic acid. Gels were stained for 30 mins before destaining with an aqueous solution containing 10% (v/v) methanol, and 5% (v/v) acetic acid. Gels were vacuum dried.
2.3.7 Protein transfer to nitrocellulose filters

Gels, nitrocellulose membrane (BDH) and 3MM paper (Whatman) were soaked in transfer buffer for 15 mins prior to transfer of protein from the gel to the membrane using a Biorad semi-dry blotter according to manufacturer's instructions. Minigels were transferred for 21 mins at 12V and large gels for 50 mins at 18V. Membranes were blocked of non-specific binding sites with 5% non fat milk in PBS at rt for ~1h.

2.3.8 Immunoblotting

Between 0.1 and 1µg of mouse mAb or rabbit polyclonal antiserum per ml of blotting buffer were incubated with nitrocellulose filters for 1-3 h at RT. Filters were washed 4 times with PBS-T before incubation for 1-2 h with horse radish peroxidase (HRP) conjugated secondary antibody. A 1/1000 dilution of rabbit anti-mouse IgG-HRP was used for detection of primary mouse mAb and a 1/1000 dilution of goat anti-rabbit IgG-HRP was used for detection of primary rabbit polyclonal antiserum. Nitrocellulose membranes were then washed again four to five times with PBS-T before incubation for 1 min with enhanced chemiluminescence (ECL) reagent (Amersham Life Science). Reactive species were visualised by exposure to X-ray film for between 5s to 15 min.

2.3.9. Metabolic labelling

Cells were washed in RPMI lacking cysteine and methionine and resuspended in this medium containing 20µCi/ml [35S]-translabel at 10^6 cells/ml. Cells were incubated at 37°C in 5% CO\textsubscript{2} for 5 h before being washed in RPMI and lysed as described in section 2.3.3. Precipitated proteins were separated by SDS-PAGE and visualised by autoradiography of dried gels.
2.3.10 *In vitro* kinase assay

Immunoprecipitates and fusion protein precipitates (see sections 2.3.4 and 2.6.1) were performed as described up to the stage where beads had been washed 3 times. All the liquid was removed from the beads using a Hamilton syringe (Hamilton Co.) being careful not to remove any beads. Beads were then resuspended in 30μl of kinase buffer containing 5μCi of [γ³²P]-ATP/sample. Reaction mixtures were incubated for 10 mins at RT. The beads were washed twice with NP40 lysis buffer to remove surplace unincorporated radioactive ATP, and the beads suspended in 2X SDS sample buffer. Sample were boiled and resolved by SDS-PAGE analysis. Incorporated phosphate was visualised by autoradiography of dried gel, or blotted onto nitrocellulose membrane and then visualised.

2.4 Production of Itk and Tec GST fusion proteins

2.4.1 Precipitation of DNA and PCR primers

cDNA was precipitated by the addition of 2.5 volumes of ethanol (BDH) and 0.1 volumes of 3M sodium acetate at -20°C for 1h to o/n. Samples were then centrifuged at 12 000g for 15 mins. Pellets were washed with 70% ethanol and recentrifuged as above. Pellets were left to dry at room temperature before being resuspended in water. PCR primers were precipitated from ammonia stock solution. Resuspended pellets were quantified by measuring the optical density (OD) at 260nm. An adsorption of 1 OD unit was taken to equal an oligonucleotide concentration of 33μg/ml.

2.4.2. Construction of fusion protein expression vectors

The SH3 domain coding regions of cDNA were amplified by PCR using the Itk and Tec primers shown in Table 2.2. The amino acid sequences of the Itk
and Tec SH3 domains are shown in Table 2.3. Reactions were performed in 50μl volumes under the following conditions:

1x 3 mins at 94°C, 30x (30 secs at 94°C, 1 min at 60°C, 30 secs at 72°C), 1x 10 mins at 72°C.

Reaction products were resolved by electrophoresis using 1-2% agarose gels. 1μg 1kb ladder (Gibco BRL) was electrophoresed to enable estimation of the size and quantity of DNA. To purify the PCR products, 60μl water was added to increase the reaction mixture volume. Any protein present was degraded by vortexing the diluted reaction mixture with 100μl phenol/chloroform/isoamylalcohol (25:24:1) (Biometra/Merck/Merck). The mixture was centrifuged for 10 mins at 12 000g and the aqueous layer was removed to a fresh tube. Any contaminating phenol was removed by the addition of an equal volume of chloroform/isoamylalcohol (Merck) in a 49:1 ratio, and the mixture vortexed and centrifuged. The aqueous layer was removed to a fresh tube and the DNA was precipitated with sodium acetate and ethanol as described in section 2.4.1. Resuspended DNA was digested with a 3 fold excess of *Bam* HI and *Eco* RI (Promega) for 2 h at 37°C in the manufacturer’s buffer. Digested products were phenol/chloroform cleaned as described above.

pGEX-2T vector (Pharmacia Biotech) was also digested with *Eco* RI and *Bam* HI and purified as above. Digested PCR products were ligated with 20 ng digested pGEX-2T. Ligations were performed using T4 ligase (Promega) in the manufacturer’s buffer at 4°C overnight.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SH3 Domain Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itk</td>
<td>VVIALYDYQTNDPQELALRRNEEYCLLDSSEIHWWRVQDRNGHEGYVPPSYL</td>
</tr>
<tr>
<td>Tec</td>
<td>VVAMYDFQAAGEHDLRLERGQELYLILEKNDVHWWRARDKYGNEYIPSNYV</td>
</tr>
</tbody>
</table>
2.4.3. Transformation of XL1-blue *E. coli*

Transformation competent *E. coli* were prepared by treatment with CaCl$_2$. *E. coli* were grown to log phase (OD 600nm=0.6) in 40 ml L-broth, centrifuged at 2 000g at 4°C for 5 mins and resuspended in 20 ml ice cold 50mM CaCl$_2$ for 50 mins. Bacteria was then pelleted by centrifugation and resuspended in 2ml ice cold 50mM CaCl$_2$ for 30 mins. 100μl of resuspended bacteria was then added to an equal volume of 100 mM Tris-HCl, pH 7.5, containing 10 ng ligation mix. The mixture was kept on ice for 10 mins and then heated to 42°C for 2 mins before cooling on ice. 1 ml SOC medium was added and cultures were incubated at 37°C for 20 mins. 200μl aliquots were plated on SOB/agar containing 100μg/ ml ampicillin and incubated at 37°C o/n. Resistant colonies were picked and grown o/n in L-broth. Cultures were diluted 1/100, grown to log phase (4h at 37°C) and a 1 ml sample taken, pelleted and lysed by the addition of 2X SDS sample buffer. Remaining cultures were induced to express fusion protein by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) (Calbiochem) to 100μM. Cultures were grown for a further 2-4 h before a 100μl sample was taken as described above. Pre and post induction samples were resolved by SDS-PAGE and stained with Coomassie brilliant blue to assay for the presence of inducible fusion protein. Cultures with inducible fusion protein were frozen as 50% glycerol stocks at -70°C.

2.4.4. DNA sequencing of transformed *E. coli*

pGEX-2T plasmid was extracted from bacterial colonies grown in ~5ml L-broth o/n using the Magic/Wizard Miniprep (Promega) system according to the manufacturer’s instructions. 3μg of plasmid was alkaline denatured by the addition of 200mM NaOH, 200μl EDTA, followed by incubation at 37°C for 30 mins and sequenced using the Sequenase version 2 kit (USB) according to the manufacturer’s instructions. For each insert, the PCR primers used for its
amplification were used to sequence it in forward and reverse directions. Reaction products were resolved by electrophoresis on 6M urea gels and visualised by autoradiography.

2.4.5. Affinity purification of fusion proteins

Selected clones were grown o/n from scrapings of frozen stocks. Cultures were then diluted and grown to log phase as discussed in section 2.4.3. Induced cultures were grown for 4 h at 37°C. Bacteria were then pelleted by centrifugation at 2000g for 10 mins. The following steps were performed at 4°C. Pellets were washed with STE and resuspended in STE containing 100μg/ml lysozyme for 10 mins. Bacteria were lysed by the addition of N-laurylsarcosine to 1.5% (w/v). After 20 min lysates were centrifuged at 15000g for 1 h to remove insoluble material. Triton X-100 was added to the supernatants to a final concentration of 2% (v/v). Glutathione-Sepharose 4B (Pharmacia Biotech) beads were added and incubated with rolling for 1 h to bind fusion protein. Beads were pelleted and treated with 50mM Tris-HCl, pH 7.4, 2mM ATP, and 10mM MgSO₄ for 10 mins at 37°C to rid it of bacterial dna K protein, then washed 5 times with PBS and stored as a 10% (v/v) mixture with FP storage buffer at -70°C. Analysis of purified samples was performed on thawed aliquots to account for the affects of freezing.

2.5 Tyrosine Phosphorylation of the Btk GST-SH3 domain fusion protein

2.5.1. Production of baculovirus Btk

*Spodoptera frugiperda* (Sf9) cells were maintained as described by Summers and Smith 1987. Cells were cultured in IPL4-1 supplemented with 19% FCS, 2% yeastolate, 1% lipids concentrate, 1% Fungizone and 0.1% Gentamycin. Plasmid containing human Btk cDNA was transferred to a modified version of the baculoviral *Autographa californica* multiple nuclear
polyhedrosis virus genome (Baculogold, Pharminigen) using lipofectin. Sf9 cells were transfected with Baculogold DNA and recombinant transfer plasmid. Recombinant virus was isolated by plaque assay, harvested, and used to reinfect Sf9 cells from which kinase active Btk was extracted. (Work performed by S. Hinshelwood, this laboratory, and K. Salim, Ludwig Institute for Cancer Research, Middlesex Hospital, London).

2.5.2 Phosphorylation of the Btk GST-SH3 fusion protein with baculovirus Btk

Btk GST-SH3 fusion protein was washed in 1ml of phosphorylation buffer. Fusion protein was resuspended in ~0.5 ml phosphorylation buffer and baculovirus Btk was added to a concentration of 30 ng per 1µg of fusion protein. The mixture was incubated with rolling at room temperature for 1h. The fusion protein was washed twice in NP40 lysis buffer, once in 2% Triton X-100, 20mM Tris-HCl pH 8.0, 130 mM NaCl, and once in 1M NaCl. Fusion protein was tested for phosphotyrosine content by immunoblotting with the anti-phosphotyrosine monoclonal antibody, 4G10.

2.5.3 Purification of phosphorylated Btk GST SH3

Fusion protein was eluted from glutathione Sepharose 4B beads (Pharmacia Biotech) by incubation with glutathione elution buffer. Beads and buffer were rolled together at 4°C for at least 1h. A second round of elution increased the yield of eluted fusion protein. A third round however failed to elute anymore significant quantities of fusion protein from the beads.

Eluted fusion protein was diluted 10X into binding buffer. 1ml of protein A Sepharose beads to which 4G10 mAb was covalently linked (Upstate Biotechnology Inc.) was incubated with rolling with the eluted fusion protein in binding buffer at 4°C for 1-2 hr. The protein A Sepharose:4G10 beads were then washed 5 times in fresh binding buffer before being eluted of phosphotyrosine containing proteins with phenolphosphate phosphotyrosine elution buffer. The column was eluted of non-specific proteins with glycine.
buffer into neutralisation buffer, regenerated with regeneration buffer, and stored in PBS 0.02% azide.

The phosphotyrosine containing phenolphosphate eluate was diluted 10X in more binding buffer and incubated with fresh glutathione Sepharose 4B beads, rolling for 1 h at 4°C. The beads were washed three times in binding buffer, once in PBS and were stored in FP storage buffer at -70°C. Recovered tyrosine phosphorylated fusion protein was quantitated by resolution by SDS-PAGE and staining with Coomassie brilliant blue.

2.6 Analysis of fusion protein binding ligands

2.6.1 Fusion protein precipitation

5-10μg (unless otherwise stated) of fusion protein attached to glutathione Sepharose beads was incubated with cell lysates from 4X10^6 (unless otherwise stated) cells, which may have been metabolically labelled (section 2.3.9), obtained as described in sections 2.3.1-2.3.3, at 4°C for 2 h, rolling. Beads were washed with NP40 lysis buffer and either resolved by SDS-PAGE and stained with Coomassie brilliant blue, dried and laid against X-ray film, or immunoblotted as described in sections 2.3.6-2.3.8. Fusion protein precipitates were subjected to in vitro kinase assays as were immunoprecipitates (section 2.3.10). Fusion protein ligands are thus detected in the same manner as proteins which are immunoprecipitated.

2.6.2 Peptide competition assays

Peptides (Alto Biosciences), see Table 2.4, were incubated with cell lysates from 4X10^6 stimulated cells for 30 mins. Peptides were added such that the final peptide concentrations varied from 2-66μM. Phosphorylated Btk GST-SH3 fusion protein was used to precipitate from the peptide containing lysates as normal, section 2.6.1. Ligands attached to the washed fusion protein were
subjected to an *in vitro* kinase assay (section 2.3.10), resolved by SDS-PAGE and detected by autoradiography of the dried gel.

Table 2.4 **Peptides derived from Btk SH3 domain.** Phosphorylated tyrosine is bold and underlined.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence (residues 220-231 of Btk, excluding GGG)</th>
<th>Position of phosphotyrosine within full length Btk</th>
</tr>
</thead>
<tbody>
<tr>
<td>pep</td>
<td>GGGALYDYMPMNAN</td>
<td>-</td>
</tr>
<tr>
<td>pY-223 pep</td>
<td>GGGALYDYMPMNAN</td>
<td>Y223</td>
</tr>
<tr>
<td>pY-225 pep</td>
<td>GGGALYDYMPMNAN</td>
<td>Y225</td>
</tr>
</tbody>
</table>

2.7. Investigating Wiskott-Aldrich Syndrome patients for expression of WASP

2.7.1 Patient material

Blood samples of between 3-5ml were collected in EDTA coated tubes from patients diagnosed as having WAS on the basis of sex, thrombocytopenia with small platelets, eczema, immunodeficiency and in some cases family history. Samples were also collected from normal controls. Blood samples were diluted 1:1 in PBS and mononucleated cells were isolated by Ficoll (Pharmacia Biotech) separation. EBV transformed cell lines of patients 9 and 11 (lines GB and PM (Lau et al., 1989)) were cultured in RPMI supplemented with 10% (v/v) FCS, 5mM L-glutamine and 10μg/ml ciprofloxin (Bayer) at 37°C with 5% CO₂.

2.7.2 WASP immunoblotting

Cells from blood samples or from EBV cell line cultures were counted after washing in PBS and lysed in 1ml NP40 lysis buffer per 3X10⁷ cells, on ice
for 10 mins. Lysates were cleared by centrifugation. A portion of each sample was diluted 1:1 in 2X SDS sample buffer and resolved by SDS-PAGE analysis (section 2.3.5). Each lane contained 10μl of lysate, equivalent to 3X10^5 cells. Protein was transferred to nitro-cellulose membrane as described in section 2.3.7 and immunoblotted with both SK3 and JD1 anti-WASP antisera, and anti PKCβI (section 2.3.8).

2.7.3 Enrichment for WASP using Btk GST-SH3

Remaining patient lysates, up to 0.5ml when available, were incubated with 10μg Btk GST-SH3 or 10μg GST fusion protein. Fusion protein was washed, resuspended in 2X SDS sample buffer and resolved by SDS-PAGE analysis. Gel lanes contain precipitates from the lysate of 1.5X10^7 cells (0.5ml lysate). Protein was transferred to nitrocellulose membrane and immunoblotted with SK3 anti-WASP antiserum.
Chapter 3

Btk is not activated in human B cells on stimulation through CD22 and CD38

3.1 Introduction

Btk, as discussed in section 1.3.1.2, is activated through several receptors in mouse cell lineages. No activation of Btk has been reported in human lymphoid cells however, apart from that seen in B cells on stimulation through the BCR (de Weers et al., 1994; Hinshelwood et al., 1995). As the defects associated with XLA suggest that Btk is likely to play a role in multiple signalling pathways in B cells, the activation state of Btk following stimulation via other surface receptors on human B cells was investigated. Two receptors that were considered to be likely and interesting candidates for the stimulation of Btk in human B cells were CD22 and CD38.

3.1.1 CD22

CD22 is a 135kD B lineage restricted phosphoglycoprotein. It was first reported as a protein expressed in the cytoplasm of bone marrow derived human pre-B cells (Dorken et al., 1986). The surface expression of CD22 on mature B cells remains upon cell activation but is lost on differentiation of the B cell into an antibody secreting plasma cell (Dorken et al., 1986). CD22 is an
important cell surface molecule playing a role in cell adhesion mediated by its extracellular domains, and acting as a signalling molecule using its intracellular regions, (Law et al., 1994).

Several signalling responses to human B cell stimulation through the BCR are potentiated when the cells are co-stimulated with antibodies to CD22. Although stimulation of CD22 alone results in no signalling responses in the B cell, co-ligation of CD22 and the BCR result in increased cell signalling as observed by proliferation, induced entry into the cell cycle, and increased calcium levels (Pezzutto et al., 1987) (Fig. 3.1).

3.1.2 CD38

A second B cell surface receptor considered to be a likely candidate through which the activation of Btk may be mediated is CD38. CD38 is a 42kD transmembrane glycoprotein which is widely distributed on haematopoietic cells of mouse and human, (Lund et al., 1995a; Shubinsky, 1997). As well as acting as an adhesion molecule (Dianzani et al., 1994) and a signalling molecule (Funaro et al., 1990; Santos Argumedo et al., 1993; Zupo et al., 1994; Kumagai et al., 1995; Lund et al., 1995a), CD38 is also a bifunctional enzyme (Howard et al., 1993) which catabolises NAD$^+$ to cADPR, and cADPR to ADPR (Fig. 3.1). cADPR is capable of inducing IP$_3$ independent calcium mobilisation via ryanodine receptor calcium channels (Meszaros et al., 1993), and ADPR may result in protein modifications (Grimaldi et al., 1995). Both products of CD38 enzymatic activity therefore have the potential to play roles as second messengers in CD38 signalling.
Figure 3.1 The Roles of CD22 and CD38 relevant to signalling via Btk. CD38 is a transmembrane signalling molecule, which when ligated results in several cellular responses, as well as a catabolic enzyme. Its enzymatic actions result in products which have the potential to act as signalling second messengers, although how they could act inside the cell has not been determined. CD38 dependent proliferation is not observed in mouse B cells with mutated Btk (xid). CD22 has been found in complex with several signalling molecules in murine B cells, both positive (Syk and PLCγ) and negative (SHP) regulators. A small percentage of membrane bound CD22 is found associated with the BCR. Although ligation of CD22 has no effect on the cell, it may be required for BCR mediated calcium mobilisation in human B cells, and when co-ligated with the BCR, the effects normally seen on BCR ligation are enhanced.
Several cell lines show the same unresponsiveness to ligation by both CD38 and IgM, and one of these cell lineages is mouse xid splenic B cells (Lund et al., 1995b, Santos-Argumendo et al., 1996) (Fig. 3.1). The inability of xid B cell to proliferate on CD38 ligation, plus evidence that the BCR and CD38 may utilise an overlapping group of signalling proteins, implies that Btk may be an effector of CD38 induced signal transduction.

The activity of Btk was therefore investigated in the human B cell lymphoma cell line Daudi, on stimulation through CD38 and CD22. Ligation of these cell surface receptors using monoclonal antibodies is believed to mimic their ligation by an in vivo ligand and allow for the detection of any specific proteins activated in the resulting signal transduction pathway.

### 3.2 Results

#### 3.2.1 Daudi cells express surface CD22 and CD38

Daudi cells are a mature B cell lymphoma cell line which express high levels of slgM and Btk (Genevier et al., 1994). Although CD22 is generally expressed on B cells at this stage in development, CD38 is not. To test the possibility that both CD22 and CD38 may be expressed on Daudi cells, the cells were stained with monoclonal antibodies that recognise CD22 and CD38, and analysed by FACS. Fig.3.2 shows that both CD22 and unexpectedly, CD38, were expressed on Daudi cells. Other cell lines including Ramos, and Raji B cell lymphomas, and Molt-4 and Jurkat T cell leukemic lines were also stained and found to express similar levels of CD38 (data not shown). Daudi B cells were selected to provide a suitable system in which to investigate the activation of Btk on stimulation through CD22 and CD38.
Fig 3.2 Cell surface expression of CD22 and CD38. Daudi B cells were stained with antibodies specific for CD22 and CD38 and analysed by flow cytometry.
3.2.2 Protein tyrosine phosphorylation following stimulation of Daudi cells

Daudi cells were stimulated through the BCR, CD22 and CD38, using antibodies that recognise extracellular epitopes of the receptors. Incubation of such antibodies with Daudi cells results in cross-linking and activation of the receptors in a manner predicted to mimic that caused by the binding of antigen to the BCR, or of \textit{in vivo} ligands to CD22 and CD38. Unstimulated cells were used as controls. Antibodies were incubated with the cells in a minimal volume of RPMI at 37°C for 5 minutes. Following lysis, cellular tyrosine phosphorylation was analysed by immunoblotting.

