Molecular Studies on Bruton's Tyrosine Kinase

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Abstract

X-Linked agammaglobulinaemia (XLA) is a humoral immunodeficiency resulting from a block in the B cell maturation pathway at the pre-B cell stage. Affected males have normal numbers of pre-B cells in the bone marrow but virtually no mature B cells in the peripheral circulation and are unable to produce immunoglobulin of all isotypes. The gene defective in XLA has been identified as a non-receptor tyrosine kinase and named Btk (Bruton's tyrosine kinase). Btk is a modular protein related to but distinct from the Src family of tyrosine kinases. However, the precise pathways in which Btk is involved and its exact role in lymphocyte maturation remain unclear.

This thesis describes a programme of work ranging from the identification of the genetic defects in Btk through to studies attempting to define the role of Btk in intracellular signalling by the use of gene transfer technology. Mutation analysis of Btk cDNA from individuals with XLA using single stranded conformational polymorphism (SSCP) analysis and direct sequencing resulted in the identification of 5 mutations. In three unrelated individuals the same mutation was identified suggesting the presence of a mutational hotspot in Btk. Subsequent studies investigated the expression and activity of Btk protein in primary cells from XLA patients in order to correlate the clinical phenotype with the genetic defect. However, it was shown that regardless of the mutation or clinical phenotype, there was complete lack of Btk expression and activity. These assays were also used to confirm the diagnosis of XLA in certain individuals in whom XLA was suspected but not confirmed by genetic analysis.

Gene transfer technology was used to create *in vitro* models with which study the function of the Btk protein. Retroviral vectors encoding a copy of wild type and kinase mutant Btk were generated and used to express Btk in a fibroblast line to study the binding of Btk to candidate signalling proteins. Retroviral transfer of Btk into an EBV transformed B cell line from an XLA patient with a null phenotype was also performed in order to study reconstitution of B cell function.

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Abbreviations

ADA	Adenosine deaminase
ADA-SCID	ADA deficient severe combined immunodeficiency
AR-CGD	Autosomal recessive chronic granulomatous disease
ATP	Adenosine triphosphate
AAV	Adeno associated virus
BAP	Btk associating protein
β-ARK	β-adrenergic receptor kinase
BCR	B cell receptor
BMT	Bone marrow transplantation
bp	Base pair
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
Ca^{++}	Calcium
CAT	chloramphenicol acetyltransferase
CFU-S	Colony forming unit-spleen
CGD	Chronic granulomatous disease
CVID	Common variable immunodeficiency
dAdo	Deoxyadenosine
dATP	Deoxyadenosinetriphosphate
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DTH	Delayed-type hypersensitivity
DTT	Dithioetheitrol
EBV-LCLs	Ebstein-Barr virus immortalised B lymphoblastoid cell lines
EDTA	Ethylene diamine tetra acetic acid (disodium salt)
FCS	Foetal calf serum
FACS	Fluorescence activated cell sorting
FITC	Fluorescence isothiocyanate
GAP	GTPase activating protein
GDP	Guanine diphosphate
GHD	Growth hormone deficiency
GST	Glutathione sepharose transferase
GTP	Guanine triphosphate
HRP	Horse radish peroxidase
HUCB	Human umbilical cord blood
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
IP	Inositol phosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IRES	Internal ribosomal entry site

IRS-1	Insulin receptor substrate 1
ITAM	Immunoreceptor tyrosine based activation motif
JAK	Janus associated kinase
kb	Kilobase
kD	Kilodalton
LB	Luria-Bertani bacterial growth medium
LTBMC	Long term bone marrow culture
LTCIC	Long term culture initiating cell
LTR	Long terminal repeat
MAP kinase	Mitogen activated protein kinase
MBL	Mannan binding lectin
MHC	Major histocompatibility complex
MLV	Moloney murine leukaemia virus
MPSV	Myeloproliferative sarcoma virus
MSCV	Murine stem cell virus
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NIH	National Institutes of Health
NK	Natural killer cell
NMR	Nuclear magnetic resonance
nrs	Normal rabbit serum
OLB	Oligolabelling buffer
ORF	Open reading frame
PBL	Peripheral blood lymphocytes
PBS	Primer binding site
PBSC	Peripheral blood stem cells
PCR	Polymerase chain reaction
PEG-ADA	Polyethylene glycol conjugated adenosine deaminase
PH	Pleckstrin homology
PKC	Protein kinase C
PLC	Phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMC	Peripheral blood mononuclear cells
PRR	Proline rich region
PTK	Phosphotyrosine kinase
pTyr	Phosphorylated tyrosine
RAG	Recombinase activating gene
RhEGF	Recombinant human growth factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAHH	S-adenosyl homocysteine hydrolase
SCID	Severe combined immunodeficiency
SCIDX1	X-linked SCID
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	Src homology
sIgM	Surface IgM
snRNA	Small nuclear RNA
SRC	SCID mouse repopulating cell
SSCP	Single stranded conformational polymorphism
STAT	Signal transducers and activators of transcription

TB	Terrific broth
TCR	T cell receptor
TdT	terminal deoxynucleotidyltransferase
TH	Tec homology
tRNA	Transfer RNA
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
X-CGD	X-linked chronic granulomatous disease
XLA	X-linked agammaglobulinaemia
XLT	X-linked thrombocytopaenia
ΨLC	Surrogate light chain
ZAP-70	ζ chain associated protein-70

Publications

The work presented in this thesis has contributed towards the following publications:

H.C.Genevier, S.Hinshelwood, **H.B.Gaspar**. K.P.Wrigley, D.Brown, S.Saeland, F.Rousset, R.J.Levinsky, R.E.Callard, C.Kinnon, R. Lovering (1994) Expression of Bruton's tyrosine kinase protein within the B cell lineage. *Eur. J. Immunol.* **24**, 3100-3105.

H.B.Gaspar, L.A.D.Bradley, F.Katz, R.C.Lovering, C.M.Roifman, G.Morgan, R.J.Levinsky, C.Kinnon (1995) Mutation analysis in Bruton's Tyrosine Kinase, The X-linked agammaglobulinemia gene, including identification of an insertional hotspot. *Hum. Mol. Genet.* **4**, 755-757.

H.B.Gaspar, L.A.D.Bradley, F.Katz, C.M.Roifman, G.Morgan, R.J.Levinsky, C.Kinnon (1995) XLA resulting from Adenosine base replication slippage. *Progress in Immunodeficiency* **V**, 131-132, (Caragol, I., Espanol, T., Fontan, G., and Matamoros, N., eds.) Springer-Verlag Iberica, Barcelona, Spain.

R.C.Lovering, S.Hinshelwood, L.A.D. Bradley, **H.B.Gaspar**, G.O.C.Cory, R.J.Levinsky, C. Kinnon (1995) Elucidation of the defects in the X-linked agammaglobulinaemia gene, Bruton's tyrosine kinase (BTK). *Progress in Immunodeficiency* **V**, 27-31, (Caragol, I., Espanol, T., Fontan, G., and Matamoros, N., eds.) Springer-Verlag Iberica, Barcelona, Spain.

H.B.Gaspar, T.Lester, R.J.Levinsky, C.Kinnon (1998) Bruton's tyrosine kinase expression and activity in X linked agammaglobulinemia: the use of protein analysis as a diagnostic indicator of XLA. *Clin. Exp. Immunol.* **111**, 334-338.

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1

Introduction

In 1952 Colonel Ogden C. Bruton reported the case of an 8 year old boy who had suffered from recurrent infections including multiple episodes of otitis media and pneumonia due to several serotypes of pneumococcus. In an attempt to treat him prophylactically he was vaccinated against a number of pneumococcal types but no evidence of a specific antibody response was found. The question then arose as to whether he was capable of producing any antibody at all and indeed on further investigation, there was no demonstrable gamma globulin fraction seen on protein electrophoresis (Fig. 1.1) (Bruton, 1952). The boy was treated with monthly intramuscular injections of human gammaglobulin with significant clinical improvement. Thus was written the first account of a primary immunodeficiency and its treatment. Although there was no family history in this initial case, subsequent studies revealed a similar clinical phenotype with an X-linked pedigree. This condition of the absence of immunoglobulin production with an X-linked inheritance was termed Bruton's disease or X-linked agammaglobulinaemia (XLA).

1.1 X-Linked agammaglobulinaemia

1.1.1 Clinical phenotype of XLA

The phenotype of X-linked agammaglobulinaemia is characterised in its classical form by the absence of immunoglobulin of all isotypes and the absence of B cells in the peripheral circulation. As a result, the majority of affected boys are prone to recurrent bacterial infection from the age of approximately 4-12 months following the disappearance of maternal immunoglobulin (Lederman and Winkelstein, 1985).