Fig 3.3 shows the tyrosine phosphorylated proteins resulting from stimulation through the various receptors. Cells that were stimulated through the BCR, whether it was using a complete anti-IgM antibody or the F(\textit{ab'})\textsubscript{2} fragment of anti-IgM (Cappel), showed the tyrosine phosphorylation of a number of cellular proteins. There are three heavily phosphorylated proteins with apparent molecular weights of ~70, 74, and 130 kD. Four less phosphorylated or less abundant proteins migrate with apparent molecular weights of ~56, 58, and 160 kD. And a further three species are represented by weaker bands migrating at ~80, 188 and 190 kD.

Co-stimulation of the BCR with an anti-CD22 antibody resulted in the same profile of tyrosine phosphorylated proteins. However, stimulation of Daudi cells using the activating anti-CD38 (IB4) (Funaro et al., 1990) or the anti-CD22 antibody alone did not show any tyrosine phosphorylation of cellular proteins as compared to the unstimulated or non-activating anti-CD38 (IB6) treated controls.
Figure 3.3 Tyrosine phosphorylation of cellular proteins on cell stimulation. Cells were stimulated by incubation with antibodies in a minimal volume of media through the BCR, using an anti-IgM antibody or the F(ab')2 fragments of anti-IgM, Cappel (Cap), through CD22, and through CD38 using the activating 1B4 anti-CD38 antibody, and the non-activating 1B6 anti-CD38 antibody. Cells were lysed, protein separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the anti-phosphotyrosine monoclonal antibody, 4G10.
A previous report had shown that in murine splenic cells stimulated through CD38, an increase in the tyrosine phosphorylation of several proteins was observed at a maximum of 15 min after stimulation (Kirkham et al., 1994). This experiment using Daudi cells was therefore repeated and samples of lysate were removed at several time points after activation between 30 s and 20 min and examined for tyrosine phosphorylation. Also, since the observed murine CD38 induced tyrosine phosphorylation was dependent on the presence of IL-4, as was the CD38 dependent proliferation of murine B cells (Santos Argumedo et al., 1995), the experiment was performed using CD38 as a stimulant in conjugation with human IL-4. Neither the increased stimulatory time nor the presence of IL-4 resulted in any observable tyrosine phosphorylation in the whole cell lysates of the CD38 stimulated cells when analysed by 4G10 immunoblotting (results not shown).

3.2.3 The activation of Btk on cell stimulation

Btk, like several other non-receptor tyrosine kinases, has been shown to become tyrosine phosphorylated and activated on stimulation of human B cells through the BCR. Fig.3.3 shows that several proteins were tyrosine phosphorylated in B cells on stimulation with anti-IgM, one of which could be Btk, for example, either of the species running with apparent molecular weights of ~74 or ~80 kD.

The activation of the catalytic activity of cellular protein tyrosine kinases on cell stimulation can be detected using an *in vitro* kinase assay. This is a very sensitive method compared to immunoblotting for detecting tyrosine phosphorylated proteins. The *in vitro* kinase assay involves mixing a specific immunoprecipitated protein with $[^{32}\text{P}]$ labelled ATP. The presence of kinase activity will result in the incorporation of a radiolabelled phosphate group onto the substrate which can then be detected by autoradiography. As Btk has been shown to autophosphorylate (de Weers et al., 1994; Hinshelwood et al., 1995), an increase in the incorporation of radiolabelled ATP into Btk during the *in
vitro kinase assay indicates an increase in both phosphorylation and autokinase activity of Btk.

Immunoprecipitated Btk from stimulated and unstimulated Daudi cells were subjected to an in vitro kinase assay. The samples were separated by SDS-PAGE and the dried gel was exposed to X ray film. The radiolabelled Btk protein (Fig. 3.4(A)) migrates with an apparent molecular weight of 77kD, indicated with an arrow. The increase observed in the size and density of the band from cells with ligated receptors compared to untreated cells indicates an increase in Btk autokinase activity. In unstimulated cells Btk showed a basal level of activity which increased significantly in cells treated with an anti IgM antibody containing only the F(ab')_2 fragments (Cappel), in agreement with previous reports showing inducible Btk activity on BCR ligation (de Weers et al., 1994; Hinshelwood et al., 1995).

Stimulation through CD22 alone resulted in no increase in the activation of Btk as maybe expected from the lack of tyrosine phosphorylation shown in Fig. 3.3 following anti-CD22 stimulation. Co-stimulation through the BCR and through CD22 resulted in no increase in the activation of Btk compared to its activation on IgM ligation alone, and in some instances, as here, the activation of Btk seems to be reduced when compared with Btk stimulated by the BCR alone. This result however, is not consistently observed. Ligation of CD38 resulted in no activation of Btk using both the activating and the non-activating anti-CD38 antibodies compared to unstimulated basal levels.

In order to discount the possibility that the increase in the intensity of the radiolabelled immunoprecipitated Btk band in Fig.3.4(A) was caused by unequal loading of Btk in the lanes rather than an increase in kinase activity, the Btk loaded into each lane was quantified. Immunoblotting a membrane onto which radiolabelled Btk immunoprecipitates were transferred,
Table 3.4: Btk Activation on B cell stimulation.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cap</th>
<th>CD22</th>
<th>CD22+ IB4</th>
<th>IB4</th>
<th>IB6</th>
<th>Cap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>205</td>
<td>116</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Btk</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitating Ab</td>
<td>H360B (αBtk)</td>
<td>NRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.4 (A) *Activation of Btk on B cell stimulation*. Btk was immunoprecipitated from lysates of Daudi cells stimulated through sIgM (Cap), CD22, and CD38 (IB4 activating, IB6 non-activating), and unstimulated cells. The Btk immunoprecipitates were subjected to an *in vitro* kinase assay, and separated by SDS-PAGE. The dried gel was exposed to X-ray film. The NRS control lanes contain all bands but that at 77kD on a longer exposure of the gel (not shown, see Fig. 5.10 (B)), indicating that these bands are due to non-specific binding of H360B.
detected Btk as a species that migrated to the same place as the 77kD radiolabelled species when the membrane was exposed to X ray film (data not shown). However, the poor quality of the immunoblots of samples having undergone an *in vitro* kinase assay made it impossible to quantitate the amount of Btk in each lane. Therefore to quantitate the amount of Btk in each lane, Btk was immunoprecipitated from stimulated and unstimulated cell lysates prepared from double the number of cells required for the *in vitro* kinase assay. Prior to the *in vitro* kinase assay, each sample was halved. One half was subjected to the *in vitro* kinase assay, the results of which are depicted in Fig.3.4(A). The other half was boiled in 2X SDS sample buffer, separated by SDS-PAGE and immunoblotted with H360B as shown in Fig.3.4(B). A sample of a lysate of Daudi cells stimulated through IgM from a previous experiment was run to show that H360B only cross-reacts with one major species in Daudi cell lysates. Immunoblotting the Btk precipitates shows that an equal amount of Btk was found in each sample, and was not present in the NRS control.

**Figure 3.4 (B) Btk protein loading control.** Btk was immunoprecipitated from lysates of Daudi cells stimulated through receptors as indicated, precipitates were separated by SDS-PAGE and immunoblotted with H360B. A sample of whole cell lysate (wcl) was taken prior to immunoprecipitation and separated with the precipitates.
3.2.4. The activation of Syk on cell stimulation

During the course of these investigations into the activation of Btk, another group published work which showed that another protein tyrosine kinase, Syk, is activated upon the ligation of CD38 of human immature B cell lines (Silvennoinen et al., 1996). Here, Syk activation on CD38 ligation in a mature B cell line was investigated.

The activity of Syk in cells stimulated through the BCR, CD22 and CD38 was investigated in a manner similar to that of Btk as described in section 3.2.3. Syk was immunoprecipitated from the lysates of stimulated and unstimulated cells, subjected to an in vitro kinase assay, separated by SDS-PAGE and the dried gel exposed to X ray film. In Fig.3.5 the activity of Syk, which is also capable of auto-phosphorylation and migrates on SDS-PAGE analysis with an apparent molecular weight of 72kD (Taniguchi et al., 1991), is seen to be increased in Syk immunoprecipitates from cells that were stimulated through the BCR and through CD38 using the activating antibody IB4. Unstimulated, CD22 and IB6 ligated cells show only a basal level of activity. Interestingly, the activity of Syk when co-stimulated with both anti IgM and CD22 is not raised above this basal level. Controlling for equal loading of Syk in each lane proved troublesome due to the extremely poor quality of immunoblots of Syk immunoprecipitates using the same antiserum. The Syk antiserum however only recognises one band in Daudi whole cell lysates (see Fig.5.11(b)), the experiments were performed identically to those investigating Btk activation, and the increased phosphorylation of Syk on stimulation of the cells with IB4 was observed when the experiment was repeated.
3.3. Discussion

Figure 3.5 Activation of Syk on B cell stimulation. Syk was immunoprecipitated from the lysates of Daudi cells which were stimulated through sIgM (Cap), CD38 (IB4 and IB6) and CD22. The immunoprecipitates were subjected to an *in vitro* kinase assay, and separated by SDS-PAGE. The dried gel was exposed to X-ray film.
3.3. Discussion

These investigations, and others (de Weers et al., 1994; Hinshelwood et al., 1995; Silvennoinen et al., 1996) show that, to date, the only B cell transmembrane signalling receptor shown to induce the activation of Btk on its ligation in human B cells, is the BCR.

3.3.1 Experimental Methods

3.3.1.1 The Daudi Cell Line

The mature B cell line Daudi was used as a system in which to study the activation of Btk. Daudi cells express high levels of sIgM (Genevier et al., 1994), and flow cytometry showed them to also express both CD22 and CD38. CD38 is not highly expressed on mature human B cells (Lund et al., 1995a, see Fig. 1.1.). Investigations in this Chapter however showed CD38 to be expressed on all mature human B cell lines tested. Why mature cell lines should express CD38 when in primary mature cells its surface expression is low is not known. Previous investigations into Btk activity showed it to be inducible on IgM crosslinking in Daudi cells (Hinshelwood et al., 1995). In other mature B cells lines such as Raji and Ramos, high levels of basal Btk activity were observed (data not shown), and as surface expression of CD22, CD38 and IgM, plus Btk expression is high in the Daudi B cell line (Genevier et al., 1994) it was considered a suitable system in which to test the induction of Btk activity.

Stimulation through the BCR, CD22 and CD38 was performed by crosslinking with monoclonal antibodies raised against the receptors believed to mimic ligation by cellular ligands.
3.3.1.2 In Vitro Kinase Assay

Btk autophosphorylates (de Weers et al., 1994; Hinshelwood et al., 1995), and as a consequence, during the process of an in vitro kinase assay, immunoprecipitated Btk will incorporate radiolabelled ATP at its site of autophosphorylation, should the kinase activity of Btk be activated in the cell prior to immunoprecipitation. As discussed in section 1.8.2.3, Btk activation cannot be initiated by autophosphorylation, but requires the presence of a Src PTK such as Lyn. As Btk has not been shown to co-precipitate with any Src PTKs, the increase in incorporation of radiolabelled ATP during an in vitro kinase assay is presumed to be due to the state of Btk activity in the cell prior to lysis, and is an indication of the stimulatory effects of the antibody incubated with the cells. Syk, on the other hand, is capable of auto-phosphorylation independent of Src PTKs, and Syk clustering is capable of inducing Syk mediated signalling (section 1.8.2.2). So although Btk cannot initiate its own autophosphorylation, and the reaction is believed to be due to an intramolecular interaction, gauging the activity of immunoprecipitated kinases, especially ones such as Syk, from unstimulated cells is an important control in order to take into account the effects of kinase clustering on autophosphorylation.

3.3.2 CD38: A transmembrane signalling molecule

CD38 has a large extracellular domain containing its catalytic region, which is also involved in adhesion, and a small cytoplasmic part (21 amino acids) (Jackson and Bell, 1990). This intracellular region contains no known motifs that could mediate interactions with signalling molecules. The murine, but not human CD38, contains one tyrosine residue within the cytoplasmic region. Two serine residues, within consensus regions recognised by cGMP dependent kinases, are conserved in mouse and human CD38 (Shubinsky et al., 1997). An enzyme with homology to CD38 is regulated by these ser/thr kinases in sea urchins (Lund et al., 1996), and the cytoplasmic tail of CD38 may
therefore serve as a regulator of the CD38 ectoenzyme, rather than, or in addition to, a tool for signal transduction.

3.3.3 Signalling through murine CD38 activates Btk

Studies on the capacity of murine CD38 to act as a signalling molecule have produced conflicting results. Signalling through CD38 transfected into a mature mouse B cell lymphoma resulted in no tyrosine phosphorylation of cellular proteins (Lund et al., 1996). However ligation of CD38 on mouse splenic B cells resulted in tyrosine phosphorylation of several proteins (Kirkham et al., 1994), including Btk (Kikuchi et al., 1995).

3.3.4 Signalling through human CD38 does not lead to Btk activation

CD38 signalling has been studied using several human cell lines. CD38 ligation on the leukemic human T cell lines Jurkat and MOLT-4, failed to induce any tyrosine phosphorylation of cellular proteins (Kontani et al., 1997). In contrast, CD38 ligation of human immature B cell lines led to the rapid and transient tyrosine phosphorylation of several cellular proteins including Syk, PLCγ, c-Cbl, and the p85 subunit of PI3-K (Silvennoinen et al., 1996). In this report, Btk activation was found to be unaffected by CD38 ligation. As this result is contradictory to studies on Btk in CD38 ligated murine B cells, it was suggested that the lack of Btk activation may be due to the immature developmental stage of the B cells examined, and that Btk may become activated downstream of CD38 in mature human B cells. Work presented in this Chapter (Fig 3.4(A)) however indicates that this is not the case, and that Btk does not appear to be involved in CD38 mediated signalling in human B cells.

CD38 ligation on immature human B cells resulted in the tyrosine phosphorylation and activation of Syk (Silvennoinen et al., 1996), and this was confirmed to also be true on signalling through CD38 in mature human B cells (Fig 3.5). It is important to bear in mind that CD38 is not normally expressed at
high levels on mature human B cells and therefore the physiological relevance of this result in hard to interpret. Nevertheless, these results indicate that Btk most likely is not involved in CD38 signalling, and that Syk activation through CD38 is not restricted to cells of the immature lineage.

### 3.3.5 CD38 and the BCR

Several murine B cell populations have been identified, including xid B cells (Fig. 3.1), that fail to respond to both CD38 and BCR ligation (Lund et al., 1995a,b). Furthermore, studies on CD38 co-ligated with the BCR in mouse reveal that the effects induced were less than additive (Lund et al., 1995b), suggesting either (i) that CD38 acts as an antagonist of BCR signalling, or (ii) that certain aspects of signalling machinery may be shared between the two receptors. Human CD38 signalling alone results in several cellular responses similar to those seen on BCR crosslinking, such as cell activation and proliferation (Funaro et al., 1990) and perhaps tyrosine phosphorylation (see above); its action as a negative regulator is therefore unlikely. Investigations into murine and human CD38 induced tyrosine phosphorylation indicate that the second scenario is more likely. Co-expression studies have shown that the BCR, and particularly the cytoplasmic tails of Igα and Igβ, are required for murine CD38 mediated signal transduction, characterised by an influx of extracellular calcium. However under these conditions CD38 ligation induces no protein tyrosine phosphorylation, and co-expression with the BCR alone is therefore not sufficient for the full signalling activity of CD38 (Lund et al., 1995a; 1996). Shared signalling components that limit the additive effects of co-ligation of CD38 and the BCR, and which are required for complete CD38 signalling in humans, could be PLCγ, PI3-K, or Syk, all of which are also involved in BCR signalling (section 1.8). It would appear that in mouse, but not human B cells, Btk is a signalling component utilised by both the BCR and CD38.
3.3.6 Mouse versus human CD38

Although CD38 in mouse shares 70% identity with its human counterpart (Harada et al., 1993), it could possibly be a closely related protein and not a direct homologue. The presence of an intracellular tyrosine in murine CD38, absent in human CD38, maybe a factor contributing to the different signalling responses induced by ligation of murine and human CD38, as phosphorylation at this residue may be important for the recruitment of SH2 domain containing proteins and the clustering of signalling proteins.

Human and mouse CD38 expression follow reciprocal patterns of expression on B cell development. In humans CD38 expression is high on the surface of B cells in early stages of development and on plasma cells. In mouse, the pattern of CD38 expression is the reverse (Lund et al., 1995a). Therefore comparing CD38 signalling between mice and humans is complicated not only by differences in the CD38 molecule itself, but also, differences in CD38 signalling in B cells during various stages in their development cannot be directly compared between mouse and man due to its variable expression pattern.

Murine and human CD38 ligation induces responses similar to those seen on BCR cross-linking as mentioned. However ligation of each of these two receptors also induces effects attributed to one or the other (Lund et al., 1995a). In both human and mouse B cells, CD38 ligation has been shown to protect cells from apoptosis (Santos Argumedo et al., 1993; Zupo et al., 1994), and the Ca++ flux from extracellular stores induced by CD38 enzyme activity is independent of IP$_3$ (Kirkham et al., 1994; Aarhus et al., 1995). Btk action on BCR cross-linking may therefore be restricted to a pathway that does not overlap with CD38 signalling, such as PLC$_\gamma$ activation and IP$_3$ dependent Ca++ mobilisation, as discussed in section 1.8.3.1.
3.3.7 CD22 and calcium mobilisation

Human and mouse CD22 molecules share 62% amino acid identity. The region of greatest homology includes the cytoplasmic tail, which includes six conserved cytoplasmic tyrosine residues (Law et al., 1994). Human CD22 is expressed in pro- and pre-B cells, but its surface expression is at its peak in cells expressing sIgM and sIgD (Fig. 1.1) (Dorken et al., 1986).

Ligation of CD22 on human B cells alone using Mabs does not induce any signalling effects (Pezzutto et al., 1987; 1988). However co-crosslinking of CD22 and the BCR increases BCR induced proliferation, and Ca^{++} mobilisation (Pezzutto et al., 1988). CD22 engagement may function to lower the threshold of BCR mediated signals required for B cell activation, as in the presence of CD22 Mabs, only 10-20% of anti-IgM antiserum was required to induce the same responses illicitcd on cross-linking of IgM alone (Pezzutto et al., 1987; 1988). Furthermore in CD22 deficient human tonsillar B cells, BCR ligation does not result in an increase in Ca^{++} levels, suggesting that CD22 may act as an essential molecule for transducing BCR dependent signals (Pezzutto et al., 1988).

Studies of the CD22 deficient mouse however do not support the theory that CD22 is crucial for BCR mediated calcium mobilisation, as all BCR mediated signalling responses investigated, including calcium mobilisation, are augmented in the B cells of these animals (O'Keefe et al., 1996), indicating that in wild type cells CD22 acts to suppress BCR mediated signals.

Btk has been shown to be required for BCR dependent Ca^{++} mobilisation in the avian B cell line DT40 (Takata and Kurosaki, 1996)(sections 1.3.1.2, 1.8.3.1), however results presented in this Chapter indicate that the actions of Btk are most likely not affected directly by CD22 ligation, but that its activation is a result of the signal resulting from BCR cross-linking, required for the IP_{3} dependent increase in Ca^{++} levels. Should CD22 play a role in human BCR
mediated calcium mobilisation as suggested by the work of Pezzuto et al, the mechanism by which this is achieved remains unexplained.