Fig 1.1 Agammaglobulinaemia

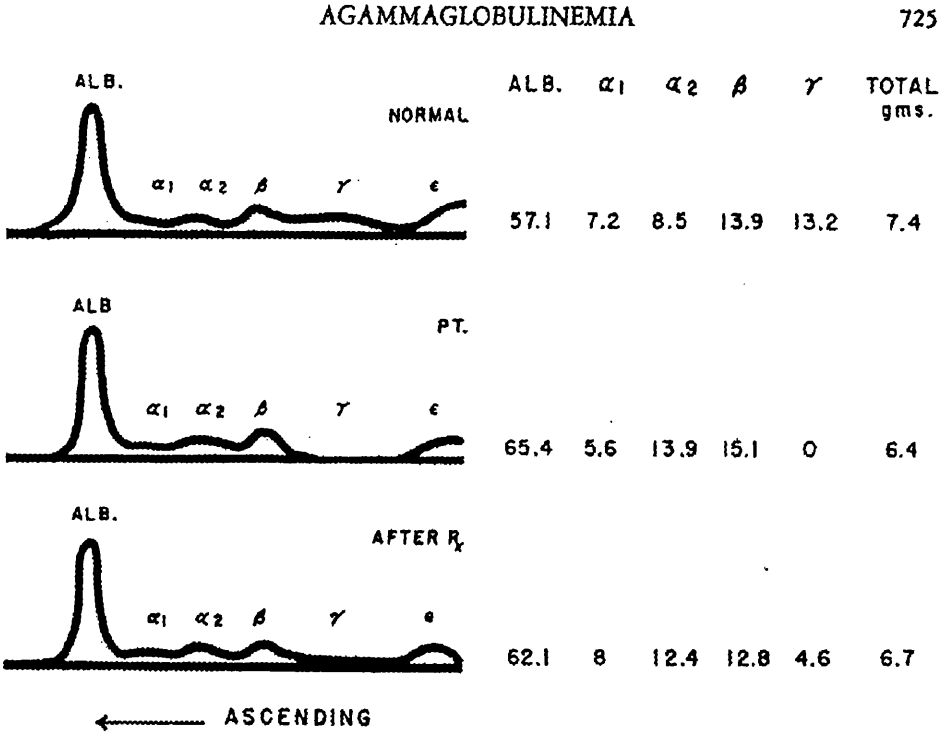


CHART 2. To show normal electrophoretic pattern compared with that of patient before and after giving gamma globulin.

The absence of the γ globulin fraction in protein in Bruton's original description of agammaglobulinaemia (Bruton 1952).

In a significant number of cases (21% in a survey of 44 patients) the initial presentation may occur later at 3-5 years of age (Hermaszewski and Webster, 1993). The infections are usually caused by pyogenic bacteria with *Staph. Aureus*, *Strep. Pneumoniae*, *H. Influenzae* and *Pseudomonas* being the most common species (Lederman and Winkelstein, 1985), although many other types have also been reported. The site of infection varies considerably with sinusitis, otitis media and lower respiratory tract infections being the most common site of infection (Hermaszewski and Webster, 1993). Resistance to viral infection remains predominantly intact except for a susceptibility to enteroviral infection which has resulted in chronic meningoencephalitis or poliomyelitis in a significant number of cases. Indeed the major cause of mortality in the series reported by Hermaszewski and Webster (1993) was as a result of enteroviral meningoencephalitis.

The disease has always been considered to be confined to the B lymphocyte compartment but it has also been documented that in acute infective episodes XLA patients suffer from neutropaenia suggesting there may also be an abnormality in the myeloid response to infection (Farrar et al., 1996).

Effective management for XLA is now available in the form of prophylactic immunoglobulin replacement and the use of aggressive antibiotic therapy for active infection. This is not without complications, since a significant number of individuals with primary antibody deficiency have developed hepatitis C infection as a result of viral transmission from the immunoglobulin replacement product (Quinti et al., 1995). Overall, however, with compliance and regular follow up the outlook and prognosis for XLA is now good with early diagnosed individuals surviving into adulthood.

1.1.2 Gene defect in XLA

Linkage analysis of families affected by XLA had mapped the genetic locus of the condition to the long arm of the X chromosome at Xq22. Subsequent studies had closely linked the gene to a number of polymorphic markers including DXS178 (O'Reilly et al., 1992; Lovering et al., 1993). Finally in 1993, two groups demonstrated that XLA was caused by defects in a gene designated Btk (Tsukada et al., 1993; Vetrie et al., 1993) (see section 1.3).

1.1.3 Atypical XLA

It is well recognised that an atypical or 'leaky' form of XLA exists. In this phenotype there is presence of B cells in the peripheral circulation, albeit in significantly reduced numbers, and there may be evidence of immunoglobulin production. Reports also exist of variation in the clinical phenotype within the same pedigree suggesting that factors other than the genetic defect may influence the phenotype of XLA (Kornfeld et al., 1997). There is some overlap of the clinical phenotype with other conditions such as common variable immunodeficiency (CVID) which tends to present later with varying degrees of humoral and in some cases cellular immunity (Spickett et al., 1997). However, with the advent of mutation analysis in Btk it has been possible to show that some of these individuals previously labelled as CVID or atypical XLA have mutations in Btk indicating that there is considerable heterogeneity in the XLA phenotype (Jones et al., 1996; Hashimoto et al., 1996).

In addition to the 'leaky' phenotype described above, an additional variant has been reported. A pedigree was initially described in which hypogammaglobulinaemia with growth hormone deficiency was inherited in an X-linked recessive manner (Fleisher et al., 1980). Following this original report, three further families were also identified (Sitz et al., 1990; Monafo et al., 1991; Osborne et al., 1990). In all cases there was laboratory evidence of lack of growth hormone production. Linkage analysis of affected families showed that the defect mapped to the same region of the X chromosome as XLA (Osborne et al., 1990) suggesting that this condition might be an allelic variant or a small contiguous gene deletion syndrome encompassing both the gene for XLA and an adjoining gene responsible for growth hormone production. Subsequent mutation analysis of Btk in an individual with this condition has shown this syndrome can result from a mutation in Btk and is therefore an allelic variant of XLA (Duriez et al. 1994). The original family described by Fleisher et al. (1980) has however been investigated and no abnormality in the cDNA sequence nor mRNA and Btk expression was found, thus leaving open the possibility of an alternative gene on the X chromosome responsible for some cases of XLA/GHD (Stewart et al., 1995).

1.1.4 Diagnosis of XLA

Prior to the identification of the Btk gene, the diagnosis of XLA was made on the basis of an X-linked pedigree, reduced peripheral B lymphocyte numbers and lack of immunoglobulin production. The diagnosis can be more difficult to make in the first few months of life if there is no family history because of the presence of maternal IgG. However, the diagnosis may be suggested by the markedly reduced numbers of peripheral B lymphocytes.

Carrier status can be carried out using a number of methods. Obviously in cases where the mutation has been defined in an affected individual or pedigree, females in the family can be investigated for the mutation in genomic DNA either by direct sequencing or by restriction digest analysis, if possible. In other cases where a mutation cannot be found, X inactivation analysis in maternal B lymphocytes can be performed (Fearon et al., 1987 ;Puck et al., 1987). Normal lyonisation in females results in the random inactivation of one or other X chromosome in all somatic cell types. In B lymphocytes of carrier XLA females, only B cells in which the X chromosome with wild type Btk is active will develop normally since normal Btk expression is required for B lymphocyte development. In the same way, B lymphocytes in which the X chromosome carrying mutant Btk gene is active will fail to mature. Thus, in carrier females of XLA non-random X inactivation in the B cell lineage is displayed. Random X inactivation is seen in T cells and monocytes since presumably Btk expression is not necessary for normal development of these haematopoietic lineages.

1.2 B lymphocyte development in XLA

The molecular defect in XLA results in an abnormality in the development of B lymphocytes. To fully understand the precise defect in XLA a comprehensive understanding of normal B lymphocyte development is necessary. This is outlined below.

1.2.1 Normal B cell development and differentiation

The differentiation of B lymphocytes from haematopoietic stem cells is a complex and tightly regulated process which occurs in response to numerous signals from the bone marrow microenvironment, soluble cytokines, interaction with antigen and T

lymphocytes. Lymphocyte development, in both T- and B- lineages, begins with the commitment of multipotent progenitors to the lymphoid lineage. It is likely that these early stages are in part dependent upon extracellular and stromal signals, though the exact mechanisms of early lymphoid commitment are not well defined. The transcription factor Ikaros appears to play an important role in these initial stages, since mice with targeted deletions of the Ikaros gene lack T, B and natural killer cell development (Georgopoulos et al., 1994).

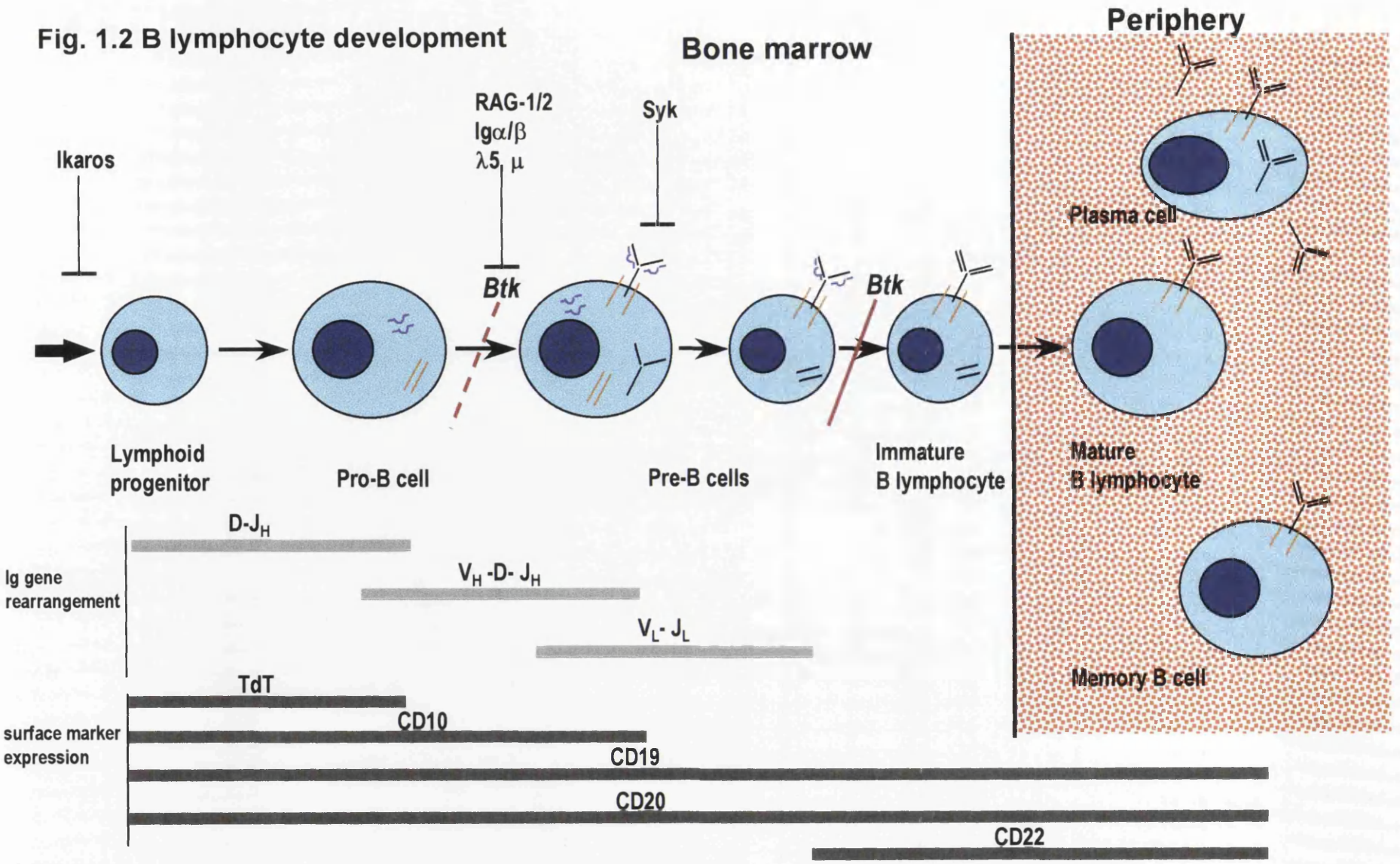
In mammals, B lymphocyte development occurs primarily in the foetal liver and adult bone marrow. Bone marrow stem cells predominantly develop into conventional B cells whereas foetal liver stem cells can also develop into a unique subset termed B-1 lineage cells. This population is located mainly in the peritoneum and cells express the surface antigen CD5. Importantly, it is this population that is primarily affected in the *xid* mouse (see section 1.2.3 below). The stages of B cell differentiation are described below with respect to surface marker expression, recombination of immunoglobulin (Ig) variable region genes, dependence on antigenic stimuli and the expression of individual genes essential for normal B lymphocyte maturation (reviewed in Melchers et al., 1995; Owen and Venkitaraman, 1996; Satterthwaite and Witte, 1996; Burrows and Cooper, 1997). Figure 1.2 summarises this process.

The earliest stages of normal B cell development are antigen independent and occur in the bone marrow in response to stromal cell contact and cytokines. Cells express the surface marker CD34 which is characteristic of early progenitor cells of all lineages. One of the earliest B cell specific markers to be expressed is CD19, a signal transduction molecule, which remains expressed throughout the B lymphoid lineage up to the plasma cell stage (Saeland et al., 1992). Other early markers are CD10, a neutral endopeptidase (LeBien et al., 1990), and terminal deoxynucleotidyltransferase (TdT) (Campana et al., 1985).

Fig 1.2 B lymphocyte development. *The stages of B lymphocyte development in the bone marrow and periphery are outlined. Expression of the surface markers Tdt, CD10, CD19, CD20 and CD22 are shown. Also highlighted is the temporal order of immunoglobulin gene rearrangenment. D-J_H heavy chain rearrangement is followed by V_H-D-J_H heavy chain rearrangement. Successful assembly of the heavy chain then allows light chain (V_L-J_L) rearrangement to proceed. The cytoplasmic and surface expression of the various components of the BCR and pre-BCR are also illustrated.*

The developmental block most commonly seen in individuals with XLA is shown by a bold line at the pre-B to immature B lymphocyte transition. An earlier developmental arrest has also been noted and is illustrated by a broken line. The knowledge from knockout mouse studies and from the study of other individuals with congenital autosomal recessive agammaglobulinaemia has allowed the understanding of the role of other genes in B cell development. The maturation arrest arising from abnormalities in these other genes is also demonstrated.

Fig. 1.2 B lymphocyte development



A critical feature of B cell development is the formation of a functional pre-B cell receptor (pre-BCR) and subsequently a mature B cell receptor (BCR) after which antigen dependent maturation can continue. These receptors are in essence surface immunoglobulin molecules (sIg) consisting of an Ig heavy (IgH) and Ig light (IgL) chain, coupled to accessory signal transduction proteins (Igα and Igβ). Successful rearrangement of IgH and IgL chains results in cell surface expression. The variable regions of the immunoglobulin are assembled from gene segments termed variable (V), diversity (D) and joining (J) (for the heavy chain locus) or V and J (for the light chain locus). However, in the earliest stages of B cell development immunoglobulin genes are retained in a germline configuration.

At the pro-B cell stage, transcription and cytoplasmic expression of Igα, Igβ and the surrogate light chain proteins (ΨLC) V-preB and λ5 (in mice) or λ-like (in humans) are detectable. The rearrangement of genes coding for the variable region of the immunoglobulin heavy chain is also initiated at this stage with DJ rearrangements being the first to occur. The protein products of the recombinase activating genes RAG-1 and RAG-2 (Schatz et al., 1989; Oettinger et al., 1990) are crucial for the rearrangement process. Abnormalities in these genes result in a failure of B and T lymphocyte development and as a consequence give rise to a severe combined immunodeficiency (SCID) phenotype in both mice and humans (Schwarz et al., 1996b; Mombaerts et al., 1992).

Completion of VDJ_H rearrangement results in pre-B cell expression of intracellular μ heavy chain. At this stage μ heavy chain (μ_H) can associate with the ΨLC and the Igα and Igβ accessory molecules to form a pre-BCR which can then be expressed on the cell surface. Following this, further rearrangement of μ_H is prevented and Ig light chain rearrangement is initiated. These events may be regulated by signalling through the pre-BCR (Melchers et al., 1994). Once light chain rearrangement has occurred, a mature IgM molecule can be assembled and expressed on the surface (sIgM) together with the accessory molecules to form the BCR.

These immature B cells now leave the bone marrow and migrate to the periphery where antigen dependent BCR signalling results in self reactive B cells being

eliminated or anergised. Further B cell development occurs in an antigen dependent manner. When peripheral mature B cells expressing both sIgM and sIgD encounter antigen in the context of T cell help, they are activated to proliferate and differentiate into antibody secreting plasma cells or memory B cells (MacLennan and Chan, 1993; Forster and Rajewsky, 1990). This involves class switching and somatic hypermutation of Ig genes to create B cell populations secreting antibody of high affinity.

1.2.1.1 Role of pre-BCR and BCR in B cell development

Targeted mutations of genes encoding for the various components of the pre-BCR and the BCR provide strong evidence for the critical roles of these proteins in the B lymphocyte differentiation process. Mutant mice with targeted deletion of the transmembrane portion of the μ_H chain locus show a complete block of development at the pre-B cell stage (Kitamura et al., 1991). Further studies demonstrated that μ_H expression on the surface of mouse pre-B cells is required to turn off V_H to DJ_H rearrangement at the second allele and to allow proliferative expansion and further development of pre-B cells (Kitamura and Rajewsky, 1992b). Further studies in which the λ5 gene of the λ5/Vpre-B surrogate light chain (ΨLC) locus was mutated resulted in mice with a partial abnormality in B cell development (Kitamura et al., 1992a). In these mutants, there was a dramatic reduction in the numbers of late pre-B cells and delayed appearance of $sIgM^+$ immature B cells. However, Ig light chain and heavy chain rearrangement was still possible thus allowing further B cell maturation.

The Igα/Igβ are also essential components of both pre-B and B cell antigen receptors. Their critical role in development is illustrated by a series of gene knockout studies. In the absence of both of these signal transducing units, Ig molecules fail to reach the cell surface resulting in a developmental block at the pre-B cell stage (Gong and Nussenzweig, 1996). It has also been shown that the cytoplasmic tails of Igα and Igβ are interchangeable in allowing allelic exclusion and further B cell development (Papavasiliou et al., 1995a). The cytoplasmic tail of Igβ is sufficient to allow pre-B cell signalling but this is dependent on the presence of intact tyrosine residues in its ITAM (immunoreceptor tyrosine based activation motif) region (Papavasiliou et al., 1995b). Indeed, if the entire cytoplasmic tail of Igα is

deleted, then B cell development in the bone marrow is only reduced 2-4 fold but there is a dramatic decrease in the number of peripheral B cells (Torres et al., 1996). The implication from this study is that the Igβ cytoplasmic tail can compensate for Igα mediated signalling in B cell development but there is a further developmental checkpoint which prevents B cells with defective B cell antigen receptors from leaving the bone marrow.

1.2.1.2 Signalling molecules essential for B cell development

The requirement of the Ikaros transcription factor and the RAG-1 and RAG-2 recombinase genes in B lymphoid differentiation has already been discussed. Following the assembly of functional pre-B and B cell antigen receptors, signal can now be transduced from the cell surface to the nucleus. Essential to this are a series of signalling molecules which if affected result in abnormal B cell development.