### 3.3.8 CD22 and the BCR

CD22 associates with sIgM and is rapidly phosphorylated on tyrosine residues on CD22 and BCR ligation (Schulte et al., 1992; Peaker and Neuberger, 1993). Consistent with a role in Ca\(^{++}\) mobilisation, tyrosine phosphorylated CD22 recruits a number of intracellular signalling molecules, including Syk and PLC\(\gamma\)1, (Law et al., 1996b) (Syk is required for PLC\(\gamma\) activation which produces IP\(_3\) resulting in Ca\(^{++}\) mobilisation (section 1.6.2, 1.8.3.1)). Other molecules also recruited to CD22 include Lyn, PI3-K (Tuscano et al., 1996) and SHP-1 (Doody et al., 1995).

The protein tyrosine phosphatase SHP-1 (also termed PTP-1c, SHPTP1 and HCP) contains two SH2 domains and is mutated in the viable motheaten (me\(^{v}\)) mouse (Kozlowski et al., 1993). B cells of me\(^{v}\) mice induce a release of intracellular Ca\(^{++}\) in response to lower concentrations of antigen than do normal B cells indicating SHP-1 is a negative regulator of BCR mediated signalling (Cyster and Goodnow, 1995). Recruitment of SHP-1 to CD22 therefore implicates a negative signalling role for CD22 in cell signalling. The ligation of CD22 has been suggested to result in sequestration of CD22 away from the BCR, and hence on crosslinking of both IgM and CD22, an inhibitory effect of CD22 is released from the vicinity of the BCR (Doody et al., 1995; Tuscano et al., 1996). The associations between CD22 and PI-3K, Lyn and Syk, and the reported dependence of human BCR mediated calcium mobilisation on the presence of CD22, suggest however that CD22 is also involved in positive signalling.

Whether CD22 acts as a negative inhibitor, or a positive regulator, the ligands of CD22 that recognise its extracellular domain in vivo, (a population of sialoglycoproteins (Powell et al., 1993), see Fig.1.8), are expressed in secondary
lymphoid organs and would be expected to lower the threshold for B cell activation when exposed to antigen within these structures.

It is interesting to note that the results presented here suggest the possibility that the BCR induced activation of Btk and Syk is reduced in cells co-stimulated with anti-CD22 and anti-IgM (Fig. 3.4(A) and 3.5). This observation was not always observed however, and as attempts to co-precipitate CD22 and Btk from NP40 and digitonin lysates of Daudi B cells were unsuccessful (data not shown), it cannot be concluded from these results that Btk is involved in CD22 mediated signalling.
Chapter 4

SH3 domain interactions of
Itk, Tec and Btk, members
of the Btk family of protein
tyrosine kinases

4.1. Introduction

In order to determine the specific role of signalling proteins in B cells, it is important to understand the nature of their ligands. Potential cellular ligands may be found in the immunoprecipitates of proteins, providing the interaction between the immunoreactive protein and its ligand is not disrupted by the conditions of cell lysis or the binding of the precipitating antibody.

In vitro systems may also be used in order to determine the protein ligands of a particular protein of interest. The yeast two hybrid system (Fields and Song, 1989) and fusion protein analysis (Smith and Johnson, 1988; Frangioni and Neel, 1993) are examples of such in vitro approaches. For the latter, proteins or structural domains within proteins, can be produced in bacterial systems fused to a protein which binds coated Sepharose beads, and these fusion protein coated beads can be used to affinity purify protein ligands directly in cell lysates (Fig 4.2).

A fusion protein containing the Btk SH3 domain was found to bind two cellular proteins identified as c-Cbl (section 1.4.4.1) and WASP (section 1.4.4.2)
Chapter 4

(Cory et al., 1995, 1996). The work presented in this Chapter investigates the binding properties of the SH3 domains of the closely related proteins Itk and Tec. Although similar, these proteins are involved in separate, as well as common, signalling pathways (section 1.3.1.2). Investigations into the domain ligand binding specificity of these proteins may help explain the common and divergent actions of, and requirements for, this family of PTKs in lymphoid signalling.

4.2 Results

4.2.1. Construction and isolation of SH3 domain fusion proteins

The Btk SH3 domain fusion protein was constructed as described previously (Cory et al., 1995). The Itk and Tec SH3 domain sequences were amplified from human cDNA using primers designed from the published sequences of Itk (Gibson et al., 1993) and Tec (Sato et al., 1994) (see Table 2.1, and Fig.4.1(A)). The amplified products were ligated in frame into the pGEX plasmid downstream of the inducible Tac promoter. Ampicillin resistant colonies were selected and grown in a 5 ml culture.

Plasmid from cultures that showed inducible expression of Tec fusion protein (and Itk fusion protein, data not shown) on addition of IPTG (Fig. 4.1 (B), lanes 3 and 4) was extracted and sequenced. Clones containing plasmid with the SH3 domain inserted with no mutations arising from the PCR or cloning procedures were grown to large volumes. Fusion protein expressed on induction by addition of IPTG to the large cultures was extracted and adsorbed to glutathione Sepharose beads via an interaction between the GST moiety of the fusion protein and the glutathione molecule covalently attached to the Sepharose bead, summarised in Fig.4.2 stages (i)-(iv). In order to estimate the purity of the recombinant fusion protein beads (Fig. 4.2, stage iv) were boiled
Figure 4.1 Preparation of Itk and Tec GST-SH3 domain fusion proteins. (A) Human cDNA was PCR amplified using primers designed to flank the SH3 domain encoding regions (Table 2.2). The PCR products were visualised with EtBr under UV light, and the size was estimated using 1Kb ladder. (B) pGEX-SH3 transformed ampicillin resistant E.coli colonies were grown in 5ml cultures, lysed prior to and after IPTG induction in 2X sample buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue. A band running at ~30kD in the cell lysate, as seen in lanes 3 and 4, and indicated with an arrow, indicates fusion protein expression. Sample taken prior to induction with IPTG has not expressed any fusion protein (lane 1), the culture failed to express fusion protein when induced with IPTG (lane 2). (C): Increasing quantities of GST-SH3 domain fusion proteins, attached to glutathione Sepharose 4B beads were boiled in 2X sample buffer, separated by SDS-PAGE and stained with Coomassie brilliant blue.
(i) SH3 domain amplified from human cDNA by PCR

(ii) Amplified SH3 domain fragment ligated downstream of the GST gene

(iii) E.coli transformed with plasmid and induced to express fusion protein

(iv) Fusion protein purified using glutathione Sepharose bead

(v) Beads coated with fusion protein are incubated with Daudi cell lysate

(vi) Beads are washed and fusion protein bound ligands are resolved by SDS-PAGE.

Figure 4.2. Protocol for the construction of a GST-SH3 domain fusion protein, and its use to isolate cellular ligands.
4.2.2. Cellular ligands of the Btk family of SH3 domains

Fusion proteins were used to affinity purify ligands of the SH3 domains of Btk, Itk and Tec as described in section 2.6.1, and summarised in Fig.4.2 stages (v)-(vi). The Btk GST-SH3 domain and GST only fusion proteins attached to glutathione Sepharose beads were incubated with cell lysates from a methionine labelled culture of Daudi lymphoma B cells. The fusion protein precipitates were washed and separated by SDS-PAGE. The dried gel was exposed to X ray film, and the resulting ligands of Btk GST-SH3 can be seen in Fig.4.3(A). Btk GST-SH3 is seen to bind several proteins from the cell lysate that are not bound by GST alone. Major species bound are labelled with an arrow and migrate with apparent molecular weights of ~45kD, a doublet at ~65/67kD, ~70kD, and ~120kD. Other more minor species can also be observed.

Tec GST-SH3 and Itk GST-SH3 bind a similar profile of cellular proteins to Btk GST-SH3 from a Daudi B cell lysate, Fig.4.3(B). The fusion protein precipitates were separated by SDS-PAGE and the gel was stained with Coomassie brilliant blue. There appears therefore to be no detectable differences in the nature of the ligands bound by the Btk, Itk and Tec SH3 domains using this in vitro system.
Figure 4.3 Btk, Itk and Tec GST-SH3 domain ligands in Daudi lysates. The GST-SH3 domain fusion proteins from the family members indicated were incubated with Daudi cell lysate. (A): Cells were metabolically labelled with [35S] methionine, lysed and incubated with Btk GST-SH3 and GST only fusion proteins. Precipitates were separated by SDS-PAGE, and the dried gel exposed to X-ray film. (B) Cells were lysed and incubated with Itk and Tec GST-SH3 and GST only fusion protein. The fusion protein precipitates were separated by SDS-PAGE and the gel stained with Coomassie brilliant blue. The major species precipitated by the GST-SH3 fusion proteins are indicated with arrows.
4.2.3. WASP is a ligand for Itk and Tec SH3 domain fusion proteins

Previous work performed in this laboratory has identified the 65kD species bound to Btk GST-SH3 as the Wiskott-Aldrich Syndrome Protein (WASP) (Cory et al., 1996). Due to the degree of homology between the SH3 domains of Btk and its related kinases Itk and Tec (Table 4.1), and the similarity of the profiles of bound protein from all three (Fig. 4.3), the presence of WASP in the Itk and Tec GST-SH3 precipitates was investigated using a specific anti-WASP antiserum, SK3. Equal amounts of Btk, Itk and Tec GST-SH3 domain (5μg) and GST only fusion proteins were incubated with lysates from both BCR stimulated and unstimulated Daudi B cells. Fig. 4.4 shows that WASP was detected as a species migrating with an apparent molecular weight of 65kD in the whole cell lysate, and was also present in the precipitates of all the fusion proteins except GST alone. The stimulatory state of the cell had no effect on the binding of the fusion proteins to WASP, nor does there appear to be any major differences in the amount of WASP bound by Btk, Itk, or Tec GST-SH3.

Table 4.1 The percentage homology between the Btk, Itk and Tec SH3 domains. Numbers in italics indicate the number of identical residues between any two SH3 domains.

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<th>Itk</th>
<th>Tec</th>
</tr>
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<tbody>
<tr>
<td>Btk</td>
<td>100%</td>
<td>50.9%</td>
<td>59.4%</td>
</tr>
<tr>
<td>Itk</td>
<td>26</td>
<td>100%</td>
<td>44.6%</td>
</tr>
<tr>
<td>Tec</td>
<td>30</td>
<td>23</td>
<td>100%</td>
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4.2.4 Itk and Tec GST-SH3 domain fusion proteins also bind c-Clb.

Another ligand of the SH3 domain fusion protein of Btk previously identified by work in this laboratory is c-Clb (Cory et al., 1995). Immunoblotting of the same membrane as seen in Fig. 4.4 using an anti-c-Clb antibody revealed c-Clb bound to Btk GST-SH3, and also to Itk and Tec GST-SH3.

Figure 4.4 Itk and Tec GST-SH3 domain fusion proteins bind WASP in both stimulated and unstimulated Daudi B cells. Daudi cells were lysed prior to and after stimulation through the BCR. Lysates were incubated with equal amounts of GST-SH3 fusion protein from the proteins indicated and with GST alone. The bound ligands were separated by SDS-PAGE and immunoblotted with the anti-WASP antibody SK3.
4.2.4 Itk and Tec GST-SH3 domain fusion proteins also bind c-Cbl.

Another ligand of the SH3 domain fusion protein of Btk previously identified by work in this laboratory is c-Cbl (Cory et al., 1995). Immunoblotting of the same membrane as seen in Fig. 4.4 using an anti-c-Cbl antibody reveals c-Cbl bound to Btk GST-SH3, and also to Itk and Tec GST-SH3 (Fig. 4.5). Itk GST-SH3 however, binds c-Cbl to a considerably reduced extent as compared to Btk or Tec GST-SH3 (Fig. 4.6 (A)), as c-Cbl can only be observed on a long exposure of the membrane of another precipitation where the quantities of fusion protein were increased from 5 to 10 μg, (Fig. 4.5 (B)). This may reflect a difference in the degree of sequence homology seen between the three SH3 domains, with Itk showing less homology to Btk than does Tec (Tables 4.1 and 4.2, section 4.3.2).

As reported previously, the association between Btk GST-SH3 with c-Cbl is decreased in the lysate of stimulated cells as compared to c-Cbl bound by Btk GST-SH3 in unstimulated cell lysate. This same pattern was observed for the binding of c-Cbl by Tec GST-SH3 and Itk GST-SH3 (Fig. 4.5 (A)).
4.2.5. Titration of the Btk GST-SH3 domain fusion protein shows WASP is bound more readily than c-Cbl

The Itk, and Tec GST-SH3 fusion proteins, as well as Btk GST-SH3, bind c-Cbl in both BCR stimulated and unstimulated B cells. Daudi cell lysates were incubated with GST-SH3 domain fusion proteins from the PTKs indicated and GST alone. Ligands were separated by SDS-PAGE. (A): Blots were first probed with SK3, and subsequently with anti-c-Cbl antiserum. (B): Increased quantities of fusion protein and a longer exposure were required in order to visualise c-Cbl bound to Itk GST-SH3.
4.2.5. Titration of the Btk GST-SH3 domain fusion protein shows WASP is bound more readily than c-Cbl

By reducing the amount of fusion protein that is added to the lysates of Daudi B cells, the ligand preferentially bound may be identified as that which binds to reduced concentrations of fusion proteins, when the other available ligands can no longer be detected. By reducing quantities of fusion protein in cell lysate, the binding sites will become saturated with ligands, and different ligands must compete for these sites.

10, 5, 2, 1 and 0.25 μg of Btk GST-SH3 fusion protein was incubated with five equal volumes of Daudi cell lysate. The fusion protein precipitates were washed, separated by SDS-PAGE, and the protein transferred to nitrocellulose membrane. The membrane was cut in half and the top immunoblotted with anti-c-Cbl and anti-WASP antisera (Fig. 4.6(A)), the lower half was immunoblotted with an anti-GST antibody (Fig. 4.6 (B)). As the amount of fusion protein added decreases (as can be seen from the anti-GST blot in (B)), the quantities of c-Cbl bound by Btk GST-SH3 are seen to disappear very rapidly, with no trace of the protein detected by immunoblotting once the fusion protein added was less than 5μg. WASP however is clearly seen bound to fusion protein when it is present in considerably lower quantities. 1μg of Btk GST-SH3 still binds a clearly detectably quantity of WASP from the cell lysates. Although the sensitivity of the anti-WASP and anti-c-Cbl antisera may differ, causing the differences observed using this method to arise not due to differential binding of ligands by the fusion proteins, but to differences in detection by two different antibodies, the fact that both the WASP and the c-Cbl bands detected in the whole cell lysate are of equal intensity indicates that such a difference in sensitivity would not cause the large differences in the quantities of WASP and c-Cbl bound by Btk GST-SH3 observed.
Similarly, the Itk and Tec GST-SH3 domain fusion proteins bound WASP to a greater degree than c-Cbl (data not shown).

Figure 4.6 Titration of the Btk GST-SH3 domain fusion protein. Daudi cell lysates were incubated with 10-0.25 µg of Btk GST-SH3, and 5 µg GST fusion protein. Precipitates were separated by SDS-PAGE. (A): The top half of the membrane was immunoblotted with anti-c-Cbl, and anti-WASP (SK3). (B): The bottom half was immunoblotted with anti-GST antiserum.
in 2X sample buffer, separated by SDS-PAGE and stained with Coomassie brilliant blue.

Fig 4.1(C) shows that the major species in the preparation is the recombinant fusion protein migrating with an apparent molecular weight of ~32kD, and demonstrates that the glutathione affinity purification procedure yielded fusion protein of high purity. The Btk GST-SH3 domain fusion protein migrates with a slightly larger apparent molecular weight than Itk and Tec when resolved by SDS-PAGE, despite containing fewer SH3 domain residues (Btk SH3=49 residues, Itk SH3=52 residues, Tec SH3=51 residues). The contaminating band seen in the lane loaded with 4μl of Tec GST-SH3 running with an apparent molecular weight of ~70kD is the bacterial dnaK protein which was simply removed as described in section 2.4.5. The smaller contaminating protein seen running at ~18kD has not been identified, but may represent degraded fusion protein.
Figure 4.1 Preparation of Itk and Tec GST-SH3 domain fusion proteins. (A) Human cDNA was PCR amplified using primers designed to flank the SH3 domain encoding regions (Table 2.2). The PCR products were visualised with EtBr under UV light, and the size was estimated using 1Kb ladder. (B) pGEX-SH3 transformed ampicillin resistant E.coli colonies were grown in 5ml cultures, lysed prior to and after IPTG induction in 2X sample buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue. A band running at ~30kD in the cell lysate, as seen in lanes 3 and 4, and indicated with an arrow, indicates fusion protein expression. Sample taken prior to induction with IPTG has not expressed any fusion protein (lane 1), the culture failed to express fusion protein when induced with IPTG (lane 2). (C): Increasing quantities of GST-SH3 domain fusion proteins, attached to glutathione Sepharose 4B beads were boiled in 2X sample buffer, separated by SDS-PAGE and stained with Coomassie brilliant blue.
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</table>
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Another ligand of the SH3 domain fusion protein of Btk previously identified by work in this laboratory is c-Cbl (Crey et al., 1993). Immunoblotting of the same membrane as seen in Fig. 4.4 using an anti-c-Cbl antibody reveals Cbl bound to Btk GST-SH3 and also to Itk and Tec GST-SH3 (Fig. 4.5 (A)). This binding is seen between the fusion proteins and c-Cbl in both stimulated and unstimulated cell lysates. This same pattern was observed for the binding of c-Cbl by Tec GST-SH3 and Itk GST-SH3 (Fig. 4.5 (A)).

Figure 4.4 Itk and Tec GST-SH3 domain fusion proteins bind WASP in both stimulated and unstimulated Daudi B cells. Daudi cells were lysed prior to and after stimulation through the BCR. Lysates were incubated with equal amounts of GST-SH3 fusion protein from the proteins indicated and with GST alone. The bound ligands were separated by SDS-PAGE and immunoblotted with the anti-WASP antibody SK3.
4.2.4 Itk and Tec GST-SH3 domain fusion proteins also bind c-Cbl.

Another ligand of the SH3 domain fusion protein of Btk previously identified by work in this laboratory is c-Cbl (Cory et al., 1995). Immunoblotting of the same membrane as seen in Fig.4.4 using an anti-c-Cbl antibody reveals c-Cbl bound to Btk GST-SH3, and also to Itk and Tec GST-SH3 (Fig. 4.5). Itk GST-SH3 however, binds c-Cbl to a considerably reduced extent as compared to Btk or Tec GST-SH3 (Fig. 4.6 (A)), as c-Cbl can only be observed on a long exposure of the membrane of another precipitation where the quantities of fusion protein were increased from 5 to 10 µg, (Fig. 4.5 (B)). This may reflect a difference in the degree of sequence homology seen between the three SH3 domains, with Itk showing less homology to Btk than does Tec (Tables 4.1 and 4.2, section 4.3.2).

As reported previously, the association between Btk GST-SH3 with c-Cbl is decreased in the lysate of stimulated cells as compared to c-Cbl bound by Btk GST-SH3 in unstimulated cell lysate. This same pattern was observed for the binding of c-Cbl by Tec GST-SH3 and Itk GST-SH3 (Fig. 4.5 (A)).
Figure 4.5 The Itk, and Tec GST-SH3 fusion proteins, as well as Btk GST-SH3, bind c-Cbl in both BCR stimulated and unstimulated B cells. Daudi cell lysates were incubated with GST-SH3 domain fusion proteins from the PTKs indicated and GST alone. Ligands were separated by SDS-PAGE. (A): Blots were first probed with SK3, and subsequently with anti-c-Cbl antiserum. (B): Increased quantities of fusion protein and a longer exposure were required in order to visualise c-Cbl bound to Itk GST-SH3.
4.2.5. Titration of the Btk GST-SH3 domain fusion protein shows WASP is bound more readily than c-Cbl

By reducing the amount of fusion protein that is added to the lysates of Daudi B cells, the ligand preferentially bound may be identified as that which binds to reduced concentrations of fusion proteins, when the other available ligands can no longer be detected. By reducing quantities of fusion protein in cell lysate, the binding sites will become saturated with ligands, and different ligands must compete for these sites.