The presence of the ITAM motifs on the cytoplasmic tails of Igα and Igβ and the absolute requirement of this motif for normal development suggests that signalling via this pathway is essential for development. The non receptor tyrosine kinase Syk is a molecule that consists of two SH2 domains and one SH1 (catalytic) domain (see section 1.5.4.). It is structurally analogous to the T cell specific kinase ZAP-70 which if defective results in abnormal T cell development (Valerio et al., 1983; Chan et al., 1994). Syk is primarily expressed in B cells and biochemical and genetic studies show that it plays a critical role in B cell development. sIgM stimulation results in the phosphorylation of the tyrosine residues on the ITAM motif (DeFranco et al., 1995). Syk is able to bind to these phosphorylated residues via its double SH2 domains and in doing so is itself activated (Kimura et al., 1996). Targeted deletions of Syk in mice result in perinatal lethality due to excessive haemorrhage during embryogenesis (Cheng et al., 1995; Turner et al., 1995). Therefore, B lymphocyte development in Syk knockout mice has been studied using RAG-$1^{-/-}$ radiation chimaeras. The transplantation of $Syk^{-/-}$ foetal liver stem cells into sublethally irradiated RAG-$1^{-/-}$ mice allows analysis of lymphopoiesis of $Syk^{-/-}$ cells. These studies demonstrated that although T cell development is normal, B cell development is arrested at the pro-B cell to pre-B cell transitional stage (Cheng et al., 1995; Turner et al., 1995) with the complete absence of sIgM positive cells. In addition it has also

been shown that Syk is required for the development of B-1 cells (Satterthwaite and Witte, 1996).

The role of the proto oncogene Vav in B cell development has also been investigated using the RAG-$2^{-/-}$ blastocyst complementation system. Embryonic stem cells from wild type and $Vav^{-/-}$ mice were used to generate RAG-$2^{-/-}$ (Tarakhovsky et al., 1995; Zhang et al., 1995). The numbers of peripheral B cells were reduced in $Vav^{-/-}$ chimaeras but this may be related to the degree of chimaerism. More significantly however, the development of the B-1 lineage was completely inhibited. B cell proliferation in response to sIgM stimulation was also shown to require the presence of Vav.

The advances in gene knockout technology have now resulted in an enormous number of murine models in which lymphopoiesis can be studied. In a recent review over 50 knockout mice with abnormalities of B cell development were described (Kokron et al., 1997). More pertinently, a number of the Src like tyrosine kinases have been studied. Of these only Syk, Lyn and Btk show significant abnormalities in B cell lymphopoiesis. Indeed in $Lyn^{-/-}$ mice, these abnormalities are less dramatic with decreased numbers of peripheral B cells but no abnormality of B cell development in the bone marrow (Hibbs et al., 1995). More importantly, $Lyn^{-/-}$ mice have circulating autoantibodies and suffer from autoimmune disease as a result of IgG complex deposition in the kidneys. These experiments suggest that Lyn may have an important role in tolerance induction.

The role of Btk in B cell differentiation has been studied extensively, both in murine models and in patients with XLA, although in both cases the natural models existed long before the discovery of the gene responsible.

1.2.2 Block of B cell development in XLA

The stage of maturation arrest in B cells has been identified by a number of studies as being from the pre-B lymphocyte stage to immature B lymphocytes. Examination of bone marrow from affected individuals suggests that there are normal numbers of pre-B cells in the bone marrow but there is a lack of mature B lymphocytes in the bone marrow, lymphoid tissues and peripheral circulation (Pearl et al., 1978). In

addition there is a lack of mature plasma cells from sites where they are normally present i.e. bone marrow, lymph nodes and the lamina propria. Alternative studies have also demonstrated that there may be another earlier block to B cell development. In a study of bone marrow from XLA patients, it was found that there was a predominance of cytoplasmic μ-, terminal deoxynucleotidyl transferase (TdT)+, pro-B lymphocytes with fewer cytoplasmic μ+ pre-B lymphocytes than expected suggesting a block in the transition from pro-B to pre-B cells (Campana et al., 1985).

Studies have also been performed on the small number of B cells that can be detected in the peripheral circulation. Surface IgM positive B cells detected in XLA patients were shown to have a very different phenotype to peripheral B cells in a control population (Conley, 1985). The XLA B lymphocytes showed a significantly more intense staining for IgM with normal IgD staining (IgD^{lo}/IgM^{hi}). This phenotype is similar to that described for immature B cells in the *xid* mouse (see below). The production of immunoglobulin in XLA patients has also been examined. A number of studies report detectable levels of immunoglobulin production of one or other subtype in the serum of affected patients studied (Lederman and Winkelstein, 1985; Levitt et al., 1984). The above studies were performed prior to the identification of Btk and so the exact diagnosis may be in doubt, but reports subsequent to 1993 in patients with known Btk mutations show that immunoglobulin can indeed be detected (Jones et al., 1996). The results of the studies described above and other reports suggest that the block in B lymphocyte development in XLA can occur at multiple stages. Furthermore this block is incomplete with small numbers of B cells, capable of immunoglobulin production, being detected in the periphery.

1.2.3 The *xid* mouse is a murine analogue of XLA

The *xid* mouse has a humoral immunodeficiency which is significantly different from the human immunophenotype but a number of features of the *xid* strain suggest that the two conditions may be analogous (Scher, 1982). In contrast to XLA, the B lymphocyte population in *xid* is relatively normal in number but detailed analysis of this population suggests that it is highly abnormal both phenotypically and functionally. The B-1 population of murine B cells (equivalent to the CD5 population

in humans), commonly found in the peritoneum, is lacking in *xid* mice. Furthermore surface staining of peripheral and splenic B cells displays an immature phenotype with increased numbers of cells displaying the IgD^{lo}/IgM^{hi} phenotype and decreased numbers displaying the mature IgD^{hi}/IgM^{lo} phenotype. Functionally these cells are considerably abnormal with impaired responses to T-independent type 2 antigens and a failure of IgM production. Abnormal responses to various B cell stimuli such as CD40 (Hasbold and Klaus, 1994) and CD38 (Santos Argumedo et al., 1995) have also been demonstrated.

Despite the differences in immune function, XLA and *xid* share a number of common features. The surface phenotypes of B cells in both are similar with a high intensity of IgM staining suggesting an immature population. Both arise from an intrinsic B lineage defect since carrier females of both XLA and *xid* show non-random X inactivation in B cells. Finally linkage analysis showed that the gene defect in *xid* mapped to a region of the murine X chromosome that shares homology with the human XLA locus at Xq21.3-Xq22 (Malcolm et al., 1987; Kwan et al., 1990). Following the identification of the genetic defect in XLA, Btk was also shown to be the abnormal gene in *xid*. The murine defect was identified as being a missense mutation R28C in the PH domain of Btk thus confirming that *xid* is indeed a murine analogue of XLA (Thomas et al., 1993).

1.3 Bruton's tyrosine kinase

The genomic organisation of the human Btk gene spans approximately 37.5 kb and consists of 19 exons, 18 of which code for the protein. The murine gene has a similar organisation and is 43.5kb in length, the difference being due to the size of various introns (Sideras et al., 1994). The coding sequence of the gene is highly conserved with 99.3% homology between the murine and human genes. The cDNA comprises 2560 nucleotides and has an open reading frame encoding a 659 amino acid polypeptide beginning with the initiation codon at position 133.

1.3.1 Expression of Btk

Btk expression has been found in cells of the B lymphocyte, monocyte and myeloid and mast cell lineage but no expression is seen in T lymphocytes (Genevier et al., 1994; Smith et al., 1994). The expression in B cells is seen from an early CD34

expressing cell line MIK-ALL through to mature B cells (Genevier et al., 1994). However, no expression of either protein or mRNA was detectable in two terminally differentiated plasma cell lines. A number of T cell lines and primary tonsillar T cells have been analysed but no expression was seen. These studies indicate that Btk plays a critical role in B cell development from early B lymphocyte through to mature cells. They also imply that Btk is functionally redundant in T cells and also in plasma cells suggesting that continued expression of Btk is not necessary for immunoglobulin production in plasma cells. The expression in myeloid cells is surprising since no overt defect of myeloid function is found in XLA. The recent report by Farrar et al. (1997) (see section 1.1.1), however might signify a role for Btk in this lineage.

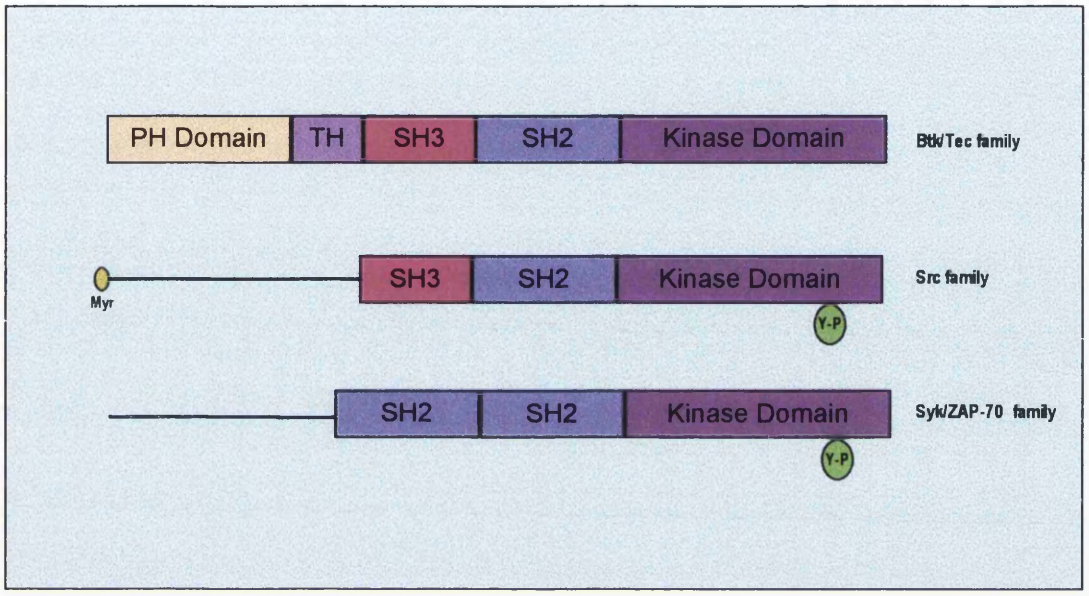
1.3.2 The Btk protein

The Btk protein belongs to a family of non receptor tyrosine kinases. It is a modular protein consisting of 5 identifiable domains (Fig. 1.3). It has structural homology with the family of Src tyrosine kinases but shows important differences. Unlike the Src kinases, Btk has a characteristic N-terminal region consisting of a pleckstrin homology (PH) domain followed by the unique proline rich Tec homology (TH) domain. Furthermore, Btk lacks a N-terminal myristoylation signal which is necessary for constitutive membrane association. This suggests that Btk is found mainly in the cytoplasm although translocation of Btk from the cytosol to the membrane may play an important role in its activation. Finally, the catalytic Src homology 1 (SH1) domain of Btk lacks the residue equivalent to tyrosine 527 of c-Src. This tyrosine is conserved in all Src subfamily members and has been implicated as a mechanism for negatively regulating the kinase activity of the Src proteins (Superti et al., 1993; Courtneidge et al., 1993) (see section 1.4).