10, 5, 2, 1 and 0.25 μg of Btk GST-SH3 fusion protein was incubated with five equal volumes of Daudi cell lysate. The fusion protein precipitates were washed, separated by SDS-PAGE, and the protein transferred to nitrocellulose membrane. The membrane was cut in half and the top immunoblotted with anti-c-Cbl and anti-WASP antisera (Fig. 4.6(A)), the lower half was immunoblotted with an anti-GST antibody (Fig. 4.6 (B)). As the amount of fusion protein added decreases (as can be seen from the anti-GST blot in (B)), the quantities of c-Cbl bound by Btk GST-SH3 are seen to disappear very rapidly, with no trace of the protein detected by immunoblotting once the fusion protein added was less than 5μg. WASP however is clearly seen bound to fusion protein when it is present in considerably lower quantities. 1μg of Btk GST-SH3 still binds a clearly detectably quantity of WASP from the cell lysates. Although the sensitivity of the anti-WASP and anti-c-Cbl antisera may differ, causing the differences observed using this method to arise not due to differential binding of ligands by the fusion proteins, but to differences in detection by two different antibodies, the fact that both the WASP and the c-Cbl bands detected in the whole cell lysate are of equal intensity indicates that such a difference in sensitivity would not cause the large differences in the quantities of WASP and c-Cbl bound by Btk GST-SH3 observed.
Similarly, the Itk and Tec GST-SH3 domain fusion proteins bound WASP to a greater degree than c-Cbl (data not shown).

Figure 4.6 Titration of the Btk GST-SH3 domain fusion protein. Daudi cell lysates were incubated with 10-0.25μg of Btk GST-SH3, and 5μg GST fusion protein. Precipitates were separated by SDS-PAGE. (A): The top half of the membrane was immunoblotted with anti-c-Cbl, and anti-WASP (SK3). (B): The bottom half was immunoblotted with anti-GST antiserum.
4.3 Discussion

The three members of the Btk family of protein tyrosine kinases investigated in this study, Btk, Itk and Tec, are expressed in the haematopoietic system of man. In lymphoid lineages, Btk is expressed in B cells but not T cells, Itk is expressed in T cells but not B cells, and Tec is expressed in both (see Table 1.1). The structural similarity of all three and the inducible activation of Btk and Itk in B and T cells has lead to the proposal that their functions in the cell lines in which they are expressed are also similar.

This study has shown that the overall pattern of protein ligands bound by the Btk, Itk and Tec GST-SH3 domain fusion proteins from B cell lysates in vitro is similar. Furthermore the Btk SH3 domain ligands, WASP and c-Cbl, also associate with the SH3 domain fusion proteins of both Itk and Tec, albeit the interaction of c-Cbl with Itk being weaker than it is with Btk and Tec. The degree of homology observed between the three SH3 domains is consistent with this result as the Itk SH3 domain shows the greatest degree of divergence, (Table 4.2). If there are differences in the ligands bound by these SH3 domains due to divergent binding specificity, such specificity does not affect the binding of c-Cbl or WASP.

In order to establish the role played by signalling proteins in B cell function, an important aspect to consider are the in vivo ligands of particular signalling proteins. Proteins contain regulatory domains which have evolved to bind to particular protein motifs, and the integrity of the binding surfaces of these domains are crucial for the transduction of signals from the cell surface to the nucleus. As discussed, the regulatory domains of both Btk and Src family PTKs (section 1.7.3) are involved in intramolecular interactions which are important for the regulation of the catalytic activity of the protein. Regulation of the protein kinase activity due to intramolecular interactions is also dependent upon intervening interactions involving the same regulatory regions.
partaking in intermolecular interactions with other cellular ligands. Protein:protein interactions are therefore important in signal transduction through the cytoplasm, and also in the regulation of the protein mediators of such transduction, essential for, and integral to the signalling process itself.

### 4.3.1 Experimental Approach

#### 4.3.1.1 Immunoprecipitation

Interactions between proteins within cell lysates may be detected by co-immunoprecipitation studies. The immunoprecipitating anti-Btk antibody H360B, was used repeatedly to try to co-precipitate c-Cbl and WASP with Btk from NP40 and digitonin lysates of stimulated and unstimulated Daudi cell lysates, to no avail (data not shown; Cory, 1997). Interactions between Btk and ligands may be disrupted in the process due to the nature of the detergent, even digitonin, or the binding of H360B to Btk may result in the disruption of protein:protein interactions (H360B was raised against residues 168-218 of Btk, comprising TH domain sequences (Genevier et al., 1994)). H360B was also used in cytostaining procedures in order to determine should Btk be observed to co-localise with other proteins, such as sIgM, on the stimulation of whole cells, also without success (data not shown).

#### 4.3.1.2 Fusion Protein Precipitation

In order to determine in vitro cellular ligands of Btk, Itk and Tec the approach undertaken was to use SH3 domain GST fusion proteins to affinity purify bound proteins from cell lysates. Using fusion proteins in this manner can be a highly efficient method for identifying in vivo cellular ligands, and has successfully led to the identification of WASP as an in vivo ligand for Nck and Fyn (Rivero Lezcano et al., 1995; Banin et al., 1996). Large quantities of fusion protein can be produced and incubated with cell lysates, so that even ligands with low affinity for the domain may be isolated and identified. However, using fusion proteins comprising individual protein domains will not take
account of synergistic interactions that may involve other regions of the protein, such as the SH2 domain, as discussed in section 1.7.3.1.1. Effects of intramolecular interactions on ligand binding will also not be taken into account using single domain fusion proteins. These features of the fusion protein system when used to identify ligands result in the binding of protein to a part of Btk presented in a context distinct from that of the native protein. The SH3 domain fusion protein however, is believed to form a native conformation in solution, and should display the same amount of ligand specificity as an SH3 domain in the context of a whole protein, not taking competing intramolecular interactions, or co-operative intermolecular interactions involving other regions of the protein into account. Therefore ligands identified using fusion protein provide valuable information concerning protein:protein interaction occurring in cell lysates, which may represent \textit{in vivo} interactions occurring in the cell.

4.3.2 Conserved SH3 domain residues and ligand binding

Comparing the residues in the Src SH3 domain shown to be involved in ligand interactions (Erpel et al., 1995) with their equivalent residues in the Btk family of PTKs shows that six residues forming the SH3 domain hydrophobic ligand binding patch on Src SH3 are all conserved in both Btk and Itk (BtkY223, Y225, W251, Y263, P265, Y268, where the first Btk SH3 domain residue is V119, Table 4.2), only one in Tec differs, and this is a conservative substitution of a phenylalanine in Tec for a tyrosine residue at position 225 in Btk. These residues are highly conserved across most SH3 domains (Yu et al., 1994; Erpel et al., 1995) as they are fundamental for the binding of the PPII helix.

The aspartic acid residue at position 99 in the Src SH3 domain responsible for interacting with the RXL motif of SH3 domain ligands (section 1.4.3.2) is also conserved in Btk (D232), and Tec SH3 domains, as indicated in Table 4.2. In Itk a conservative substitution of a glutamic acid is found at this position. This substitution in Itk may result in the decreased affinity of the Itk GST-SH3 domain fusion protein for c-Cbl as compared to the Btk and Tec GST-
SH3 domains, as Src Asp99 has been shown to be important in forming a salt bridge with an arginine residue in the SH3 ligand (see section 1.4.3.2). As glutamic acid and aspartic acid are residues of similar structure, this could also explain why the interaction is not totally disrupted, but only reduced.

Table 4.2. The amino acid sequences of the Btk, Itk and Tec SH3 domains. Residues conserved in all three SH3 domains are in blue. The 6 conserved hydrophobic residues believed to be involved in ligand binding are underlined. The residue equivalent to Src-Asp 99 is red.

<table>
<thead>
<tr>
<th>Species</th>
<th>RT loop</th>
<th>n-Src loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btk</td>
<td>V.V.A</td>
<td>L.YDYNPM</td>
</tr>
<tr>
<td>Itk</td>
<td>V.V.I.</td>
<td>L.YDYC</td>
</tr>
<tr>
<td>Tec</td>
<td>V.V.A</td>
<td>M.YDFQ</td>
</tr>
</tbody>
</table>

Ligand specificity is determined by differences within the SH3 domain primary sequence, especially at the regions comprising the RT and n-Src loops (Feng et al., 1995; Lee et al., 1995, 1996)(see Table 4.2) that border the hydrophobic binding patch in the three dimensional structure of the domain, as discussed in section 1.4.3.2. Comparison of the amino acid sequences of the RT and n-Src loops of Btk, Itk and Tec (Btk residues L221-E240, and E246-P250, respectively, see Table 4.2) show that only a small proportion of amino acids are conserved in these regions of all three SH3 domains (5/18 in the RT loop, and 0/6 in the n-Src loop), however many residues are conserved between two proteins out of the three, and the differences observed include many conservative substitutions, consistent with the similarity of their ligand binding profiles shown in Fig.4.3. The decreased binding of c-Cbl to Itk, compared to
Btk and Tec GST-SH3 domain fusion proteins, may be a result of conservative amino acid substitutions occurring within the n-Src and RT loops (including the substitution of E for D at the position equivalent to Src Asp 99 within the RT loop of Itk). While differences may not completely prevent the binding of c-Cbl to the Itk SH3 domain, neither does the amino acid sequence form the optimal SH3 domain binding surface for binding the conformation of the c-Cbl PPII helix.

As c-Cbl but not WASP binding is affected by differences between Itk and Tec/Btk SH3 domains, possibly due to differences within the RT and n-Src loops, WASP and c-Cbl may be bound by different interactions involving their proline rich regions and the SH3 domain (see also sections 5.2.3 and 5.2.4). For example, c-Cbl may rely on RXLPXXP for binding, whereas peptide binding studies indicate that WASP may bind Tec family SH3 domains via PXXP motifs within a different sequence (Cory et al., 1996, Finan et al., 1996). As titration experiments show that WASP is the preferred ligand for the Tec family SH3 domains, the observed binding of c-Cbl may be an artifact. Alternatively the Btk SH2 and SH3 domains may act synergistically to bind c-Cbl, and the interaction with the SH3 domain alone is relatively weak.

4.3.3 SH3 domain binding and protein function

Although in these experiments the Itk SH3 domain fusion protein was used to bind cellular ligands from lysate of mature B cells in which Itk is not expressed, both c-Cbl and WASP are expressed in T cells as well as B cells. T cells are affected in Wiskott-Aldrich syndrome patients, and c-Cbl is implicated in signalling through the TCR (Donovan et al., 1994), a receptor that utilises many proteins common to both T and B cells in a highly homologous manner (Weiss and Littman, 1994; Borst et al., 1996). Indeed, it is proposed that Btk and Itk perform analogous functions in BCR and TCR mediated signalling, an idea strengthened by the results reported here showing similar interactions of their SH3 domains with cellular ligands identified as WASP and c-Cbl, plus the
unidentified proteins with the same apparent molecular weights bound by the SH3 domains of all three members of the Btk family of PTKs (section 4.2.2, Fig. 4.3).

The similarities in the *in vitro* cellular ligands of Itk, Btk and Tec may indicate the likelihood that intramolecular interactions within these proteins are similar, and thus that Btk and Tec SH3 domain, and proline rich region accessibility may be regulated in a manner similar to that shown for Itk (section 1.7.3.1.1).

The functional redundancy of Tec and Btk in B cells, and Tec and Itk in T cells is not known. Tec function does not appear to be able to compensate for Btk function in XLA patients, nor for Itk function in Itk deficient mice (Liao and Littman, 1995) and therefore differences that arise between these proteins, apparently not including the SH3 domains ligands, give rise to their specific functions. Btk deficient DT40 cells could be investigated for the possible restoration of Ca**+** flux on the introduction of Itk or Tec expression constructs, and it would be interesting to observe the requirement for the Tec family SH3 domains in such a system.

### 4.3.4 SH3 domains and B cell activation

B cell activation by ligation of the BCR does not induce any changes in the binding of the SH3 domain to WASP, and although c-Cbl binding is reduced by cell activation, it is not abrogated. Thus changes in WASP and c-Cbl, such as their phosphorylation at tyrosine residues, known to occur at c-Cbl on BCR ligation (Cory et al., 1995), or other modifications, do not result in a drastic change in their capacity to bind the SH3 domains of the Btk family of PTKs. However changes that might occur at the SH3 domain, such as phosphorylation at Y223 within the Btk SH3 domain (Park et al., 1996) (a residue conserved in Itk and Tec) that are a result of B cell activation will not be represented in these experiments. Btk autophosphorylation is an intramolecular event (Mahajan et al., 1995), and as the SH3 domain fusion protein contains no
catalytic function, phosphorylation at Y223 cannot occur. Alternatively should the fusion protein be a substrate of any other enzymes it would not have been compartmentalised in the cytoplasm on cell activation to be in the correct location. Moreover, should the fusion protein be a substrate for any enzymes, Btk or otherwise, capable of activity in cold NP40 lysis buffer, the amount of Btk GST-SH3 domain fusion protein introduced into the cell lysate would be far in excess of the normal amount of Btk in the cell and any modification that may result in a change in the ligand binding capacity of the SH3 domain fusion protein of Btk (or Itk or Tec) would surely be overwhelmed by remaining unmodified fusion protein.

4.3.5 Titration of SH3 domain ligands

The stronger binding of WASP by the SH3 domains tested compared to that of c-Cbl may be interpreted as suggesting that WASP is the most likely *in vivo* ligand (D.Cantrell, personal communication). However it must be remembered that the binding between domain and ligand seen as a result of these experiments is a static reflection of protein interactions at a single time point and will not reflect the dynamic interactions taking place within the cell. Both WASP and c-Cbl being physiologically relevant ligands of the SH3 domains of Btk, Itk and Tec is not inconsistent with there suggested roles as important messengers in T and B cell signalling.
Chapter 5

Phosphorylation of the Btk SH3 domain alters its ligand binding properties

5.1 Introduction

The exact role played by Btk in B cell signalling remains elusive. However, investigations into Btk phosphorylation on cell stimulation has produced important results concerning how such phosphorylation may regulate the activation of Btk.

As discussed in section 1.7.3.1.2, phosphorylation of Btk at Y551 within the activation loop of the catalytic domain results in activation of the enzymatic activity of Btk, which autophosphorylates at a second site mapped to Y223, summarised in Fig.5.1. Sequence comparisons with SH3 domains of known structures (Noble et al., 1993; Andreotti et al., 1997), or on which mutational studies have been performed (Erpel et al., 1995), indicate that Btk Y223 is one of the highly conserved SH3 domain residues that cluster together in the tertiary structure of the domain to form the hydrophobic patch which interacts with the PPII helix of the ligand. Thus Btk autophosphorylation at this position may alter the affinity of the SH3 domain for its cellular ligands (or intramolecular binding regions). Btk autophosphorylation may therefore have the potential to act as a controlling switch for the function of Btk on cell activation.
It has been shown that in activated cells the SH3 domain of activated Btk is phosphorylated at Y223 (Park et al., 1996). Immunoprecipitation of Btk from stimulated and unstimulated cells using H360B anti-Btk does not result in coprecipitation of any other proteins (see Fig.3.4(A), and Cory, 1997). No Btk ligands or changes in the population of Btk ligands that may occur on cell activation can thus be detected using this method. However as presented in Chapter 4, Btk GST-SH3 domain fusion protein can be used as a tool to detect in vitro ligands of this particular region of Btk and these may be seen as candidate in vivo ligands.

In order to investigate the possibility of changes in the specificity of binding of the Btk SH3 domain on cell activation, an in vitro assay was established to mimic the phosphorylation of Y223 on a Btk GST-SH3 fusion protein. Ligands bound by phosphorylated SH3 domain fusion protein in B cell lysates were compared with those bound by the normal, non-phosphorylated Btk GST-SH3 domain fusion protein, in order to compare and contrast their ligand specificities.
Figure 5.1 Model of Btk phosphorylation on B cell stimulation. (i) B cell is stimulated through the B cell receptor by cross-linking. The Src PTK, Lyn which is found constitutively associated with sIgM, is activated and (ii) transphosphorylates Btk at tyrosine 551 (Y551). Phosphorylation at this site is believed to alter the conformation of the kinase domain to an active state which results in (iii) Btk autophosphorylation at another tyrosine residue, Y223 which lies within the SH3 domain. Dotted lines indicate proposed phosphorylation events.
5.2 Results

5.2.1 Phosphorylation of the Btk GST-SH3 domain fusion protein

The GST-SH3 domain fusion protein of Btk was phosphorylated in vitro by a purified baculovirus preparation of Btk in kinase assay buffer containing an optimised concentration of ATP, plus MgCl₂ and MnCl₂. A similar approach (Park et al., 1996) showed that a Btk GST-SH3 domain fusion protein was tyrosine phosphorylated on Y223 by recombinant Btk, mimicking the tyrosine phosphorylation that occurs following BCR ligation. Fig.5.2 (A), shows an increase in tyrosine phosphorylation of Btk GST-SH3 domain when incubated with increasing concentrations of recombinant Btk. To control for the presence of fusion protein in the lanes where no phosphorylation was seen, the membrane was immunoblotted with anti-GST (B).

![Image: Figure 5.2 Phosphorylation of the Btk GST-SH3 domain fusion protein by baculovirus Btk. 1 μg of fusion protein was incubated with either 3, 10 or 30 ng of baculovirus Btk in kinase buffer and an optimised amount of ATP for 1 hr at room temperature. The fusion protein was boiled in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with (A) anti-phosphotyrosine (αpY) and (B) anti-GST. Arrows point to fusion protein.]

Fusion Protein: SH3

Increasing ng Btk

A

α pY

46 —

32 —

B

α GST

46 —

32 —

Fusion Protein: SH3

Increasing ng Btk

Figure 5.2 Phosphorylation of the Btk GST-SH3 domain fusion protein by baculovirus Btk. 1 μg of fusion protein was incubated with either 3, 10 or 30 ng of baculovirus Btk in kinase buffer and an optimised amount of ATP for 1 hr at room temperature. The fusion protein was boiled in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with (A) anti-phosphotyrosine (αpY) and (B) anti-GST. Arrows point to fusion protein.
On incubation of a GST only fusion protein with Btk and ATP in kinase buffer no tyrosine phosphorylation was observed (Fig. 5.3), indicating that the GST moiety of the fusion protein is not a substrate of Btk and that phosphorylation most likely occurs within the SH3 domain moiety of the fusion protein using the same conditions as previously (Park et al., 1996).

![Diagram showing phosphorylation](image)

Figure 5.3 The GST moiety is not phosphorylated by Btk. Btk GST-SH3 and GST fusion proteins, before (SH3, GST), and after (SH3YP, GST'yp') incubation with Btk, were analysed by SDS-PAGE and immunoblotted with (A) anti-phosphotyrosine (αpY), and (B) anti-GST.

### 5.2.2 Purification of phosphorylated Btk GST-SH3 domain fusion protein

The phosphorylated Btk GST-SH3 domain fusion protein (SH3YP) was purified over an anti-phosphotyrosine column as described in Fig. 5.4. Fig. 5.5 shows samples taken from each stage of phosphorylation and purification.
Figure 5.4. Purification of phosphorylated SH3 domain fusion protein (SH3YP). (A) GST-SH3 domain fusion protein of Btk (SH3), attached to glutathione Sepharose 4B beads was incubated with baculovirus Btk and ATP (see fig 5.2) in a buffer containing MnCl₂ and MgCl₂, for 1 hour at room temperature. (B) The fusion protein, which comprised phosphorylated and unphosphorylated forms was eluted from the beads with glutathione. (C) The eluted fusion protein in binding buffer solution was poured through a column packed with α-phosphotyrosine (αpY) monoclonal antibody, 4G10, attached to protein A Sepharose beads. The column was washed thoroughly with more binding buffer, before being eluted of tyrosine phosphorylated fusion proteins (SH3YP) with phenol phosphate, a phospho-tyrosine analog. It was then washed of non-specifically bound proteins with glycine. (D) The phenol phosphate eluate containing SH3YP was diluted in more binding buffer and incubated with fresh glutathione Sepharose 4B beads.
resulting in a preparation of fusion protein containing only phosphorylated Btk GST-SH3, with no contaminants (also see Fig.5.9). The recovery of SH3YP is about 10% of the total fusion protein initially added to the phosphorylation reaction. The anti-GST immunoblot shows no degradation of the fusion protein indicating that it remains stable after phosphorylation and purification.

**Figure 5.5 Steps in the recovery of Btk GST-SH3YP on purification through an anti-phosphotyrosine (apY) column.** Fusion protein from various stages of purification was resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with (A) anti-GST and (B) anti-phosphotyrosine (apY). The amount of SH3YP collected on its rebinding to glutathione Sepharose 4B beads was approximately 10% of the amount of fusion protein originally added to the phosphorylation reaction. Loss is accounted for mainly due to (i) inefficiency of glutathione elution of fusion protein from glutathione Sepharose 4B beads; sample run shows fusion protein remaining bound to beads after three rounds of elution, and is also due to (ii) fusion protein remaining unphosphorylated, seen as a sample of flow through from the column, (iii) residual SH3YP not eluted by phenol phosphate from the apY column, seen in a sample of the glycine eluate, (iv) SH3YP not rebound to glutathione Sepharose 4B beads. SH3YP was collected as a clean preparation (see also Fig.5.8), as the major species rebound to glutathione Sepharose 4B beads.
5.2.3 Precipitation of c-Cbl with Btk GST-SH3 and Btk GST-SH3YP

Previous work from this laboratory has shown that the proto-oncogene, c-Cbl binds the SH3 domain fusion protein of Btk in vitro (Cory et al., 1995) (and shown in Chapter 4, this thesis). In order to determine if phosphorylation of the Btk SH3 domain fusion protein had any effect upon its binding of c-Cbl, equal amounts (10μg) of Btk GST-SH3 domain fusion protein, Btk GST-SH3YP domain fusion protein, and GST only control, attached to glutathione Sepharose 4B beads, were incubated with NP40 lysates from both BCR ligated (using Cappel, see section 3.2.2) and unstimulated Daudi cells. Fig. 5.6 shows c-Cbl was present in the sample of whole cell lysate and in the precipitates of both phosphorylated and unphosphorylated Btk GST-SH3 fusion proteins, migrating with an apparent molecular weight of 120 kD from both stimulated and unstimulated cells. It is absent in the GST controls.

Since GST incubated with Btk under phosphorylating conditions was not phosphorylated (GST'yp') (Fig. 5.3), and its binding profile was not altered when incubated with cell lysate (data not shown), it was not included in future experiments.
Figure 5.6 Phosphorylation of Btk GST-SH3 does not affect its capability to bind c-Cbl in B cells. Lysates of stimulated and unstimulated Daudi cells were incubated with Btk GST-SH3 (SH3), phosphorylated Btk GST-SH3, (SH3YP), GST, and Btk treated GST (called GST 'yp', as it is not in fact phosphorylated). Fusion protein precipitates were washed, resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with anti-c-Cbl antibody. A sample of whole cell lysate (wcl) was removed prior to fusion protein incubation and separated with the precipitates.

5.2.4 WASP is no longer bound to the SH3 domain of Btk when the fusion protein is phosphorylated

The SH3 domain fusion protein of Btk has previously been reported to also bind WASP in vitro (Cory et al., 1996) (shown in Chapter 4, this thesis). Equal amounts of phosphorylated and unphosphorylated Btk GST-SH3 fusion protein and GST only were incubated with stimulated and unstimulated Daudi cell lysates as in section 5.2.3. Anti-WASP immunoblots are shown in Fig.5.7 (A). WASP can be seen as a protein migrating with an apparent molecular weight of 65 kD in the whole cell lysate and in the Btk GST-SH3 domain fusion protein precipitates before and after stimulation. However on phosphorylation of the Btk GST-SH3 domain fusion protein, WASP is no longer bound from the cell lysate. In (B) the lower part of the membrane containing precipitates from
stimulated cells was immunoblotted with an anti-phosphotyrosine antibody to confirm the presence of SH3YP. Fig 5.7(B) also demonstrates that the Btk GST-SH3 domain fusion protein does not become phosphorylated when incubated with cell lysate, as compared with the \textit{in vitro} phosphorylated Btk GST-SH3 domain which remains clearly tyrosine phosphorylated, apparently unaffected to any major extent by cellular phosphatases.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Phosphorylation of Btk GST-SH3 abrogates its ability to bind WASP in B cells. Daudi cell lysates were incubated with equal amounts of Btk GST-SH3 (SH3), Btk GST-SH3YP (SH3YP), and GST fusion proteins. Half the cells prior to lysis were stimulated through the BCR. The fusion protein precipitates were washed and resolved by SDS-PAGE analysis, and transferred to a nitrocellulose membrane. The membrane was cut in half; (A) the top half of the membrane was immunoblotted with anti-WASP (SK3), and the bottom half with anti-phosphotyrosine (B).}
\end{figure}
5.2.5 An *in vitro* kinase assay of Btk GST-SH3 fusion protein precipitates shows distinct species bound only by SH3YP

Equal amounts of Btk GST-SH3, SH3YP, and GST fusion proteins (10 µg) were incubated with cell lysates from both BCR stimulated and unstimulated Daudi cells and the precipitates were subjected to *in vitro* kinase assays. In Fig. 5.8, Btk GST-SH3YP can be seen to bind two proteins from stimulated cells that are labelled with $[^{32}\text{P}]-\text{ATP}$ in the *in vitro* kinase assay. A large signal is seen migrating with an apparent molecular weight of ~73 kD and a second smaller one with ~66kD. These proteins are referred to as p73 and p66. p66 however was not always present in these experiments. In future experiments the amount of fusion protein used to precipitate from the cell lysates was decreased from 10 to 5 µg, and p66 was no longer seen. Neither band was visible in either the non-phosphorylated fusion protein or GST precipitates or in precipitates from unstimulated cells.

Figure 5.8 Btk GST-SH3YP binds two proteins, called p73 and p66 from stimulated B cells, which are phosphorylated in an *in vitro* kinase assay. Stimulated and unstimulated Daudi cells were lysed and incubated with Btk GST-SH3YP, Btk GST-SH3 and GST fusion proteins. The precipitates were subjected to an *in vitro* kinase assay, separated by SDS-PAGE and the dried gel exposed to X-ray film. The three fusion proteins alone were run as controls.
5.2.6 The Btk GST-SH3YP ligand, p73 is phosphorylated on tyrosine residues.

Duplicate samples from an in vitro kinase assay performed as described above (section 5.2.5) but using 5 µg of fusion protein were separated by SDS-PAGE and protein was transferred to nitrocellulose membrane. Fig. 5.9 (A) shows an X ray film exposure of the membrane clearly showing p73 as a phosphorylated protein (p66 is absent). The membrane was then immunoblotted with an anti-phosphotyrosine antibody, Fig. 5.9 (B). p73 is clearly tyrosine phosphorylated and it migrates with the same apparent molecular weight as a major tyrosine phosphorylated species in the whole cell lysate of stimulated cells. On a longer exposure of this membrane to film after application of ECL reagent, more tyrosine phosphorylated cellular proteins are observed in the whole cell lysate, and a species of 120kD, presumably c-Cbl, was detected in the Btk GST-SH3/YP precipitates from stimulated cell lysates (results not shown).

Figure 5.9 p73 is phosphorylated on tyrosines. Stimulated cells were incubated with Btk GST-SH3, Btk GST-SH3YP and GST fusion proteins. The precipitates were washed and subjected to an in vitro kinase assay. Ligands were separated by SDS-PAGE, transferred to nitrocellulose membrane, and (A) exposed to X-ray film, and (B) immunoblotted with anti-phosphotyrosine (a-PY). Fusion protein alone was run as a control.
GST precipitates and fusion protein which had not been incubated with any cell lysate were treated and run as controls. Subjecting fusion proteins alone to an \textit{in vitro} kinase assay shows that radiolabelled species bound by SH3YP are not contaminants from the recombinant Btk preparation.

This finding that the phosphorylated SH3 domain of Btk bound what appeared to be the predominant cellular substrate of BCR induced tyrosine phosphorylation further implicates Btk in BCR signal transduction. That this interaction requires tyrosine phosphorylation of Btk GST-SH3 provides a clue as to the temporal regulation of Btk and protein/protein interactions following BCR signalling. It was therefore decided to identify p73.

5.2.7 \textit{p73 interacts directly with Btk GST-SH3YP, and can be re-precipitated with an anti-Syk antiserum.}

Due to the fact that p73 appears to be tyrosine phosphorylated following BCR ligation, is potentially capable of auto-phosphorylation, and migrates to approximately the correct molecular weight, likely candidates for this protein were Btk itself and another PTK, Syk. In Fig.5.10 (A) a Btk immunoprecipitate and a Btk GST-SH3YP precipitate from stimulated Daudi cells were subjected to an \textit{in vitro} kinase assay, separated by SDS-PAGE and the dried down gel exposed to X-ray film. Run on a large 8% gel, p73 is clearly migrating further than Btk (at 77kD), and Btk is therefore an unlikely candidate for p73. This was confirmed by re-precipitating the Btk GST-SH3YP precipitate with Btk. As can be seen in Fig.5.10 (B) after boiling a Btk precipitate in 1% SDS to disrupt any protein/protein interactions, Btk can be re-precipitated with more Btk antiserum. However p73 from the SH3YP precipitate could not be re-precipitated with Btk antiserum. Fig.4.10 (C) shows that the interaction between Btk GST-SH3YP and p73 is direct, as p73 can be re-precipitated with more Btk GST-SH3YP after an initial precipitation with the same. Should the interaction not be direct, and involve an intermediary linker, the chances that the association between Btk GST-SH3YP and p73 would re-associate outside of
the cellular environment and when diluted in lysis buffer to such an extent is highly unlikely.

Figure 5.10 p73 is not Btk, and it interacts directly with Btk GST-SH3YP. (A) p73 has a lower molecular weight than Btk. A Btk GST-SH3YP precipitate and a Btk immunoprecipitate from stimulated Daudi B cells were subjected to an in vitro kinase assay, and resolved by SDS-PAGE on a large 8% gel. The dried gel was exposed to X-ray film. (B) Btk cannot be re-precipitated from a Btk GST-SH3YP precipitate. Btk GST-SH3YP, GST, Btk and NRS immunoprecipitates from stimulated Daudi cell lysates were subjected to an in vitro kinase assay, boiled in 1% SDS and re-precipitated with H360B anti-Btk antiserum. Btk and NRS immunoprecipitates, and the re-precipitates were separated as in (A), and the dried gel exposed to X-ray film. (C) A Btk GST-SH3YP precipitate from stimulated Daudi cells was subjected to an in vitro kinase assay, boiled in 1% SDS, and p73 re-precipitated with fresh Btk GST-SH3YP, indicating the interaction between p73 and Btk GST-SH3YP is direct.
Fig. 5.11 shows an experiment similar to that in Fig 5.10 except that the dissociated SH3YP precipitates were re-precipitated with anti-Syk antiserum. *In vitro* kinase assays of Btk GST-SH3YP and Syk precipitates show that p73 and Syk migrate to almost the same position on the gel, with Syk migrating at 72 kD. On reprecipitation of Btk GST-SH3YP and Syk precipitates with Syk antiserum, Syk is clearly re-precipitated from both, although Syk re-precipitated from an SH3YP precipitate continues to migrate with a higher molecular weight than Syk in either the primary or the secondary precipitates. This difference may be explained by the existence of different post-translationally modified forms of Syk with distinct affinities for SH3YP and anti-Syk antisera. This is addressed further in the discussion. NRS re-precipitates were also performed and loaded as controls.

Figure 5.11 **p73 can be re-precipitated with an anti-Syk antibody.** Syk, NRS and Btk GST-SH3YP precipitates from stimulated Daudi B cells were subjected to an in vitro kinase assay, boiled in 1% SDS, and re-precipitated with Syk and NRS. The precipitates and re-precipitates were resolved by SDS-PAGE and the dried gel exposed to X-ray film.
5.2.8 Syk is detected in Btk GST-SH3YP precipitates from stimulated cell lysates by immunoblotting

In order to confirm an interaction between SH3YP and Syk, Btk GST-SH3, Btk GST-SH3YP, and GST fusion protein precipitates, having undergone an *in vitro* kinase assay, were separated by SDS-PAGE and the protein transferred to a nitrocellulose membrane.

In Fig. 5.12 (A) the membrane was first exposed to an X-ray film showing the phosphorylated protein in the Btk GST-SH3YP precipitate. The same membrane was then immunoblotted with an anti-Syk antiserum (B). The phosphorylated protein in (A) and the Syk band in the Btk GST-SH3YP precipitate from stimulated cells in (B) migrate with the same mobility. Interestingly Btk GST-SH3YP does not bind Syk in detectable quantities in unstimulated cells confirming that the absence of a radiolabelled species corresponding to Syk in kinase assays of SH3YP precipitates is due to an absence of Syk rather than detectable kinase activity.

Again Syk in the Btk GST-SH3YP precipitate runs with an apparently higher molecular weight to Syk in the whole cell lysate from both stimulated and unstimulated cells. The membrane was immunoblotted with anti-c-Cbl to control for protein in the unstimulated cell lysate (Fig. 5.12 (C)), and the membrane was also immunoblotted with an anti-GST antibody to control for equal fusion protein loading (D).

Thus the results in Fig 5.12 show that SH3YP inducibly associates with Syk in lysates of stimulated B cells, which is particularly intriguing as Btk containing phospho Y223 is also found following BCR ligation.
Figure 5.12 p73 cross-reacts with anti-Syk antibody on immunoblotting, and is seen only in precipitates from stimulated cells. Whole cell lysates of stimulated and unstimulated B cells were incubated with Btk GST-SH3, Btk GST-SH3YP and GST fusion proteins. Precipitates were subjected to an in vitro kinase assay, and separated by SDS-PAGE. The protein was transferred to nitrocellulose membrane, which was (A) exposed to X-ray film, (B) blotted with anti-Syk, (C) blotted with anti-c-Cbl (to control for protein in the unstimulated cell lysate) and (D) immunoblotted with anti-GST.
5.2.9 Phosphorylated peptides inhibit the binding of Btk GST-SH3YP to Syk

As the SH3 domain of Btk contains more than one tyrosine where it could potentially have been phosphorylated in this \textit{in vitro} system, see Fig. 5.13 (A), a peptide was designed to include a phosphate group at the mapped site of autophosphorylation within the SH3 domain (Y223) (Park et al., 1996). This peptide was used to compete with the Btk GST-SH3YP fusion protein for the binding of Syk. Control peptides were either unphosphorylated, or phosphorylated at Y225 which had been found to remain unphosphorylated on phosphopeptide sequencing of autophosphorylated Btk (Fig 5.15(B)) (Park et al., 1996).

Peptide was added to stimulated Daudi cell lysate to various concentrations prior to its incubation with fusion protein. The fusion protein precipitates were washed and subjected to an \textit{in vitro} kinase assay. The peptide phosphorylated at Y223 inhibits Syk binding to Btk GST-SH3YP at the lowest molarity whereas the non-phosphorylated peptide does not compete with SH3YP for Syk. (Fig. 5.14 (A)). In Fig.5.14 (B), the molarity of the phosphorylated Y223 peptide added was decreased further. It competes at a very low molarity of between 2 and 4\mu M. The control peptide phosphorylated at the position equivalent to Y225, also competed for Btk GST-SH3YP binding of Syk (Fig. 5.14 (C)).
Figure 5.13 Peptides designed from the SH3 domain of Btk. (A) Shows a diagram of the residues and the structural features that comprise the SH3 moiety of the Btk GST-SH3 domain fusion protein. The blue arrow delineates the region on which the peptide was designed. The red arrows point to the five tyrosine residues in the domain. (B) Shows the peptides used in competition experiments, (i) is not phosphorylated, (ii) is phosphorylated at residue Y223, and (iii) is phosphorylated at Y225. GGG was inserted at the N terminus for peptide stability and solubility.
Figure 5.14 Phosphorylated peptides compete with Syk for Btk GST-SH3YP binding (A) Stimulated Daudi cell lysates were incubated with either peptide phosphorylated at position Y223 (pY-223) or non-phosphorylated peptide (pep), prior to addition of Btk GST-SH3YP fusion protein. GST precipitates were run as controls. Fusion protein precipitates were subjected to an in vitro kinase assay, and separated by SDS-PAGE. The dried gel was exposed to X-ray film. (B) The molarity of pY-223 peptide preincubated with cell lysates was reduced, and the experiment performed as in (A). (C) Peptide phosphorylated at position Y225 (pY-225) was also used to compete with Btk GST-SH3YP for Syk binding as described in (A).
5.2.10 Btk GST-SH3YP fails to bind a phosphoprotein co-migrating with Syk in a Syk negative mast cell line

p73 bound by Btk GST-SH3YP in stimulated B cells migrates less than Syk on SDS-PAGE analysis. Despite p73 being recognised by immunoblotting and immunoprecipitation by an anti-Syk antiserum, the possibility was explored that another protein containing the epitope recognised by the anti-Syk antibody that migrates to this position but is not Syk, binds SH3YP. To do this we used a Syk negative primary mast cell line (kind gift of P. Costello, NIMR).

Syk in wild type mast cells is highly susceptible to proteolytic degradation resulting in a truncated protein which migrates on SDS-PAGE analysis at ~40 kD, illustrated in Fig. 5.15 (B). In Fig. 5.15 (A), the 40kD truncated Syk is detected in wild type mast cell lysates as the predominant species to cross react with anti-Syk on immunoblotting; only a very weak band is detected running at 72 kD which cross reacts with anti-Syk (lane 6). Neither 40kD nor 72kD species are detected in the Syk deficient cells on immunoblotting with anti Syk (lane 7). Daudi cell lysate was also immunoblotted with anti-Syk in order to control for the position of 72kD Syk (lane 5).

Btk GST-SH3YP was incubated with lysates from Syk deficient and wild type mast cells which were stimulated through the IgE receptor, FcεRI, which is also known to result in the phosphorylation of both Btk and Syk (Benhamou et al. 1993; Kawakami et al., 1993), (cells were lysed in buffer containing 10X normal concentrations of protease inhibitors in order to reduce Syk degradation). Precipitates were washed, subjected to an in vitro kinase assay, separated by SDS-PAGE, and radiolabelled species detected by autoradiography. Fig. 5.15 (A) lane 3 shows that in stimulated wild type (Syk positive) mast cells, Btk GST-SH3YP binds a 73kD species, subject to phosphorylation in an in vitro kinase assay, similar to that seen bound in stimulated Daudi B cells. This band is not observed in Btk GST-SH3YP
precipitates from Syk deficient cells (lane 1) indicating that the p73 ligand of Btk GST-SH3YP is indeed Syk, and that no other proteins subject to phosphorylation under the same conditions, co-migrates with it in mast cells. In unstimulated mast cells, as in unstimulated B cells, this interaction was not observed (data not shown). This result also suggests that the potential interaction between Syk and Btk is a common feature of BCR and FceRI signalling.

5.2.11 Btk GST-SH3YP interacts with the N-terminus of Syk which does not contain either Syk SH2 domain

Interestingly in Btk GST-SH3YP precipitates from stimulated Syk+ wild type mast cells (Fig. 5.15 (A) lane 3), a 40 kD species is also labelled with \([\gamma^{32}P]^{-}\)ATP after the *in vitro* kinase assay. This band runs to the same position as 40kD Syk in the mast cell lysate (lane 6) and is not precipitated by Btk GST-SH3YP in Syk deficient cells (lane 1), indicating that Btk GST-SH3YP not only binds full length Syk in stimulated B and mast cells, but it also binds a truncated 40kD Syk protein that retains its catalytic activity, and represents the C-terminus of Syk, which interestingly does not contain either of the SH2 domains.
Figure 5.15 SH3YP precipitates no 72kD protein from mast cells expressing no Syk. (A) Syk<sup>+</sup> and Syk<sup>-</sup> primary mouse mast cells stimulated through FcεRI were lysed in NP40 lysis buffer containing 10X protease inhibitors. Lysates were incubated with Btk GST-SH3YP (lanes 1 and 3) and GST fusion proteins (lanes 2 and 4). Fusion protein precipitates were washed in lysis buffer, incubated with [γ<sup>32</sup>P]-ATP] and separated by SDS-PAGE (lanes 1-4). The gel was dried and exposed to X-ray film. Syk is seen as a 72kD band and also a 40kD protease degraded version (tSyk) precipitated by Btk GST-SH3YP in lane 3. Samples of whole cell lysate (wcl) from wild type, Syk deficient and Daudi B cells were immunoblotted with anti-Syk antibodies (lanes 5-7). (B) The site of proteolytic degradation resulting in a fragment with a predicted molecular weight of ~40 kD is shown in a cartoon of the Syk protein.
5.2.12 Btk and Syk co-precipitation

In order to investigate the possibility of an \textit{in vivo} association between Btk and Syk, it was attempted to co-precipitate them from NP40 lysates of stimulated Daudi B cells. In Fig. 5.16, lanes 2 and 4 show Syk and Btk precipitates respectively. The precipitates were subjected to an \textit{in vitro} kinase assay. This system of detection was used, as kinase assays are the cleanest and most sensitive way of detecting a species known to be capable of auto-phosphorylation, as are both Btk and Syk. If Btk and Syk did co-precipitate a second closely running band would be seen in the precipitate of one or the other. However in the Btk precipitate no band was seen to run at the position of Syk (lane 4), and in the Syk precipitation no band ran at the position of Btk (lane 2).