These important differences have resulted in the designation of a new subfamily of cytoplasmic non receptor tyrosine kinases, known as the Btk or Tec subfamily. To date this family consists of Btk, Tec (*t*yrosine kinase preferentially *e*xpressed in liver and hepatocellular *c*arcinoma cell lines) (Mano et al., 1995), Itk (*I*nterleukin-2 inducible *T*-cell specific *k*inase) (previously known as Tsk or Emt) (Heyeck and Berg, 1993; Yamada et al., 1993), Bmx (Tamagnone et al., 1994), Txk (Haire et al., 1994) and a Drosophila protein Src28C. These proteins share a high degree of

homology and contain a PH domain followed by TH, SH3, SH2 and SH1 domains, respectively, with the exception of Txk which lacks a PH domain. In general, there is a high degree of tissue specificity and with the exception of Tec and Drosophila Src28C there is restricted expression in haematopoietic cells.

Fig 1.3 Modular structure of the Src family



The Btk/Tec family structure is shown in comparison to the structure of two other classes of tyrosine kinases. In the N-terminal region of Btk there are the unique features of the PH and TH domains. The post-translational myristoylation signal is also lacking. At the C terminal end of the protein, the kinase (SH1) domain is homologous to that of the other Src family kinases but the Btk family lack the negative regulatory tyrosine.

1.3.3 Modular domains of Btk

Intracellular signalling proteins are often constructed from combinations of modular domains. These conserved sequences can each fold into a compact and functional module independently of surrounding sequences. The domains may function in an independent manner and each imparts a particular function to the protein. SH2, SH3 and PH domains are involved in intermolecular protein-protein interactions while the catalytic activity of the protein resides in the SH1 domain. In addition, it is now recognised that intramolecular association between respective domains can also exist to regulate protein activity. The binding domains are constructed with a common core recognition ability coupled to more variable sequences which are responsible for the high affinity and specificity binding. The general properties and the specific interactions of the various domains of Btk are discussed.

1.3.3.1 PH Domain

PH domains are a family of protein modules defined by sequences of approximately 100 amino acids. The initial sequence was originally found in pleckstrin, a major protein kinase C (PKC) substrate in activated platelets (Tyers et al., 1989). The solution structure of the PH domains of β-spectrin and the crystal structure of the dynamin PH domain have now been resolved (Zhang et al., 1995; Timm et al., 1994). Despite the low amino acid identity among the PH domains of the three molecules, the 3D structures remain highly conserved. The core of the PH domain consists of seven anti-parallel β sheets and forms a putative ligand binding pocket. The C-terminal portion of the domain contains a highly conserved tryptophan residue and is folded into an α-helix.

The function of PH domains is not entirely clear but studies of PH domains from other proteins show an association with different phosphoinositides. A study of IP_3 (inositol-1,4,5-trisphosphate) binding proteins revealed an association with phospholipase C-δ1 (PLC-δ1) (Kanematsu et al., 1992). More detailed analysis of the binding site showed that the binding was dependent upon the N-terminal region of the PH domain of PLC-δ1 (Yagisawa et al., 1994). In a study of a number of PH domains including pleckstrin and PLC, it was demonstrated that the N-terminal region of PH domains bound to PIP_2 (phosphatidylinositol-4,5-bisphosphate) (Harlan et al., 1994). While the N-terminal region of PH domains appears to bind to phosphoinositides, the C terminal region appears to have different membrane binding properties. Studies on β-ARK (β-adrenergic receptor kinase) and a number of other PH domains from other proteins including Btk, showed that there is binding of the C-terminal region (and a few amino acids beyond) to the βγ subunits (Gβγ) of heterotrimeric G proteins (Touhara et al., 1994). Gβγ is membrane localised by isoprenylation of Gγ and as such, proteins binding to Gβγ must also be localised to the membrane for interaction to occur. These lines of evidence suggest therefore that PH domains may act as signal dependent membrane localisation units. In the absence of post translational myristoylation seen in some Src family kinases such as Btk, PH domains may compensate for this function.

1.3.3.2 TH Domain

The TH domain is unique to the Btk/Tec family of non receptor tyrosine kinases. The region consists of a region of approximately 80 residues and includes a conserved stretch of 27 amino acids (termed the Btk motif) and a proline rich region (PRR). The function of the whole domain remains unclear but binding studies have provided certain clues. Part of the Btk motif and the C terminal region of the PH domain bind to the Gβγ subunits of heterotrimeric G proteins as discussed above. The proline rich region has been shown to bind to the SH3 domains of the Src family kinases Fyn, Hck and Lyn (Wolos et al., 1993 ;Yang et al., 1995). This evidence has come from studies using the GST fusion proteins containing the SH3 domains of the Src kinases, but no association between full length Btk and these proteins has been demonstrated. A point mutation in the TH domain abolishes Src kinase SH3 mediated binding and impairs tyrosine autophosphorylation of Btk (Yang et al., 1995) suggesting that this proline rich region is crucial not only for Btk interaction with other Src protein kinases such as Lyn and Fyn but also with itself. In a more recent study, an intramolecular association was shown to exist between the proline rich region of the structurally homologous Itk TH domain and its own SH3 domain (Andreotti et al., 1997). This association may act as a negative regulatory switch in the activation of the Btk family of Src kinases (section 1.4).

1.3.3.3 SH3 Domain

SH3 domains are small (55-70 amino acid) protein modules that are found in many intracellular signalling proteins. The structure of Btk SH3 has been modelled and is similar to those found in other signalling molecules (Yu et al., 1992; Musacchio et al., 1994; Zhu et al., 1994a). The common architecture of SH3 domains consists of five anti-parallel β strands that pack to form two perpendicular β sheets. A hydrophobic patch containing a cluster of conserved aromatic residues and surrounded by two charged, variable loops forms the ligand binding pocket (Yu et al., 1992). SH3 domains bind to proline rich peptides of approximately 10 amino acids. Structural and mutagenic analysis demonstrates that SH3 binding peptides adopt a left handed polyproline type II helix. The core binding motif consists of P-X-X-P where X is any amino acid, although the first X is often another proline (Yu et al., 1994). The specificity of SH3 binding is conferred by interactions between the non-

proline residues in the ligand and the two variable SH3 domain loops that flank the main hydrophobic binding surface.

Ligands associating with the SH3 domain of Btk have been studied by a number of groups and *in vitro* data using GST fusion proteins containing the Btk SH3 domain have demonstrated an association with a variety of different proteins. The protein product of the proto oncogene c-Cbl as well as the Wiskott-Aldrich syndrome protein (WASP) have both been shown to bind the Btk-SH3 domain in studies conducted in haematopoietic cells *in vitro* (Cory et al., 1995; Cory et al., 1996). However, no *in vivo* binding of Btk to either of these proteins has been shown and thus the physiological significance of these interactions remains unclear.

SH3 domains of the Src kinases are involved not only in binding to other proteins but also in intramolecular associations that regulate catalytic protein activity (section 1.4).

1.3.3.4 SH2 Domain

SH2 domains form an integral part of the Src related tyrosine kinases. The structure of the SH2 domain is conserved and consists of a large antiparallel β sheet with two flanking α helices (Waksman et al., 1993; Cohen et al., 1995). Early work suggested that *in vivo*, SH2 domain containing proteins bound to phosphorylated tyrosine (pTyr) containing sites on activated receptors and cytoplasmic phosphoproteins (Anderson et al., 1990; Matsuda et al., 1990). Subsequently, it was shown that *in vitro*, isolated SH2 domains were capable of stably associating with tyrosine phosphorylated, but not unphosphorylated, receptor tyrosine kinases (Mayer et al., 1991). Although all SH2 domains bind to phosphotyrosyl containing proteins, ligand specificity is determined by the three residues immediately carboxyl to the phosphorylated tyrosine (Songyang et al., 1994). The phosphopeptide binding site in SH2 is bipartite. A conserved pocket lined by basic residues binds the pTyr. The pocket contains an invariant arginine at its base which binds the pTyr through formation of hydrogen bonds with the two pTyr phosphate oxygens. A second, more variable, binding surface allows specific recognition of the ligand through association with the immediately carboxyl amino acids (Songyang et al., 1993).

Binding of the SH2 domain to pTyr allows a protein to transduce signal in a number of different ways. Binding may alter the subcellular localisation of the protein bringing it closer to its substrate or closer to a protein that modifies it. Secondly binding may cause a conformational change in the protein thereby altering its catalytic activity.