As Btk GST-SH3YP represents the state of the SH3 domain of Btk upon its phosphorylation after Btk activation, and the increase in the apparent molecular weight of Syk precipitated by SH3YP may be due to an increase in Syk tyrosine phosphorylation (see discussion), it was thought that an \textit{in vivo} interaction may be more to likely to be observed were one or other of the proteins in a highly phosphorylated state. Co-precipitation was therefore attempted using Btk and Syk that had been precipitated from stimulated cell lysates and then incubated with ATP, MnCl$_2$ and MgCl$_2$, producing highly auto-phosphorylated and activated Btk and Syk, indicated as Btk$^+$ and Syk$^+$. Btk$^+$ and Syk$^+$ still attached to protein A Sepharose beads were washed and incubated with the lysate of more stimulated Daudi cells (Fig. 5.16, lanes 3 and 5). The precipitates were then washed, subjected to an \textit{in vitro} kinase assay, separated by SDS-PAGE and radiolabelled species detected by autoradiography as were the Btk and Syk precipitates in lanes 2 and 4. However in none of the precipitates were two bands that could be Btk and Syk co-precipitating observed.
Btk+ and Syk+ are not however fully phosphorylated by incubation with ATP in kinase buffer, as further \(^{32}\text{P}\)-ATP was incorporated into both kinases in the *in vitro* kinase assay. Btk and Syk may therefore fail to co-precipitate in this experiment as using such a technique it is not possible to produce sufficiently tyrosine phosphorylated proteins.

Figure 5.16 Btk and Syk fail to co-precipitate in stimulated B cells. Stimulated B cells were lysed in NP40 lysis buffer, and immunoprecipitated with anti-Btk, anti-Syk and NRS. To test the possibility that Btk and Syk may interact when Btk, and possibly Syk, are highly phosphorylated, washed Btk and Syk (and NRS) immunoprecipitates were incubated with ATP in kinase buffer, and then highly phosphorylated Btk and Syk (Btk+ and Syk+), still attached to protein A Sepharose beads, were incubated with fresh stimulated cell lysate. All precipitates were incubated with \(^{32}\text{P}\)-ATP, washed, and resolved by SDS-PAGE. The gel was dried and exposed to X-ray film.
5.3 Discussion

Mapping of the site of Btk autophosphorylation on stimulation of Btk autokinase activity to a site within the SH3 domain poses interesting questions concerning the effects such phosphorylation may have upon the binding capabilities and specificity of the SH3 domain. Y223 is predicted to be important in SH3 domain interactions as (i) it is equivalent to one of six conserved residues shown by mutational studies to be involved in forming the PPII helix binding hydrophobic patch in the Src SH3 domain (Erpel et al., 1995). (ii) From comparisons with structural studies of the SH3 domain of Fyn, Btk Y223 is believed to lie on the surface of the SH3 domain ligand binding groove (Noble et al., 1993; Park et al., 1996), and (iii) the Itk residue Y180, which corresponds to Btk Y223, also lies on the SH3 domain binding surface (Andreotti et al., 1997).

Alteration in the binding of the Btk SH3 domain on Y223 phosphorylation could regulate Btk function on cell activation should it result in the binding of an alternative subpopulation of ligands not bound by the unphosphorylated SH3 domain. The SH3 domain of Src also contains a site of tyrosine phosphorylation (Y138), and phosphorylation at Y138 has been reported to result in a decrease in the affinity of this domain for proline rich peptides (Broome and Hunter, 1997). As Itk Y180 appears to be involved in the intramolecular interaction between the SH3 and TH domain of Itk that regulates the accessibility of these domains to cellular ligands, and the Src SH3 domain appears to utilise equivalent SH3 domain residues in inter- and intramolecular interactions (Erpel et al., 1995), phosphorylation at Btk Y223 may also be critical in the destabilisation of regulatory intramolecular interactions within Btk (see section 1.7.3.1.1).

SH2 domains have reportedly higher affinities for their ligands than do SH3 domains for theirs (Pawson, 1995), and they bind tyrosine phosphorylated proteins which are likely to arise upon cell stimulation. SH2 domains are
therefore prime candidates for providing a switch mechanism which regulates proteins on cell stimulation as a result of cellular protein tyrosine phosphorylation dependent on/off ligand binding. However, the discovery that the SH3 domain of Btk contains a site of tyrosine phosphorylation indicates that the SH3 domain may also act as such a molecular switch. The *in vitro* evidence here shows that indeed upon tyrosine phosphorylation, Btk SH3 domain's binding fidelity changes in several ways.

The evidence described in this Chapter is to my knowledge the first demonstration that tyrosine phosphorylation of an SH3 domain confers on it the ability to bind a different ligand and provides information regarding the temporal regulation of Btk and Syk following BCR ligation.

### 5.3.1. Altered ligand binding on phosphorylation of the SH3 domain of Btk

Using an *in vitro* method to produce a purified preparation of Btk GST-SH3 domain fusion protein phosphorylated by Btk (Btk GST-SH3YP), ligands bound by the Btk SH3 domain in its resting, unphosphorylated state were compared with those bound by phosphorylated Btk GST-SH3, mimicking the *in vivo* autophosphorylated Btk SH3 domain (Park et al., 1996). The results presented show that not only does the phosphorylated state of Btk GST-SH3 make a difference to the ligand specificity of the domain, but the ligands bound also depends on the activation state of cells prior to lysis from which they are precipitated.

#### 5.3.1.1 c-Cbl

Btk GST-SH3 binds c-Cbl (Cory et al., 1995). Phosphorylation of Btk GST-SH3 does not appear to interfere with this interaction, despite it being assumed to occur due to an interaction between the six conserved residues of the Btk SH3 domain which form the hydrophobic ligand binding patch, of
which Y223 is one, and the PPII helix formed by the proline rich regions of c-Cbl which contain PXXP motifs.

5.3.1.2 WASP

The interaction between WASP and Btk GST-SH3, on the other hand, was disrupted on phosphorylation of the Btk SH3 domain fusion protein. No WASP, or levels so low as to be undetectable by immunoblotting, bound Btk GST-SH3YP from Daudi lysates. WASP appears to interact with the Btk SH3 domain via its two proline rich regions (Cory et al., 1996) (see section 1.4.4.2), and presumably the interaction between Btk GST-SH3YP and the PPII helices of WASP was disrupted by the presence of a phosphate group attached to the ligand binding hydrophobic patch of the Btk SH3 domain. The binding of WASP by both Btk GST-SH3 and Btk GST-SH3YP appeared to be unaffected by cell stimulation through the BCR.

WASP is a protein believed to be important in the organisation of the cytoskeleton and is also involved in an unknown capacity with protein tyrosine kinases (Banin et al., 1996, section 1.5.3). As a small proportion of the cellular population of Btk has been reported to localise to the cell membrane on cell activation (Kawakami et al., 1994) and the activated Btk mutant, Btk*, is also membrane associated (Li et al., 1995), membrane association may be an important step in Btk activation. Releasing Btk from an interaction with the cytoplasmic protein WASP in activated cells may be involved in this membrane localisation. As the dissociation of WASP and Btk is proposed to occur after Btk activation and phosphorylation at Y223, it is unlikely be a critical step in initial events leading to Btk activation (as implied by work showing that Y223 is not essential for Btk mediated BCR signalling (Kurosaki and Kurosaki, 1997)) but may augment events leading to maximal Btk activity on cell stimulation.

WASP is bound more readily by Btk GST-SH3 than c-Cbl (Fig. 4.6), but this association is readily disrupted by phosphorylation at Y223 within Btk GST-SH3, whereas c-Cbl and Btk GST-SH3YP still interact. This indicates that WASP and c-Cbl may be bound due to different interactions involving the Btk
SH3 domain (see also section 4.3.2). It may also imply that the WASP:Btk GST-SH3 interaction may be more specific and hence more physiologically relevant than the interaction of the Btk SH3 domain with c-Cbl.

5.3.1.3 Syk

A consequence of the phosphorylation of the SH3 domain of Btk is that it could potentially be turned into an SH2 domain binding protein. Indeed the amino acid sequence in which Y223 lies, YDYM (where the first Y corresponds to Y223) is the motif that is preferentially bound by the SH2 domain of the p85 subunit of PI3-kinase (Songyang et al., 1993). PI3-kinase was treated as a candidate for binding SH3YP, but was not detected in the precipitate of the phosphorylated fusion protein by immunoblotting (data not shown).

Although it may be possible that Syk interacts with the phosphorylated SH3 domain fusion protein of Btk in activated cells via an SH2 domain interaction, this seems unlikely as (i) for Syk activation \textit{in vivo}, both Syk SH2 domains are required to bind phosphorylated ITAM motifs within receptor subunits on cell activation, (ii) the binding consensus of the Syk C terminal SH2 domain does not match the amino acid sequence in which Y223 lies (Songyang et al., 1994) (the N terminal SH2 domain was not tested) and (iii) most significantly, data presented here shows that Btk GST-SH3YP binds the 40 kD truncated version of Syk from the mast cells which does not include either of the Syk SH2 domains.

The interaction between Syk and the Btk SH3 domain fusion protein is not observed when the fusion protein is not phosphorylated, nor is it observed in unstimulated cells. This implies that an interaction that may occur \textit{in vivo} between these two proteins depends not only upon the activation and autophosphorylation of Btk, but also on some modification to Syk that occurs on cell activation. Syk is tyrosine phosphorylated on stimulation of B cells through the BCR (see section 1.8.2.2) and evidence that the interaction between Btk GST-SH3YP and Syk may depend on Syk tyrosine phosphorylation is that Syk precipitated by the phosphorylated Btk SH3 domain fusion protein
migrates with retarded mobility compared to the normal 72 kD protein. A similar increase in the molecular weight of Syk, described as being due to a high degree of tyrosine phosphorylation, has been observed in several reports (Kurosaki et al., 1994; Kimura et al., 1996).

Interestingly, highly phosphorylated Syk has been shown to take on a different conformation to unphosphorylated Syk in unstimulated cells, which somehow involves the Syk C-terminal tail (Kimura et al., 1996). The increase in Syk phosphorylation could be induced by the addition to a Syk immunoprecipitate of a peptide designed as a doubly phosphorylated ITAM which binds the dual Syk SH2 domains, and also by the addition of an antibody raised against the C-terminal tail of Syk (Kimura et al., 1996). (A second report observed however, that the activation of Syk induced by the addition of SH2 domain binding peptides could be inhibited by an antibody raised against the same residues of the Syk amino terminal tail (Shiue et al., 1995). This discrepancy is discussed in section 1.7.3.3).

Btk GST-SH3YP is proposed to interact with the portion of Syk C-terminal of the proteolytic site as depicted in Fig.5.15 (B). This region of Syk contains the kinase domain, unlikely to be involved in protein:protein interactions, and the C-terminal tail, thought to be involved in the regulation of Syk phosphorylation and activation (Shiue et al., 1995; Kimura et al., 1996).

It is unknown whether Btk GST-SH3YP interacts with a sub-population of Syk which is heavily tyrosine phosphorylated, or if the interaction between the two proteins results in a direct increase in Syk tyrosine phosphorylation. The former is unlikely as Syk in lysates of stimulated cells does not appear as a doublet on immunoblotting with an anti-Syk antiserum, as would be expected were two differentially phosphorylated populations of Syk present in the lysate, (Fig. 5.12 (B)). The latter may be true and may possibly be caused by an interaction involving the Syk C-terminal tail with Btk GST-SH3YP, resulting in Syk activation similar to that induced by the anti-Syk C terminal tail antibody (Kimura et al., 1996). Btk GST-SH3YP may not interact with Syk in
unstimulated cells, due to the conformation of unphosphorylated Syk where the Syk C-terminal tail is proposed to be masked from the Syk C terminal tail antibody (Kimura et al., 1996), and perhaps from binding ligands such as Btk GST-SH3YP.

Comparing the data presented here with studies of Syk activation due to interactions involving its C-terminal tail, it suggests that Btk may be involved in the regulation of Syk activation. Activation of Syk due to C-terminal tail interactions may require initial activation steps involving other regions of the protein such as the SH2 domains, known to be critical for Syk activation, which are initiated on cell stimulation. Without these initial steps the Syk C-terminal tail is not accessible to ligands, as seen in unstimulated cells where Syk is poorly recognised by the C-terminal tail antibody (Kimura et al., 1996), and observed in data presented here, where Btk GST-SH3YP binds no Syk in resting cells. In activated cells where Syk is tyrosine phosphorylated and active, Btk GST-SH3YP may bind due to induced accessibility of the C-terminal tail, and result in increased Syk activity and hyperphosphorylation. The proposed role of Btk acting to enhance the activity and tyrosine phosphorylation of Syk, but being unable to initiate it, is in accordance with studies that propose regulatory interactions between Btk and Syk are involved in the activation of PLCγ, and that although the activation of Syk is not dependent on the presence of Btk (Takata and Kurosaki, 1996), optimal Syk activity does require the presence of Btk (Wan et al., 1997), albeit this latter result being observed on the stimulation of Btk through a G protein coupled receptor.

Syk hyperphosphorylation is not observed in stimulated cell lysates immunoblotted with anti-Syk antibodies, as Syk from resting and stimulated cell lysates run to the same position when analysed by SDS-PAGE (Fig. 5.12 (B)). The physiological significance of hyperphosphorlyated Syk is not known, however it may not be detected in cell lysates if it is of a highly transient nature, or if in vivo only very small quantities, too low for detection by immunoblotting, are produced. Such a scenario might be expected from studies
showing that only a very small proportion of total cellular Syk is tyrosine phosphorylated (Kimura et al., 1996) or induced to associate with the BCR upon its ligation (Hutchcroft et al., 1992).

An interaction between Btk and Syk occurring on cell stimulation, could also act as a mediator of Btk membrane association. The induced accessibility of the Syk C-terminal tail, due to a conformational change in Syk upon its activation, caused by, or coinciding with its association with the doubly phosphorylated ITAM of Iγα or Iγβ, could localise autophosphorylated Btk to the membrane and the BCR complex.

An in vivo association between Btk and Syk, implicated by the in vitro data presented in this Chapter, would provide further evidence for the requirement of both PTKs in the same pathway leading to BCR induced activation of PLCγ. In particular, results presented implicate a role for Btk in the regulation of Syk, which may be required for maximal Syk activation, downstream of initial events at the BCR upon its ligation, which result in the tyrosine phosphorylation and activation of numerous cellular proteins, including Btk.

5.3.4 In vivo interactions between Syk and Btk

Both Btk and Syk have been shown to be required for the activation of PLCγ-2, as discussed in section 1.8. Attempts to show an in vivo association between Btk and Syk by co-precipitation experiments in stimulated and unstimulated cells (data not shown) however, have not proved successful. This could be a result of disruption in protein conformation which may be vital for protein:protein interactions due to antibody binding at the epitope. Both anti-Btk and anti-Syk antisera were raised against the unique regions of the proteins, which may be required for the maintenance of protein conformations important for protein:protein interactions. Alternatively, an interaction of very transient nature may be not picked up in, or may be disrupted by a detergent cell lysate (Btk/Syk interactions were also sought in digitonin lysates of B cells,
without success, data not shown). Other systems such as overexpression of membrane associated Syk and Btk, co-precipitation of epitope tagged proteins, or immuno-staining of whole cells may provide evidence for an \textit{in vivo} interaction between Syk and Btk, should such an interaction occur.

\subsection*{5.3.5 Phosphorylation at residues other than Y223}

Peptide competition experiments showed that the interaction between Syk and Btk GST-SH3YP could be abolished by the addition of small quantities of competing peptide containing the amino acid sequence around Y223, with a phosphate group attached to Y223 (Fig. 5.14 (A) and (B)), the mapped site of autophosphorylation (Park et al., 1996). Unphosphorylated peptide did not compete with Btk GST-SH3YP for Syk binding. The interaction therefore between Btk GST-SH3YP and Syk can be said to require a tyrosine phosphorylated residue at position Y223. However as the same peptide with the equivalent of Y225 phosphorylated also competed with Btk GST-SH3YP for Syk binding, the possibility is not excluded that Btk GST-SH3YP produced by, and used in these experiments, is phosphorylated at Y225 (found to remain unphosphorylated on mapping of the site of Btk autophosphorylation (Park et al., 1996)). Y225 is another conserved residue within the Btk SH3 domain believed to make up the hydrophobic ligand binding patch (see Table 4.2). If the conformation of the competing peptides containing phosphorylated Y223 and Y225 maintain a similar conformation, it is perhaps not surprising that both can disrupt the association between Btk GST-SH3YP and Syk, as the phosphate group may lie in a similar region of the binding groove and bind Syk. The site of Btk autophosphorylation however was mapped to Y223, and not Y225, in a microsequenced Btk GST-SH3 fusion protein phosphorylated by Btk under the same conditions as used here, presumably resulting in the same pattern of tyrosine phosphorylation. Moreover, Btk GST-SH3 was incubated with one PTK only, Btk, and thus any sites of tyrosine phosphorylation on Btk GST-SH3YP must be attributed to the actions of Btk. Microsequencing of Btk GST-SH3YP would resolve this issue.
Chapter 6

Absent expression of the Wiskott-Aldrich syndrome protein in Wiskott-Aldrich syndrome patients

6.1 Introduction

Work performed previously in this laboratory has shown that the SH3 domain of the Btk family of PTKs binds the proline rich protein WASP (Cory et al., 1996). Mutations in WASP result in another primary immunodeficiency, the Wiskott-Aldrich Syndrome (section 1.5). WASP is expressed in all haematopoietic cell lineages, with T cells, B cells and platelets being most severely affected by disease causing mutations (Ochs et al., 1980). Btk, although expressed in all haematopoietic lineages except T lineages, when mutated only affects B lineages as observed in XLA patients. It was therefore of interest to examine whether the interaction between Btk and WASP may be disrupted in the B cells of WAS patients. Such a disruption could define a common underlying cause of the disease pathology.

The regions of WASP which interact with the SH3 domain of Btk were suggested to be the two proline rich regions within the protein, one near the N-
terminus (residues 160-181) and the other larger region towards the C terminus (residues 312-417) (Derry et al., 1994a) (see Fig. 6.4 (B)), confirmed by peptide competition experiments (Cory et al., 1996) (Fig 6.4(D)). Characterisation of the mutations in WAS patients however, has not revealed any mutations within these proline rich regions (Schwarz et al., 1996). This may indicate that interactions involving these regions are not of great importance to the occurrence of disease, and that mutations that may arise within these regions do not result in the disease phenotype but rather result in genetic polymorphisms. Due to the large number of proline residues, there may be redundancy of function, and the mutation of one proline may be compensated for by the presence of the others. The evolution of such a large number of proline residues within these regions resulting in such redundancy of function could indicate that proline rich region: SH3 interactions are in fact of great importance to the cell.

As the pathology of WAS was therefore unlikely to be due to the disruption of interactions of WASP with SH3 domain containing proteins such as Btk, WASP expression was investigated in WAS patients to determine whether patients with known mutations in the WAS gene express WASP in their haematopoietic system.

### 6.2 Results

#### 6.2.1 No WASP is detectable in the whole cell lysates of eleven WAS patients

The mononuclear cells from a 3-5ml blood sample from nine of the patients and a positive control were isolated using Ficoll extraction. The cells from two patients (7 and 9), were EBV transformed B cell lines cultured in supplemented RPMI medium. The primary and EBV transformed B cells were
washed, counted, and lysed in NP40 lysis buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an anti-WASP antiserum, SK3 (Fig. 6.1(A)). WASP could be seen as a species migrating with an apparent molecular weight of 65kD in the normal control, but was absent in all 11 patient samples.

The anti-WASP antiserum SK3 was raised against the C terminus of the protein (residues 487-501) (Banin et al., 1996) (see Fig. 6.4(C)). It is therefore not suitable for the detection of protein that may be truncated and lack this region. A second anti-WASP antiserum which was raised against a large portion of the protein towards the N terminus (residues 48-321) (Symons et al., 1996), was used to try to detect any protein in the patient samples (see Fig. 6.4(C)). Again WASP could be seen in the control lane running at 65kD, but was not visible in the patient samples 1-5 (Fig. 6.2(B)).