Although the general function of the domain is known and binding ligands for SH2 domains of many of the Src kinases have been determined, no specific ligand has yet been identified for the Btk SH2 domain. However, mutation analysis of Btk in patients has shown missense mutations in the SH2 binding site leading to a severe and atypical XLA phenotypes thereby emphasising the crucial role of this domain in Btk function (Bradley et al., 1994; Saffran et al., 1994)

1.3.3.5 SH1 kinase Domain

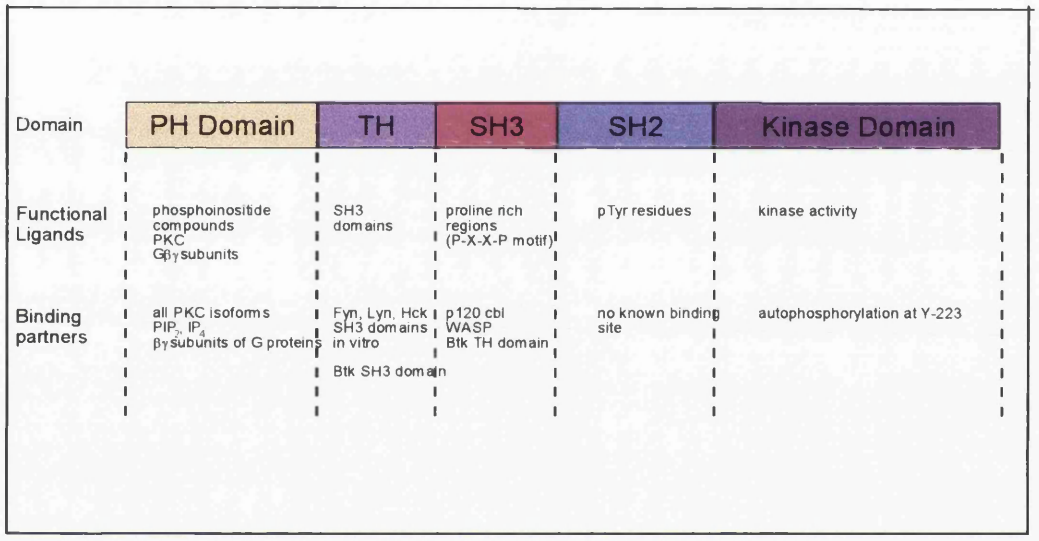
The kinase domain of Btk is the only domain with catalytic activity. It consists of approximately 250 residues and despite low overall sequence homology with other tyrosine kinases, contains several conserved sites thereby indicating functional similarities (Vihinen et al., 1994b; Mattsson et al., 1996). In summary, the domain is split into two lobes, a smaller N-terminal lobe containing a five stranded anti-parallel β sheet and one or two α helices and the C-terminal lobe which has seven helices and short β strands. The most conserved residues form the ATP binding site which lies between the two lobes. In addition there is a substrate binding site and putative catalytic residues.

As with the SH2 domain, although the function of this domain has been determined, no specific interaction with other molecules has yet been demonstrated. However, one target of Btk kinase activity is Btk itself since the activation pathway involves autophosphorylation at Y-223 in the SH3 domain of Btk (Rawlings et al., 1996) (see section 1.4.3). Once again the importance of the domain has been highlighted by the number of missense mutations in Btk which result in a severe XLA phenotype. Indeed the most commonly occurring XLA mutation, an R520Q substitution at a

CpG nucleotide is found in the activation loop of the kinase domain (Vihinen et al., 1995).

The negative regulatory tyrosine present in other Src tyrosine kinases (Y-527 in Src) is not found in Btk and the mechanism of Btk regulation may be controlled in a more complex manner. A summary of the interactions and functions of the various domains are given below.

Fig 1.4 Interactions of the Btk domains



The modular domains of Btk *together with putative ligands and actual binding partners are shown.*

1.4 Regulation of the Src family kinases

Src, in the inactive state exists in a conformation whereby the SH1 Y-527 residue, having been phosphorylated by the cytoplasmic tyrosine kinase Csk, thus binds to its own SH2 (Matsuda et al., 1990). Recent crystallographic studies suggest that in the inactive forms of the Src and the related kinase Hck, the SH3 domain together with the SH2 domain serves to inhibit catalytic activity by intramolecular association with the SH1 domain (Sicheri et al., 1997; Xu et al., 1997) thus preventing association with exogenous ligands. SH2 affinity for the phosphorylated tyrosine of another molecule has been shown to compete the SH2 domain of the Src-like tyrosine kinase Hck away from its phosphorylated Y-527 and stimulate kinase activity (Moarefi et al., 1997).

Btk activation follows a different mechanism since Btk lacks the equivalent C-terminal negative regulatory tyrosine. The regulation of catalytic activity in the Btk protein is more complex as demonstrated by the structurally homologous T cell kinase Itk which shows an intramolecular association of the SH3 domain with the proline rich segment of the TH domain thus restricting the access of the SH3 domain to potential substrates (Andreotti et al., 1997). Phosphorylation of a tyrosine residue within the SH3 domain may disrupt SH3-TH interaction thereby liberating SH3 and TH domains to form associations with alternative ligands

1.5 Function of Btk

Since the identification of Btk as the defective protein in XLA, its function in lymphocyte signalling and development has been extensively studied. However, despite the considerable attempts of a number of groups its precise role in B lymphocyte development remains elusive. What has emerged however, is relatively convincing evidence for the activation of Btk in B cells and its interaction with other signalling molecules and downstream effectors. The activity of Btk in both human and murine cells has been shown to be increased by the stimulation of a number of different receptors (see 1.5.6 below). Most studied of all is the response to BCR stimulation.

1.5.1 BCR stimulation

Experiments in mature human and murine B cell lines show that when cells are stimulated by cross linking of the BCR, there is increased activity of Btk as demonstrated by autophosphorylation activity or by activity on the exogenous substrate enolase (Aoki et al., 1994; Saouaf et al., 1994; Hinshelwood et al., 1995). Activation of the BCR also results in the tyrosine phosphorylation and increased enzymatic activity of two other classes of tyrosine kinases, namely the Src-family kinases and the Syk/ZAP-70 family (Saouaf et al., 1994). A model of B cell activation and signalling following BCR stimulation involving these three families of tyrosine kinases has now been postulated.

1.5.2 Src-PTK activation

The kinetics of BCR engagement suggest that there is a temporal sequence to the activation of the different types of tyrosine kinases. Studies performed in sIgM stimulated WEHI 231 cells showed that the Src family phosphotyrosine kinases (Src-PTKs) Lyn, Fyn and Blk were the first to be activated followed by the increased activity of Btk and Syk (Saouaf et al., 1994). The mechanism of initial Src-PTK activation is not entirely clear. It has been suggested that BCR engagement results in phosphorylation of the ITAM sequences of Igα and Igβ which then bind the Src-PTK SH2 domain thereby competing off the negative regulatory tyrosine and so resulting in Src-PTK activation. However studies, in which a Src-PTK SH2 domain was mutated so as to be incapable of binding phosphotyrosine, showed that Src-PTK activity was still present in early signalling, suggesting that activation of Src-PTKs does not require association with Igα/β ITAM (Takata and Kurosaki, 1995b). It may be therefore that the activation of Src-PTK occurs initially via an unknown SH2 independent mechanism. Subsequent Src-PTK mediated phosphorylation of ITAM residues results in recruitment and activation of further Src-PTKs in an SH2 dependent manner thereby driving the activation cascade forward (Clark et al., 1994).

1.5.3 Btk activation

The activation of Btk has been shown to be dependent upon the presence of active Src-PTKs. Studies in COS cells, EBV immortalised B lymphocytes (EBV-LCLs) deficient in Btk and fibroblast lines have all shown that Btk is phosphorylated and activated by co-expression of Src-PTKs (Mahajan et al., 1995; Rawlings et al., 1996). The use of specific mutants demonstrated that intact kinase activity of both the Src-PTK and Btk is essential for maximal Btk activation. Further studies using phosphoamino acid analysis were able to define the site of Src-PTK dependent Btk transphosphorylation as being tyrosine 551 (Y551) which is located in the active loop of the Btk catalytic domain (Rawlings et al., 1996). The sequence surrounding this site has a level of homology with the consensus Src family transphosphorylation site (Hanks et al., 1988). Transphosphorylated Btk is then able to undergo autophosphorylation at a second site which has been mapped by phosphopeptide analysis to Y223 in the Btk SH3 domain (Park et al., 1996). This proposed

mechanism is supported by evidence which identified Y551 and Y223 as being phosphorylated following BCR stimulation of B cells (Rawlings et al., 1996). It is postulated that the Y223 phosphorylation event then serves to regulate Btk activity by SH3 domain mediated binding to other molecules (Afar et al., 1996).

1.5.4 Syk activation

The other important component of the early BCR activation pathway is the Syk molecule. Again the activation of Syk has been the subject of much interest. The initially proposed model suggested a sequential mechanism of activation whereby, following Src-PTK activation, Syk is recruited to the doubly phosphorylated Igα/β ITAMs and is then activated by the Src-PTKs (Weiss and Littman, 1994). However, further studies have cast doubt upon this mechanism. Importantly it has been shown by a number of different studies that the activation of Syk is not entirely dependent upon the Src-PTKs. In a DT40 chicken B cell line that is deficient in Lyn, Syk shows a low level of activation and is able to initiate downstream events (Takata and Kurosaki, 1996). Furthermore it has been shown that Syk can associate with the BCR even prior to stimulation albeit at a low level (Hutchcroft et al., 1992). A more accurate mechanism of activation may be that although Syk activation and Igα/β recruitment is not entirely dependent upon Src-PTKS, its activity is greatly enhanced by their effects. Phosphorylation of Igα/β ITAMs and also direct phosphorylation of Syk result in enhanced activation of Syk. In addition Syk binding to the Igα/β ITAMs stimulates Syk autophosphorylation leading to further increased Syk enzymatic activity (Rowley et al., 1995).