In order to control for non-specific protein degradation in the patient samples, an antibody against PKCβ1 (Yao et al., 1994) was applied to the membranes (Fig. 6.1(C)). All the patients show PKCβ1 migrating as a doublet with an apparent molecular weight of ~80 kD, showing that the lack of WASP expression was not due to non-specific degradation of cellular proteins.
6.2.2 Enrichment for WASP from the B cell line Daudi using the Btk GST-SH3 domain fusion protein

Figure 6.1 Absence of expression of WASP in WAS patients. Peripheral mononuclear cells from patients 1-8, 10 and 11 and a normal control (+), plus EBV transformed B cells from patients 7 and 9 were lysed, separated by SDS-PAGE, and immunoblotted with (A) anti-WASP SK3. Patients 1-5 plus a positive control were immunoblotted with anti-WASP, JD1(B). All patients and control were immunoblotted with anti-PKCβ1 (C).
6.2.2 Enrichment for WASP from the B cell line Daudi using the Btk GST-SH3 domain fusion protein

Subsequent characterisation of the mutations (F. Katz, this lab) in these patients (Table 6.1) showed that in patients 1, 3-7, and 11, both proline rich regions were likely to be unaffected, in patients 8-10 the earlier proline rich region bordered by residues 160-182 was unaffected, and in patient 2, the deletion included the coding region for the N terminal proline rich region, but not the C terminal region (Fig. 6.4 (A)). As at least one of the regions presumed to be responsible for the binding of WASP to the SH3 domain of Btk (Fig. 6.4 (D)), should remain intact in all patients, it was proposed that the SH3 domain fusion protein could be used as a clean and easy tool to enrich for WASP from patient primary cell lysates and thus detect WASP should it be expressed in quantities lower than normal and too low for detection by immunoblotting.

The system of immunoblotting from whole cell lysates (wcl) in order to detect the expression of a particular protein is limited by the amount of protein within a sample of wcl that can be loaded onto an SDS-PAGE gel and the sensitivity of the antibody used to detect it. In order to determine the capability of the Btk GST-SH3 domain fusion protein to enrich for the WAS protein from cell lysates, Btk GST-SH3 was incubated with lysates containing diminishing quantities of protein from the lysate of the mature B cell line Daudi. Daudi cells cultured in supplemented RPMI media were washed and lysed in the same manner as the primary and EBV transformed cells of the eleven patients studied. Lysates were diluted with sample buffer as indicated in Fig. 6.2, a sample removed to SDS sample buffer, and the remaining lysate incubated with 10μg of Btk GST-SH3 domain fusion protein. The fusion protein precipitates were washed, separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the anti-WASP antiserum SK3.
Table 6.1 WAS patient mutations

Patients 3&4 and 5&6 are siblings, patients 8&9 are unrelated. Small case letters for base changes in patients 8, 9&10 indicates intronic change. ** indicates detection by Southern blot. * indicates new mutation not published in WASPbase (Schwarz et al., 1996).

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Exon</th>
<th>Mutation</th>
<th>Codon Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>C134T</td>
<td>R34X</td>
</tr>
<tr>
<td>2</td>
<td>Δ exon 1-7 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>G257A</td>
<td>V75M</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>G257A</td>
<td>V75M</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>T284C</td>
<td>F84L</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>T284C</td>
<td>F84L</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>G431A</td>
<td>E133K</td>
</tr>
<tr>
<td>8</td>
<td>8/9</td>
<td>g811(+1)a</td>
<td>D259 onward affected</td>
</tr>
<tr>
<td>9</td>
<td>8/9</td>
<td>g811(+1)a</td>
<td>D259 onward affected</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>ins1255GG *</td>
<td>G408 Frameshift</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>ΔC1483 *</td>
<td>S483 Frameshift</td>
</tr>
</tbody>
</table>

The samples taken from the whole cell lysate of each lysate dilution prior to incubation with fusion protein were similarly separated and immunoblotted. WASP in these whole cell lysate samples is detectable when the amount of cellular protein present is down to 10% of normal (Fig. 6.2 (A)). Using the Btk GST-SH3 domain fusion protein to enrich for WASP allows for its detection when present in the lysates at levels as low as 1% of normal (Fig. 6.2 (B)).
Figure 6.2 SH3 domain fusion protein can precipitate visible quantities of WASP from lysates containing 1% of normal quantities of protein. Daudi cells were lysed in NP40 lysis buffer at 1.5X10^7 cells/ml, giving 100% normal lysate protein levels. The lysate was diluted with more lysis buffer to give diminishing quantities of protein in 0.5ml samples. A sample of lysate was taken from each dilution, separated by SDS-PAGE, and immunoblotted with the anti-WASP serum SK3 (A). The remaining lysates were incubated with 10ug of Btk GST-SH3 fusion protein, and the precipitates were separated by SDS-PAGE and immunoblotted with SK3 (B), showing enrichment for WASP from cell lysates containing decreasing quantities of the protein.
6.2.3 Enrichment of WASP from patient B cell lysates using the Btk GST-SH3 domain fusion protein

Lysates from all 11 patients and a normal control were incubated with 10 μg of Btk GST-SH3 and GST only fusion protein, the fusion protein precipitates were washed, separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the anti-WASP antiserum SK3. WASP from the normal control can be seen to be enriched for from the lysate using the Btk GST-SH3 domain fusion protein (SH3). However, no WASP was seen in the fusion protein precipitates from any of the eleven patients (Fig. 6.3 and data not shown), confirming the results obtained from immunoblots of the whole cell lysate and showing that the protein is not being expressed even at quantities considerably reduced compared to normal controls.

Figure 6.3 Enriching for WASP from patient lysates using Btk GST-SH3 fails to detect any protein. Patient cell lysates were incubated with 10μg of Btk GST-SH3 or GST only, the precipitates were washed, separated by SDS-PAGE, and immunoblotted with anti-WASP, SK3.
6.3 Discussion

In this Chapter eleven patients diagnosed as having the classical WAS phenotype with characterised mutations in the WAS gene were investigated for expression of WASP. Mutation analysis was performed by SSCP and sequence or Southern blot analysis using genomic DNA (F.Katz, this lab). The total number of mutations found was eight; two pairs of siblings have the same mutations and one mutation appeared in two unrelated patients (Table 6.1, and Fig.6.4 (A)). As expected, the mutations occur throughout the gene, with an excess occurring in the first two exons of the gene. Of the eight mutations, two are novel (patients 10 and 11). These previously unreported mutations are a dinucleotide insertion, GG, at position 1255 in exon 10, resulting in a frameshift at glycine 408 in patient 10, and a deletion of a single nucleotide, C, at position 1483 in exon 11 which results in a frameshift at serine 483 in patient 11.

None of the patients were found to express WASP. Failure to detect WASP expression in WAS patients by immunoblot analysis does not exclude the possibility that these patients could be expressing very low amounts of the protein, significantly less than the normal control and at levels below the sensitivity of the immunoblot analysis. This problem was addressed by enriching for WASP prior to the immunoblot analysis. The SH3 domain of Btk can bind WASP in vitro (Cory et al., 1996), and was used as tool to enrich for WASP. As the mutations in the WAS patients do not affect both the proline-rich regions of the protein believed to be responsible for the binding of WASP to SH3 domains, see Fig.6.4, it was presumed that this interaction would not be affected by the specific mutations. However, no WASP could be detected in Btk GST-SH3 precipitates from the patient samples, confirming the data from immunoblots of the patient whole cell lysates, and showing there is no expression of WASP above 1% of normal.
**A:** patient mutations 1-11:

![Image](image)

**B:** PH WH1 PR-N GBD PR-C WH-2

![Image](image)

**C:** regions against which antibodies were raised:

![Image](image)

**D:** PR peptides which compete with WASP for Btk-SH3 domain binding:

![Image](image)

**Figure 6.4** Structural diagram of WASP. (A) Shows the 11 patient mutations and their position in WASP. See Table 6.1 for the nature of the mutations. (B) Shows the domains of WASP and the positions of the border residues. (C) Shows the regions of WASP against which the anti-WASP antisera was raised. (D) Shows the regions of WASP shown previously to compete with WASP for binding the Btk GST-SH3 domain fusion protein.
Numbers of both mature T and B cells in WAS patients' peripheral blood decrease in an age dependent manner. The lymphoid cells which do develop and survive do so with deleterious consequences due to WASP malfunction or absence, resulting in the WAS phenotype. This can be contrasted to XLA (Vetrie et al., 1993), where mutations in Btk, the affected protein, result in a block in B cell development and no mature B cells are found in the peripheral circulation. Previous work has shown that in a majority of XLA patients investigated, Btk was undetectable in peripheral blood mononuclear cells (Hashimoto et al., 1996; Caspar et al., 1998). This appeared to be the case regardless of the type of mutation and its position in the gene. This study shows that a similar situation exists in WAS. Lack of WASP expression was confirmed by the use of two antisera, recognising different epitopes of WASP, and by attempting to enrich for the protein.

Although WASP expression has been investigated in WAS patients in other studies (Stewart et al., 1996; Remold-O'Donnell et al., 1997; Zhu et al., 1997), it was examined in patient cell lines in all but two cases. In two of these studies, WASP expression was investigated in a total of eighteen patient cell lines by Northern and immunoblot analysis (Stewart et al., 1996; Remold-O'Donnell et al., 1997). In the third study EBV transformed cell lines from a further 48 unrelated families with mutations in the WAS gene were analysed for protein expression. Taken together both studies showed that a majority of patients lacked, or had reduced, WASP mRNA and protein. The data presented in this chapter complements these observations by investigating a more relevant cell population, peripheral blood cells, showing that a similar situation exists in these primary cells; patients appear to lack protein expression at levels estimated at as low as 1% of normal.

The other studies found that WASP expression could be detected, but at reduced quantities in cell lines derived from patients with missense mutations. However the majority of these patients were described as having a less severe phenotypes with only mild immune dysfunction. It would be interesting to
investigate primary cells from these patients. Indeed patients 3 and 4 in this study have the same mutation as patient p41 in Remold-O'Donnell et al., (1997) and patients DS/DT/JW in Zhu et al. (1997). Whereas Remold-O'Donnell et al. and Zhu et al. in their studies found WASP protein levels at about 20-30% of normal in EBV transformed cells from these patients, no protein was found in the primary cells of patients 3 and 4 in the study presented here, who had the same G257A mutation, using a system sensitive enough to detect WASP expression as low as 1% of normal. Although this discrepancy may be due to differences arising from experiments using EBV transformed cell lines compared to those using primary patient cells, it is of interest to note that patients 3 and 4 in this study have a severe phenotype in contrast to the mild phenotype of patients 41, DS, DT and JW (Remold-O'Donnell et al., 1997; Zhu et al., 1997). Hence the idea that a correlation may be drawn between the severity of the phenotype, rather than the nature of the mutation, and the protein expression levels, holds. The most likely explanation for the lack of WASP protein must be that the majority of mutations alter its stability and it is degraded quickly following synthesis. This assay may therefore be useful as an initial screening procedure prior to subsequent DNA analysis. More patients with known mutations, particularly those with milder phenotypes need to be investigated for protein expression from primary tissues such as peripheral blood before this test can be used with confidence as a primary screening procedure for WAS.
Chapter 7

Discussion

Studies presented in this thesis were performed to elucidate some of the functional aspects of the role of Btk in B cell signalling. Two main experimental approaches were undertaken. Btk activation was investigated in the mature human B cell line Daudi, in order to determine if it is affected by ligation of two cell surface receptors, CD22 and CD38. The second experimental approach investigated *in vitro* interactions between the SH3 domain of Btk, and other Btk related PTKs, Itk and Tec. The previous identification of WASP as *in vitro* ligand for the Btk SH3 domain led to investigations into the expression of WASP in WAS patients.

7.1 Btk activation *in vivo*.

Btk activity is induced on antigen ligation of the BCR in mature human B cells (de Weers et al., 1994; Hinshelwood et al., 1995) and Btk function has been implicated in the cellular responses that occur downstream of BCR ligation, including PLCγ activation and apoptosis (section 1.3.1.2).

Btk expression is not limited however to slg expressing mature B cells, although the apparent non-random X chromosome inactivation observed in carrier mothers indicate that Btk function is only essential in B cell lineages. Btk is expressed in early developmental stages of the B cell lineage (Fig. 1.1), and in
other haematopoietic lineages such as mast cells, none of which express slg. Btk function is expected to be induced on activation of cells through other receptors due to the block in B cell development prior to slg expression in XLA patients and xid mice. Indeed in murine cells Btk activation has been reported to be induced on the ligation of numerous cell surface molecules, discussed in section 1.3.1.2.

7.1.1 Btk function and B cell development

The block in the development of B cell lineages in XLA patients implies a role for Btk in a signalling mechanism required for the continuation of development through a receptor, or receptors, found on the surface of pre-B cells. Ligation of the pre-BCR may signal to the cell nucleus and trigger later developmental stages in B cell development. Signalling through the pre-BCR results in tyrosine phosphorylation events (Brouns et al., 1993), and it has been suggested that Btk may play an essential role in this process. Studies in mouse pre-B cells however, reported Btk to be constitutively active in this lineage (Aoki et al., 1994) and although this does not indicate Btk activation being induced by pre-BCR signalling, it provides evidence for the functioning of Btk kinase activity in the development of pre-B cells. How Btk may be sustained in a constitutively active state in the immature cell line under study was not addressed. Other cell surface receptors are also expressed on B cells in early and late stages of their development, and may require Btk for successful signalling pathways. Studies in human cells have however failed to produce any evidence for Btk activation on ligation of any surface molecules besides the BCR.

Experiments in this thesis (Chapter 3) investigate Btk activation in mature B cells stimulated through CD38. Btk was implicated in CD38 mediated signalling, as several BCR unresponsive cell lines, including those derived from xid mice (Santos Argumedo et al., 1995), were also found to be unresponsive to CD38 ligation. This indicated that Btk function may be crucial for CD38
mediated signalling, and that the signalling machinery required for BCR activation was the same or similar to that required for CD38 mediated signals (and may include Btk). CD38 is expressed from very early stages in the development of B cells (Fig. 1.1). Were Btk activity implicated in CD38 mediated signalling, it would suggest a possible cause for the block in B cells development observed in the XLA and xid B cell lineages. Results presented in Chapter 3 however show that Btk does not appear to become activated in the mature B cell line Daudi on ligation of the cells with anti-CD38 Mabs. Also during the course of these studies Btk activation was not observed in an immature B cell line stimulated through CD38 with the same anti-CD38 Mabs used in experiments presented in Chapter 3 (Silvennoinen et al., 1996). It would thus appear that the block in B cell development observed in XLA patients is not caused by defective CD38 signalling, although it may be partially responsible for the phenotype in xid mice (see section 3.3). Syk activity, alternatively, is induced on CD38 ligation in both immature (Silvennoinen et al., 1996) and mature (Fig. 3.5, this thesis) B cell lines, and defective CD38 signalling in Syk deficient mice may be a factor in the block in B cell development in these animals.

If Btk activity in immature B cells is constitutively high as reported by Aoki et al. (1994), this may explain the block in B cell development in Btk deficient cells, and indicate that the induction of Btk activity only takes place in mature B cells. Further studies for the requirement of Btk in immature B cells may identify Btk dependent signalling pathways disrupted in XLA patients and xid mice.

7.1.2 Btk function in mature B cells

A role for Btk in CD22 mediated signalling was proposed due to functional evidence that both CD22 and Btk are crucial for BCR mediated induction of calcium mobilisation in human B cells (section 3.3). CD22 is not expressed on the surface of early B cell lineages, and although its surface...
expression has been reported to precede that of sIg (Stoddart et al., 1997), high levels of CD22 surface expression correlate with that of sIg on mature B cells (Fig. 1.1). Activation of Btk through CD22 would not therefore provide a possible explanation for the block in the development of B cells in XLA patients, but would provide further information on the integration of signalling via the BCR and its accessory molecule CD22. Neither Btk nor Syk activity was however observed to be induced by co-ligation of CD22 and the BCR. In fact the activities of both were observed on occasions to be lower than levels observed on ligation of the BCR alone. This could be due to the proposed role of CD22 as a negative regulator and may be as a result of the activity of the protein tyrosine phosphatase SHP-1 which associates with ligated CD22 (see section 1.6.2, Fig. 1.8). The roles of Btk and Syk in the mobilisation of calcium are however reported to be as positive regulators, resulting in the activation of PLCγ. As discussed, the negative effects of CD22 and BCR co-ligation on the activity of Btk and Syk was not always observed, and as such cannot be treated as a definitive conclusion to these experiments.

Evaluation of the function of Btk has led to the proposal that in mature B cells, Btk function is essential for the mobilisation of intracellular stores of calcium. The exact role it plays in B cell development however is still unknown, although there is evidence that it is required for the suppression of apoptosis (section 7.2.2.3). No Btk substrates besides Y223 (Park et al., 1995) and BAP-135 (Yang and Desiderio, 1997) (section 1.8.3.4) have been identified.

7.2 Calcium mobilisation

The mobilisation of calcium mediated by phosphoinositide signalling is fully established in gametes and is called upon to regulate major events throughout the life span of a typical cell (Berridge, 1993). IP₃ triggers calcium fluxes in maturing bovine and mouse oocytes (Homa et al., 1991; Carroll and Swann, 1992), at fertilisation of mammalian eggs (Whitaker and Patel, 1990), and in the *Xenopus* developing embryo each mitosis seems to be associated with
Chapter 7

an increase in calcium concentration (Grandin and Charbonneau, 1991), proposed to be responsible for triggering nuclear envelope breakdown at prophase (Twigg et al., 1988). IP$_3$ mediated calcium flux is also implicated in establishing dorso-ventral symmetry in amphibians (Busa and Gimlich, 1989; Maslanski et al., 1992), and left/right symmetry in mammals (Fujinaga et al., 1992). IP$_3$ mediated calcium mobilisation is also an important signalling mechanism in the central nervous system (for review see Berridge, 1993).

In lymphocytes, antigen ligation activates the IP$_3$/calcium signalling pathway (Yamada et al., 1991) which contributes to gene transcription early in the G1 phase of the cell cycle. The transcription factors NF-AT and NF-IL2A are translocated from the cytoplasm to the nucleus in response to calcium signalling, and it also results in the activation of the protein phosphatase calcineurin (Liu et al., 1991) which plays a role in the activation of T cells. Calcium also activates MAP II kinase (Chao et al., 1992), involved in a phosphorylation cascade that results in DNA synthesis. Calcium mobilisation is thus an important factor in antigen induced lymphocyte proliferation.

IP$_3$ generation is described as arising from two separate signalling pathways which activate PLC, responsible for the hydrolysis of PIP$_2$ into IP$_3$ and DAG (these pathways may not however be totally distinct, see section 7.2.2). Generally signalling through tyrosine kinase associated receptors results in the activation of PLC$_{\gamma}$, and ligation of G protein associated receptors induces the activation of PLC$_{\beta}$ (Berridge, 1993). IP$_3$ binds IP$_3$ receptors (IP$_3$R) which are found on the membrane surface of the endoplasmic reticulum, where intracellular calcium stocks are stored, and on the cell plasma membrane (Irvine, 1990, 1991). The IP$_3$R comprises a tetramer forming a channel which spans the membrane, and which when bound by IP$_3$ undergoes a large conformational change related to channel opening (Mignery and Sudhof, 1990). Calcium can also be mobilised through ryanodine receptors which share considerable functional and structural homology with IP$_3$Rs (Tsien and Tsien, 1990). These channels are opened by the actions of chemicals such as caffeine,
and also by cADPR, produced by the actions of a family of enzymes which includes CD38 (Howard et al., 1993). The predominant second messenger for the release of intracellular calcium is however $IP_3$ (Berridge and Irvine, 1989).

Btk, not being ubiquitously expressed, cannot be a crucial component of calcium signalling in general. It is however required for PLC$_\gamma$ activation in chicken DT40 B cells (Takata and Kurosaki, 1996). Should Btk become crucial when other components required for calcium signalling in undifferentiated cells are no longer expressed, Btk deficiency may abrogate the proliferation and differentiation of B cells at that point, and result in the block in B cell development observed in XLA. The partial block in B cell development observed in xid and Btk deficient murine B cells may be explained by the capacity of another protein in mouse, but not man, being capable of compensating in part for the absent Btk function in calcium signalling. Decreased calcium mobilisation and calcium signalling could also explain the defective BCR signalling observed in mature xid B cells (Rigley et al., 1989).