The evidence for the role of Syk in Btk phosphorylation and activation is inconsistent. In earlier studies on COS cells and murine fibroblasts. it was shown that co-expression of Syk did not result Btk in tyrosine phosphorylation (Mahajan et al., 1995; Rawlings et al., 1996). However, more recent studies performed in DT40 chicken B cells which were Lyn, Syk or Lyn/Syk deficient it was shown that Syk or Lyn alone were capable of tyrosine phosphorylating Btk to some extent but that the presence of both were required for maximal Btk phosphorylation following BCR stimulation (Kurosaki and Kurosaki, 1997).

The pathway of early BCR activation can be summarised as follows. BCR crosslinking results in the activation of the Src PTKs by an as yet unknown mechanism. Src PTK mediated phosphorylation of the tyrosine residues of the ITAMs of Igα and Igβ results in enhanced activation of Syk. In addition Src-PTKs and Syk together are responsible for directly or indirectly transphosphorylating Btk which then leads to autophosphorylation of its SH3 domain. This sequence of events is illustrated in Fig. 1.5

1.5.5 Downstream effects of Btk activation

BCR activation leads to the activation of a complex but distinct set of signalling pathways. The tyrosine kinases described above have been shown to be essential in mediating a number of these events. This includes the activation of phospholipase C, Ras and the MAP kinase pathway and PI-3 kinase dependent pathways. In addition, other non-receptor intracellular signalling molecules have been demonstrated to interact closely with the tyrosine kinases and to be necessary for downstream signalling e.g cbl. To date, evidence supports the involvement of Btk in some but not all of these pathways.

1.5.5.1 Signalling via PLC-γ

Engagement of the BCR induces hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG, in turn activates the protein kinase C family of molecules (Bijsterbosch et al., 1985) while IP_3 induces the mobilisation of calcium from intracellular calcium stores by binding to IP_3 receptors (IP_3Rs) on the endoplasmic reticulum. This transient increase in calcium is then followed by sustained calcium influx as a result of opening membrane calcium channels. PIP_2 hydrolysis is catalysed by PLC-γ which exists in two isoforms, PLC-γ2 being the most common in B cells. Tyrosine phosphorylation of PLC-γ isoforms results in their activation. In the case of PLC-γ1, this can occur following binding to Syk via the PLC-γ1 SH2 domain (Law et al., 1996) to the phosphorylated tyrosine residues Y341 and Y345 of Syk (Williams et al., 1997). However, the requirements for PLC-γ2 activation in B cells are different. Using the DT40 cell line Takata and Kurosaki (1996) demonstrated the need for both Syk and Btk in the activation of PLC-γ2 (further discussed in section 6.3.2).

Fig 1.5 Proximal events following BCR signalling. *Ligation of the BCR causes clustering of the Igα and β chains and the associated Src kinases (e.g. Lyn). This leads to phosphorylation of ITAM tyrosine residues (indicated by Y-P). Src kinases then bind to phosphorylated ITAMs via their SH2 domains and in doing so undergo an increase in their kinase activity and lead to phosphorylation of more ITAM residues. Increased binding of Syk to doubly phosphorylated ITAMs via its tandem SH2 domains is then seen and results in increased Syk kinase activity. Following this, phosphorylation of Btk at Y551 in the kinase domain occurs due to the action of Lyn and possibly other kinases. Increase in Btk kinase activity leads to autphosphorylation at Y223 in the Btk SH3 domain. The downstream effects of Btk activation are discussed elsewhere.*

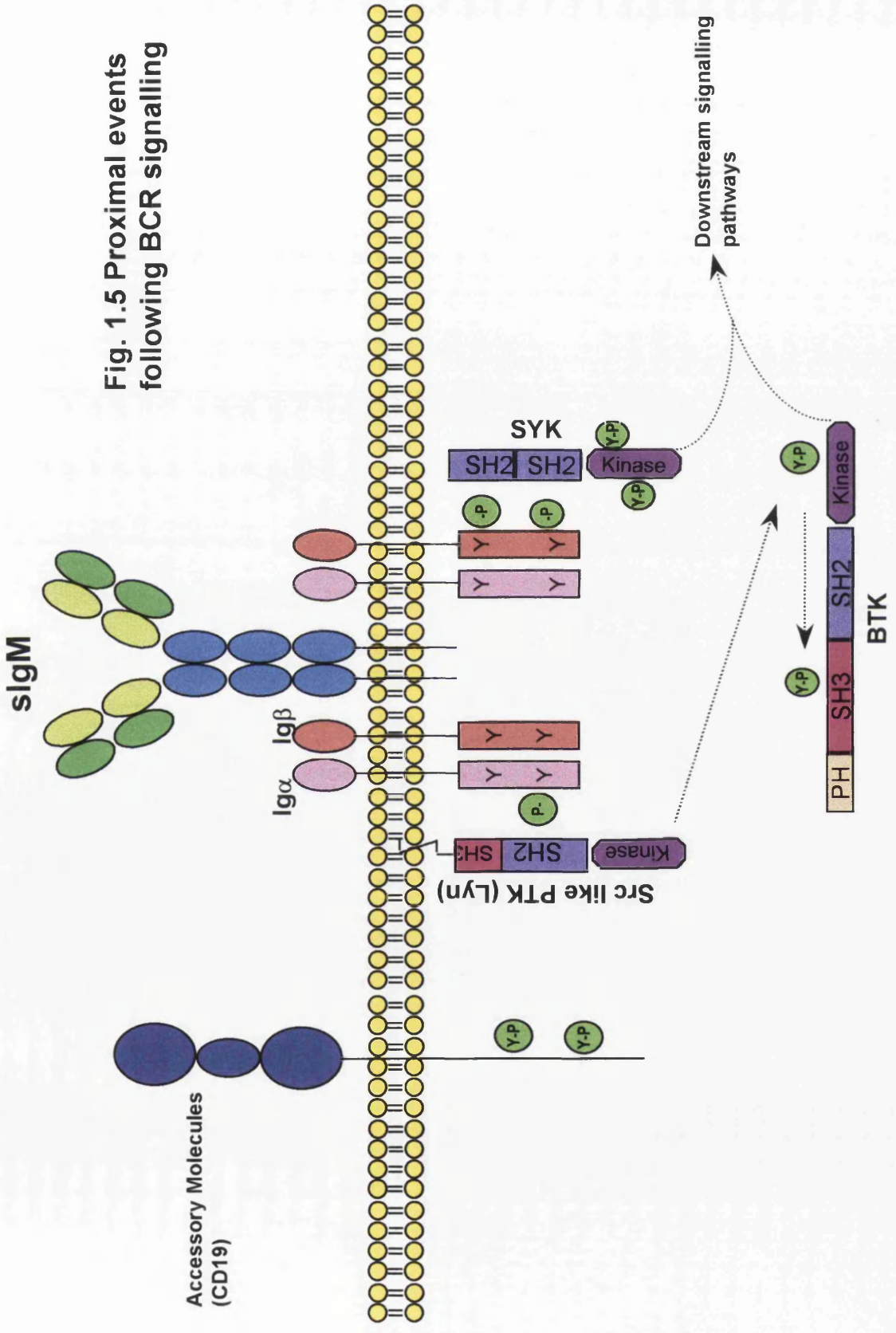


Fig. 1.5 Proximal events following BCR signalling

1.5.5.2 Signalling via Ras

Phosphotyrosine kinase activation is followed by the activation of Ras and the classical MAP kinase pathway (Lazarus et al. 1993). The MAP kinase phosphorylation cascade ultimately leads to transcription factor induction and the expression of specific genes. The importance for Ras in BCR signalling is demonstrated by studies in which transcription factor induction can be blocked by the expression of a dominant negative Ras mutant (McMahon and Monroe 1995).

The coupling of BCR stimulation to the Ras activation pathway may be mediated by PTKs by two routes. BCR engagement results in the tyrosine phosphorylation of Shc, which together with other adapter molecules Grb and SOS results in the transition of Ras-GDP to the active Ras-GTP (reviewed in DeFranco, 1997). In Syk deficient DT40 cells, tyrosine phosphorylation of Shc is abolished (Nagai et al., 1995). An alternative mechanism for PTK involvement in Ras activation may be via the Ras-GTPase activating protein (Ras-GAP). BCR stimulation results in Ras-GAP tyrosine phosphorylation and decreases its activity which in effect maintains Ras in the Ras-GTP state thus increasing Ras activity. As yet no specific PTK has been implicated in this mechanism. The involvement of Btk in Ras activation has not as yet been identified. In a Btk deficient DT40 cell line, MAP kinase activation following BCR stimulation was unaffected suggesting that Btk is not involved in the coupling of BCR stimulation to the Ras activation pathway (Takata and Kurosaki, 1996).