### 7.3 Btk ligands

#### 7.3.1 c-Cbl/ Btk interaction

The ubiquitous adaptor protein c-Cbl was reported to interact with Btk GST-SH3 (Cory et al., 1995), and with the SH3 domains of the related PTKs Itk and Tec (Chapter 3, this thesis). The interaction between c-Cbl and the SH3 domain fusion proteins appears to decrease in stimulated cells, but is not totally abrogated, discussed in section 4.3.

As discussed (section 1.4.4.1) the function of c-Cbl appears to depend on the system under study. c-Cbl appears to act as a regulator of mammalian Ras (section 1.4.3.3.), due to a mutually exclusive interaction that occurs between Grb-2 and c-Cbl or Grb-2 and Sos (Egan and Weinberg, 1993). The latter interaction results in Ras activation on cell stimulation, and it is inhibited by the binding of Grb-2 to c-Cbl. However the proposal that c-Cbl may act in a similar
fashion in the regulation of PTKs that may be activated downstream of Btk due
to a Btk: c-Cbl interaction seems unlikely in the light of the data presented here.
c-Cbl interacts with Btk GST-SH3 whether or not it is phosphorylated at Y223,
in both stimulated and unstimulated cells, and its binding does not exclude
that of other identified ligands.

The function of a Btk/Itk/Tec-c-Cbl interaction is not known. As c-Cbl
associates with the Btk family SH3 domains in resting and activated cells,
irrespective of phosphorylation of Btk SH3 at Y223 on its activation, and due to
its reported interactions with other signalling proteins (see section 1.4.4.1),
including the adaptor protein Grb-2, c-Cbl may play a role in the relocation of
Btk to the membrane upon cell stimulation, an occurrence that is correlated
with Btk activation on BCR ligation. As c-Cbl is tyrosine phosphorylated, it
may become a target for SH2 domain containing proteins which could result in
the relocation of the constitutively bound Btk PTKs on cell stimulation. Should
Btk function be required in other signalling pathways, c-Cbl may act similarly
to relocate Btk, it being involved with many proteins in various signalling
pathways (see Fig 7.1).

c-Cbl has been shown to regulate Syk as discussed in section 1.4.4.1, due
to the interaction between Syk and FceRI in mast cells, critical for Syk
activation, being competed by c-Cbl binding Syk. Should c-Cbl act in such a
way in the regulation of Btk activation is unknown. Experiments presented
here do not address this possibility, and as c-Cbl has been shown to be a
substrate for Syk and Src PTKs, but not Btk, its role in the regulation of this
PTK is less clear.

7.3.2 Syk/Btk interaction

7.3.2.1 Syk and Btk are required for PLCγ-2 activation

The reported interaction between Syk and the phosphorylated Btk SH3
domain fusion protein is consistent with reports that these two PTKs are
somehow involved in the regulation of each other in the BCR mediated pathway which leads to the activation of PLC\(\gamma\) and IP\(_3\) mediated calcium flux (Takata and Kurosaki, 1996). Btk Y551 has been reported as a substrate of Syk (Kurosaki and Kurosaki, 1997), and the interaction reported in Chapter 5 between Btk GST-SH3YP and Syk may result in increased activation of Syk due to an interaction involving the Syk C-terminal tail. Syk clustering alone has been shown to be capable of inducing the tyrosine phosphorylation of PLC\(\gamma\) (Kolanus et al., 1993), however as Btk deficient B cells show defective PLC\(\gamma\) activation, \textit{in vivo} it would appear that Btk is also required. The severity of the Syk deficient mouse phenotype compared to the Btk deficient phenotype, and the greater reduction of PLC\(\gamma\) tyrosine phosphorylation in Syk deficient B cells, compared to Btk deficient B cells (Takata et al., 1994; Takata and Kurosaki, 1996), indicates that Syk function may be more crucial than that of Btk. Furthermore, as Btk Y223 has been shown not to be critical for BCR mediated stimulation (Kurosaki and Kurosaki, 1997), should activated, phosphorylated Btk interact \textit{in vivo} with Syk and play a role in Syk activation, it would not appear to be critical in this system but may be part of a regulatory feedback loop, important for the activation of PLC\(\gamma\) in a manner not detected in the DT40 Btk Y223F cell line.

Interesting aspects of the Btk GST-SH3YP: Syk interaction are that (i) should Btk interact with Syk \textit{in vivo}, this interaction may result in an increase in the tyrosine phosphorylation of Syk, and (ii) that Syk in unstimulated cells may form a conformation that does not bind Btk. Should Btk GST-SH3YP interact with Syk from unstimulated cells that has been denatured on SDS-PAGE analysis, it would indicate that the absence of interaction between Btk GST-SH3YP and Syk in resting cells is most likely due to Syk being found in a conformation in these cells that masks the binding site, likely to be the C-terminal tail, from Btk GST-SH3YP. Btk GST-SH3YP could be used in solution to probe a nitrocellulose membrane onto which resting cell lysate has been transferred to detect whether such as an association between denatured Syk
from resting cells may interact with Btk GST-SH3YP. As the relaxation of a conformation involving the C-terminal tail of Syk has been correlated with an increase in Syk activation (Kimura et al., 1996), this could provide further evidence for a role for Btk in the regulation of Syk activation.

7.2.2.2 Btk and Syk are involved in G protein associated receptor mediated activation of mitogen activated protein kinases

A recent report has provided strong evidence for in vivo physical and further functional associations between Btk and Syk (Wan et al., 1997). Examination of PTK activity in cells stimulated through the G protein coupled m2 mACh receptor showed that overexpression of Btk in DT40 B cells led to increased Syk activity, that Btk is required for Syk activity, and that both PTKs are required for the activation of mitogen activated protein (MAP) kinases in this G protein mediated signalling pathway. An interaction between whole His6 tagged Btk purified from E.coli with agarose beads and endogenous Syk was detected in DT40 B cells. This report provides evidence for an in vivo association between Syk and Btk, complementing studies presented in Chapter 5 of this thesis. It also indicates further functional evidence for the requirement for Btk in G protein mediated signalling and for regulatory interactions between Btk and Syk in B cells. It is also the first report providing important evidence for the function of PTKs, including Syk and Btk (the latter known to be activated by G protein subunits (section 1.3.1.2)), linking G protein associated receptors with the MAP kinase signalling cascade (Seger and Krebs, 1995). A previous report indicated that MAP kinase activity in BCR stimulated Btk deficient DT40 cells was similar to that found in BCR ligated wild type DT40s (Takata and Kurosaki, 1996), indicating that Btk function in G protein mediated signalling is independent of the BCR. Thus Btk function in DT40s appears to be essential for signal transduction along a second pathway, involving interplay between Btk and Syk which somehow regulate each others' activities, a pathway which is also regulated at early stages (sections 1.4.2.2, 1.4.3.3) by another ligand of the Btk SH3 domain, c-Cbl.
7.2.2.3 Btk and Syk in apoptosis

Btk and Syk have both also been implicated in B cell apoptosis, although evidence is not always consistent and does not implicate the two PTKs acting on the same pathway. Btk deficient DT40 B cells do not undergo radiation induced apoptosis seen in wild type DT40s (Uckun et al., 1996). Whereas Syk deficient DT40s do undergo radiation induced apoptosis (Uckun et al., 1996). sIgM ligated Syk (or PLCγ) deficient DT40s show decreased levels of apoptosis (Takata et al., 1995). These studies indicate that Syk and PLCγ play a role in the apoptotic response in IgM ligated DT40 cells, as does Btk in radiation induced apoptosis in DT40 cells.

Studies in xid mice however have implicated a role for Btk in the suppression of apoptosis mediated by the products of the related proto-oncogenes Bcl-X (Anderson et al., 1996), and Bcl-2 (Woodland et al., 1996). Bcl-X is expressed in early stages of B cell development, and although levels decrease on expression of IgD, ligation of sIgM on mature B cells induces Bcl-X expression at this later stage in development (Choi et al., 1996). Mature xid B cells however show repressed Bcl-X expression on B cell activation on ligation of the BCR, and this is correlated with an increase in apoptotic death (Anderson et al., 1996). Another apoptosis suppressing protein Bcl-2 was also found at low levels in xid B cells. This low level of expression was observed neither in normal B cells, nor in xid T cells. Btk appears therefore to be required for normal levels of Bcl-2 expression in B cells, and perhaps Bcl-2 phosphorylation, as levels of phosphorylated Bcl-2 are especially reduced in xid B cells. Defective xid B cell development was rescued by expression of a Bcl-2 transgene. The mature B cells of xid/Bcl-2 mice however fail to proliferate in response to antigen ligation as do normal murine B cells (Woodland et al., 1996). This study implies that Btk may be required in two distinct pathways that when disrupted result in the xid phenotype. In the absence of Btk, low levels of Bcl-2 result in death by apoptosis during antigen independent stages of B cells development. Btk function is required however in a separate pathway mediated by antigen
ligation in mature B cells, as in the absence of Btk, Bcl-2 expression does not corrects defects in this pathway.

### 7.2.3 WASP/Btk Interaction

WASP has been implicated as playing an important role in haematopoietic cells in the regulation of the actin cytoskeleton (Symons et al., 1996) (discussed in section 1.5.3). This is consistent with the morphology of cell lineages in WAS patients, including T and B cells and platelets. The distortions observed in these patient cell lineages could result from defects in the cytoskeletal architecture (Molina et al., 1992; 1993).

#### 7.3.3.1 WASP may act to transduce messages from tyrosine kinase associated receptors with the cytoskeleton

The WH domains within WASP share homology with other proteins implicated in actin organisation, including vasodilator-stimulated phosphoprotein (VASP) (Haffner et al., 1995), and verprolin (Donnelly et al., 1993). Both these proteins are proline rich and contain regions through which they may be regulated by Rho family GTPases. Together with WASP, this group of proteins may mediate functional associations between actin organisation, Rho GTPases, and in the case of WASP, and possibly VASP and verprolin, due to their proline rich regions, SH3 domain containing PTKs.

WASP is implicated in cell signalling pathways due to its association with the PTK Fyn (Banin et al., 1996) and the Btk GST-SH3 domain fusion protein (Cory et al., 1996). As polarisation of T cells is proposed to be an important aspect of the recognition of mitogenic stimuli, and involves actin bundling (Parsey and Lewis, 1993), as does BCR ligation (Melamed et al., 1992), WASP function may provide a bridge between actin organisation and the activation of PTKs. Interestingly WASP no longer associates with Btk GST-SH3 following phosphorylation at the site of Btk autophosphorylation on Btk activation. Btk is seen to relocate to the membrane fraction of cells on activation, and its release from the cytoskeletal protein WASP may be
important for this to take place. In this model, the release of Btk from cytoplasmic WASP occurs after the activation of Btk, and is thus not implicated in the initial regulation of Btk activity. However, should the interaction between Btk and WASP be crucial for the transduction of signals from the ligation of antigen at the BCR to the cytoskeleton which must re-organise itself, the association of WASP with the unphosphorylated Btk SH3 domain may signal BCR ligation to downstream cytoskeletal effectors.

WASP may bind many signalling proteins simultaneously via its numerous PXXP motifs. As it has been reported to associate with the Btk SH3 domain, and in vivo with Fyn, tyrosine phosphorylation of Btk by Src PTKs may be made possible due to a protein such as WASP (or indeed c-Cbl) which binds both PTKs, bringing them into proximity. Both Btk and Src PTKs may be released from WASP after phosphorylation events within their SH3 domains (Broome and Hunter, 1997, Chapter 5).

A proposal for the actions of Btk in B cell activation is depicted in Fig.7.1.
Fig 7.1 (A) **Model for Btk function** In resting cells Btk associates with WASP and c-Cbl via its SH3 domain. WASP may receive signals and transmit them to the cytoskeleton via PTKs such as Btk. As it may associate with Src PTKs e.g. Fgr, Fyn (Lyn?) it may play a role in Src mediated activation of Btk. As the Btk SH3 domain no longer binds WASP on its phosphorylation (B), the association may be disrupted allowing Btk to relocate to the membrane but remain long enough on Btk activation for signals induced by on BCR ligation to be transduced to the cytoskeleton. The PH domain of Btk may be involved in the membrane association of Btk, interacting with membrane associated phospho-inositol phospholipids. It may also be involved in the negative regulation of Btk activity due to its association with PKCβ1. c-Cbl may be an intermediary between Btk and other proteins with which c-Cbl associates on BCR ligation. This may function in Btk membrane association or it may function to associate Btk with other signalling pathways in which its function has been implicated. Btk and Syk associate on Btk autophosphorylation. In this interaction Syk phosphorylation may be either upregulated due to the interaction, or a highly phosphorylated sub-population of Syk is bound. In either case both PTKs are proposed to act together in the activation of PLCγ.
7.4 Protein expression in WAS patients

Mutations that occur within specific regions of a particular protein may be speculated to cause a disease phenotype due to resulting alterations in the protein which disrupt its function, for example a point mutation at a critical proline residue may destroy the potential of a proline rich region to form a PPII helix, and thus disrupt the potential of the protein to interact with SH3 domains. Detection of mutations within regions of proteins in patients have thus been discussed as providing evidence for the importance of that particular region for the correct functioning of the protein.

As in vitro evidence indicated that two proteins, Btk and WASP, mutations in which result in two primary immunodeficiencies, interact, it was interesting to consider the possibility that mutations may be found in the regions assumed to be responsible for the interaction. Mutations in the WASP proline rich regions or the Btk SH3 domain may indicate that this association could be disrupted in patients with these disorders, and could possibly indicate a common underlying defect with consequences for the disease phenotypes.

Mutational studies of XLA patients however have not disclosed evidence for any SH3 domain point mutations that may disrupt ligands binding, and in 11 severe WAS patients investigated, none had point mutations in proline rich regions that would be assumed to disrupt the ability of WASP to bind an SH3 domain containing protein.

Predicting how a mutation may disrupt the function of a protein on the basis of where it lies, is undermined by the discovery that, as in XLA patients investigated (Hashimoto et al., 1996, Gaspar et al., 1998), the WAS patients investigated in this study expressed no detectable WASP whatsoever. Using a system to enrich for the protein, and capable of detecting WASP when expressed at quantities as low as 1%, no WASP was found in cell lysates from all patients tested. The lack of expression of WASP in all patients in the study.
could be an explanation of why patients with large deletions (patient 2) and patients with missense point mutations (patients 3, 4, 5, 6, 7), all suffer from severe WAS. Indeed no correlation was observed between the position or nature of the mutation, but was observed between the severity of the disease phenotype and a lack of expression of WASP at levels 1% of normal or above.

Other reports have reported WASP in patients with missense mutations expressed at reduced levels. The patients investigated with these low levels of WASP expression all had mild or moderate, but not severe phenotypes, consistent with the correlation observed in the data presented in Chapter 6 of this thesis. Subsequent studies on patient material in this laboratory (Yi-Chien Wang) have identified a patient which expresses WASP detected by SK3 on immunoblotting. This patient has a mild WAS phenotype strengthening the theory that a correlation may be drawn between lack of WASP expression and a severe WAS phenotype, and also providing a positive patient control.

WAS patient samples were easily obtained, and as only a small quantity of peripheral blood is required in order to detect WASP by immunoblotting mononuclear cell lysates from these samples, they were deemed a suitable and highly relevant cell population to test for protein expression. No other study has reported results using the primary cells of WAS patients, with the exception of two patients in (Zhu et al., 1997), all results were obtained from EBV transformed B cell lines. The use of a small blood sample for investigations into WASP expression is also very convenient should this method be put to use as a screening procedure for diagnosis prior to DNA sequencing.

7.4 Future prospects

In the studies presented in this thesis, no association has been reported between the whole Btk protein and WASP, c-Cbl and Syk. Indeed whole Btk has been reported to associate with only PKCβI in mice (Yao et al., 1994), and
BAP-135 in the human Ramos B cell line (Yang and Desiderio, 1997) in co-immunoprecipitation experiments from cell lysates.

_In vitro_ results obtained using the GST fusion protein system could be complimented by studies designed to show an association between whole Btk and c-Cbl or WASP, such as has been shown with Syk (Wan et al., 1997). Over-expression of tagged whole molecule constructs in a fibroblast cell line, followed by immunoprecipitation with an antibody raised against the tag may show an interaction between the two molecules that are overexpressed. An interaction between the Btk/WASP or Btk/c-Cbl products of such constructs in a haematopoietic cell line would provide further, more physiologically relevant evidence for an _in vivo_ association. Transfection of haematopoietic cells has been found to be more difficult than fibroblasts however. Should two proteins not co-precipitate in B cell lines such as Daudi due to undetectable amounts of protein actually being involved in the interaction (on/off binding rates may be very high, or only a small sub-population of proteins may be involved in the interactions of interest), or an interaction being disrupted due to the precipitating antibody interfering with a region of the protein critical for the protein:protein interaction, overexpression and the use of tags may overcome these problems of detection. Alternatively, antibodies that can be used for successful cytostaining experiments may show co-localisation of whole proteins, which may or may not be overexpressed, providing evidence for an _in vivo_ interaction.

Recent studies have focused on the regulation of Btk. However, relatively little is still known about downstream effectors of Btk. No cellular Btk substrates have been identified, besides its own site of autophosphorylation, and the protein BAP-135 of which little is known, and as discussed, few Btk interactions involving the whole protein have been reported. Due to the apparent difficulty in determining Btk interacting ligands or substrates, different approaches need to be applied to try and elucidate the function of Btk.
In the majority of XLA patients investigated, Btk is not expressed to any extent (Hashimoto et al., 1996; Gaspar et al., 1998). The importance of particular regions of Btk cannot therefore be interpreted from the position of mutations characterised in the BTK genes of these patients.

Mutant Btk can be expressed however in cell lines such as DT40s (Takata and Kurosaki, 1996), and the xid defect has been corrected by the introduction of the human Btk gene (Drabek et al., 1997, Maas et al., 1997), providing methods which could be used to introduce human Btk mutants into mice. Work performed where correction of the xid defect by expression of a YAC Btk transgene (including the Btk locus control region) concluded that in these transgenic mice, Btk is appropriately expressed in the context of native regulatory sequences, and this system may be used to study the effects Btk mutant constructs introduced into a Btk deficient background may have.

The effect of the E41K Btk PH domain mutant has been investigated by the expression of this mutant (under the control of an MHC class II gene locus control region) in Btk deficient mice. This activated Btk mutant results a phenotype that is more severe than the xid phenotype. Circulating B cells were depleted, and an absence of T cell dependent B cell responses and disorganisation of B cell areas in the spleen were observed (R. Hendriks, personal communication).

Investigations into the biochemistry and the immunological phenotype resulting from the introduction of such mutant constructs into Btk deficient mice will provide valuable information concerning the function of Btk in B cell development and function. The effects of mutations within other domains of Btk, or deletion of the domain entirely, on measurable downstream signalling events such as protein tyrosine phosphorylation and calcium flux may be determined using such a system, providing an indication of the purpose individual domains may have in BCR mediated signalling. As there is evidence that the Btk SH3 domain may be involved in the negative regulation of Btk.
(Afar et al., 1996, section 1.4.3.3), observing the development of B cells expressing Btk where the entire SH3 domain has been deleted or where a Y223F mutation has been introduced, may provide further evidence for the regulatory role of the SH3 domain.

Human bone marrow engraftment into immunodeficient mice such as SCID/NOD (Lapidot et al., 1992; Larochelle et al., 1995, 1996) may allow for the study of the function of human Btk in a human haematopoietic system, important as differences between the haematopoietic systems of mouse and man are presumed to be responsible for the difference between the mild xid and the severe XLA phenotypes. Wild type and Btk mutant constructs such as an SH3 domain construct could be transfected into human XLA bone marrow, which in turn could be introduced into the SCID/NOD mouse, and the development of the B cell population in both monitored and compared.

Future investigations into Btk will hopefully provide further details of the actions of and requirements for this protein in mature B cell signalling pathway(s), and the role it plays during B cell development in the suppression of apoptosis.
Appendix A: The protein sequence of Btk. Domain borders are indicated, and domain specific motifs are bold and underlined.
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