1.5.5.3 Signalling via PI-3 kinase

Another pathway activated following BCR stimulation and mediated by PTKs is that involving phosphatidyl inositol 3-kinase (PI-3 kinase). PI-3 kinase is a heterodimeric protein consisting of a non-catalytic 85kD p85 subunit, a catalytic 110kD p110 subunit and phosphorylated inositol lipids. It catalyses the phosphorylation of PI-4 P and PI-4,5-P_2 (PIP_2) on the D3 position of the inositol ring to yield PI-3,4-P_2 and PI-3,4,5-P_3.(PIP_3) respectively. Anti-sIgM crosslinking induced stimulation of PI-3 kinase activation was seen in murine cells at different stages of maturation and also in the mature human B cell lines, Daudi and Ramos (Gold et al., 1990). Studies have also shown that a proline rich region of the p85 subunit of PI-3 kinase is associated with the SH3 domain of Lyn and Fyn (Yamanashi et al., 1992) resulting in an increase in PI-3 kinase activity (Pleiman et al., 1994). In addition, BCR stimulation

results in the tyrosine phosphorylation of the CD19 accessory molecule. This results in the recruitment of the p85 subunit to CD19 tyrosine phosphorylated residues via an SH2 domain dependent interaction with further activation of PI-3 kinase enzymatic activity (Buhl et al., 1997). An attractive model is proposed whereby Lyn and Fyn binding to BCR ITAM sequences via SH2 domain interactions can bind p85 of PI-3 kinase via SH3 which in turn is bound to CD19 via its SH2 domain. This would result in the localisation of active PI-3 kinase to the plasma membrane where its phosphoinositide substrates are located.

To date the involvement of Btk in this pathway has been to the products of PI-3 kinase activity. Studies using biosensor assays, have demonstrated the binding of the PH domain of Btk to PIP_3 (Salim et al., 1996). This may also serve to recruit Btk to the plasma membrane where it may become activated.

1.5.5.4 Cbl and B cell signalling

Cbl is the cellular homologue of the v-Cbl oncoprotein. It encodes a 120kD protein which has been implicated in signalling through both B and T lymphocyte antigen receptors. *In vitro* evidence also suggests that it may be a possible ligand for Btk. Cbl is tyrosine phosphorylated upon BCR stimulation and has been shown to bind *in vivo* with Fyn, Lyn, Grb2 and PI-3 kinase (Fukazawa et al., 1995). In DT40 cells deficient in Lyn, Cbl tyrosine phosphorylation on BCR stimulation is defective suggesting that Cbl is a direct substrate for Lyn (Tezuka et al., 1996). Cbl has also been shown to associate with Syk in RBL-2H3 rat mast cells on FcεR1 cross-linking (Ota et al., 1996). The Cbl protein has multiple domains including proline rich regions which could potentially bind to SH3 domains. Using the GST-fusion protein system, the Btk SH3 domain has been identified as binding to Cbl in the Daudi B cell line (Cory et al., 1995). No *in vivo* data demonstrating a physical association between these two proteins has as yet been reported and the functional significance of this interaction is unclear.

1.5.6 Btk stimulation via alternative lymphocyte receptors

In addition to the BCR, Btk has been shown to be activated in response to stimulation of a number of different receptors.

1.5.6.1 FcεR1

Studies performed on murine bone marrow derived mast cells showed that Btk is tyrosine phosphorylated and shows increased kinase activity upon stimulation of the high affinity immunoglobulin E receptor (FcεRI). Analysis of subcellular fractions for Btk expression suggested that there is translocation of a small amount of Btk to the plasma membrane on cell stimulation. Btk phosphorylation and activation were not induced by phorbol esthers or by calcium ionophore suggesting that Btk functions independently or upstream of PKC activation (Kawakami et al., 1994). In the same series of experiments it was shown that Btk does not physically associate with this receptor while Lyn was clearly shown to co-immunoprecipitate with this complex. This may imply that a pattern of phosphotyrosine kinase activation similar to that following BCR engagement is present in mast cells.

1.5.6.2 IL-5R

A number of independent studies have demonstrated that Btk is activated following IL-5 stimulation . IL-5 plays an important role in the growth and differentiation of B cells and eosinophils. IL-5 stimulation of an IL-5 dependent B cell line, Y16, induced tyrosine phosphorylation and activity of Btk 2-3 fold above basal levels (Sato et al., 1994). Interestingly, no increased activity of Lyn or Fyn was seen in similar experiments suggesting that the mechanism of Btk activation may vary in response to activation by different receptor complexes. Transfection of a weakly transforming mutant of Btk into Y16 cells was shown to relieve cells of their IL-5 dependence (Li et al., 1995). Further evidence comes from studies on B cells from *xid* mice which showed abnormal responses to IL-5 stimulation (Koike et al., 1995).

1.5.6.3 IL-6R

IL-6 is important in the proliferation of haematopoietic stem cells. Stimulation of murine pro-B cells stably transfected with the gp130 component of the IL-6 receptor, showed a minimal increase in Btk and Tec activity (Matsuda et al., 1995). Btk and Tec were shown to be constitutively associated with gp130 although the mechanism of association remains to be determined.

1.5.6.4 CD38

CD38 is a transmembrane glycoprotein that has been implicated in the regulation of B cell proliferation and in the rescue of B cells from apoptosis. The response of Btk to CD38 stimulation has been studied in murine and human cells with differing results. On CD38 crosslinking, B cells from *xid* mice did not proliferate nor upregulate expression of MHC class II molecules (Santos Argumedo et al., 1995). In splenic B cells from normal mice, CD38 stimulation led to tyrosine phosphorylation of Btk but this response was abolished in cells from *xid* mice (Kikuchi et al., 1995). The mechanism of Btk phosphorylation has been shown to involve Lyn and Fyn, since this response is impaired in splenic B cells from $Lyn^{-/-}$ and $Fyn^{-/-}/Lyn^{-/-}$ mice (Yasue et al., 1997). However, no convincing evidence suggests a similar pathway in human cells. CD38 stimulation of the immature human B cell lines RS4;11 and OP-1 did not show tyrosine phosphorylation of Btk (Silvennoinen et al., 1996).

1.5.6.5 Heterotrimeric G-proteins

Following evidence that the C-terminal region of the PH domain was able to bind βγ subunits of heterotrimeric G proteins (Tsukada et al., 1994), it was later shown that $G_{\beta\gamma}$ can stimulate Btk kinase activity (Langhans Rajasekaran et al., 1995). Co-expression of Btk in HEK-293 (human embryonic kidney) cells with various subunits of $G_{\beta\gamma}$ resulted in stimulation of kinase activity.

More recently, a role for Btk in G protein mediated activation of the MAP kinase pathway has been suggested. In avian DT40 cells deficient in Btk, G_i-coupled receptors failed to activate MAP kinase, while G_q-coupled receptor mediated stimulation remained unaffected (Wan et al., 1997).

1.6 Lymphocyte signalling in primary immunodeficiencies

The study of XLA led ultimately to the discovery of the Btk gene and the realisation of its importance in B cell development and function. In an analogous manner, the study of other primary immunodeficiencies has led to greater understanding of molecules involved in immune function.

The primary immunodeficiencies are a heterogeneous group of single gene disorders of the immune system. These range from purely humoral conditions such as XLA through abnormalities in neutrophil function to severe combined immunodeficiencies in which there is abnormality of both T and B cell function. Over the past decade, the advances in the knowledge of the basic mechanisms of lymphocyte function, together with the more sophisticated methods of molecular diagnosis, have resulted in the identification of many of the genes responsible for these conditions. In the following section, the primary immunodeficiencies resulting from abnormalities of lymphocyte signalling are examined. These conditions are indeed natural human 'knockouts' and provide powerful tools with which to understand lymphocyte function and development.

1.6.1 Agammaglobulinaemia resulting from abnormalities in the B cell receptor complex

It had long been established that an autosomal recessive form of agammaglobulinaemia with absent B lymphocyte development existed. These individuals were phenotypically indistinguishable from those with XLA but were either female or had an autosomal recessive pedigree (Conley and Sweinberg, 1992; de la Morena et al., 1995). Using linkage analysis at candidate genes, two consanguineous families in which both boys and girls had panhypogammaglobulinaemia and markedly reduced numbers of B cells were studied (Yel et al., 1996). Both families were found to have mutations in the μ heavy chain gene that would result in unstable forms of the μ heavy chain protein. Further analysis of the bone marrow from one of the affected patients showed normal percentages of pro-B cells but a marked decrease in the numbers of early and late pre-B cells that start to express μ heavy chain. This is in keeping with the murine knockout previously described (Kitamura et al., 1991).

Importantly, the residual number of CD19+ peripheral B cells in these patients was compared with the residual population in patients with known Btk mutations. It was found that in XLA patients between 0.01-1% of peripheral blood lymphocytes expressed CD19 whereas in the μ chain defect patients, this population was undetectable (less than 0.01%). This may suggest that the block in B cell

development as a result of a μ chain defect is more critical than that due to Btk deficiency.

In this study the authors also commented that a number of other families with similar phenotypes and autosomal recessive pedigrees had been examined, but had not been found to have the above defect. Further analysis of one of these families demonstrated a mutation in the human lambda 5/14.1 gene which forms an integral part of the pre-B cell receptor (Minegishi et al., 1998). Both the above studies demonstrate the importance of a fully functional pre-B and B cell receptor in normal B lymphocyte differentiation.

1.6.2 Defective B- and T-cell interaction in X-linked hyper IgM syndrome

The rare X-linked hyper IgM syndrome (HIGM1) had long been seen as a variant of XLA. However, the phenotype in this disease was characteristic with affected individuals exhibiting normal or raised levels of IgM but very low or absent levels of IgA, G and E indicating a failure of immunoglobulin isotype switching (Notarangelo et al., 1992). Although the immunological abnormalities were only manifest in the humoral arm of the immune system, it was evident clinically that there was a significant cellular defect. Affected boys characteristically presented with *Pneumocystis carinii* pneumonia (Levitt et al., 1983) and more recently it has been shown that there is a high incidence of sclerosing cholangitis associated with cryptosporidial infection (Hayward et al., 1997).

The gene defect in HIGM1 had been localised by linkage analysis to Xq26.3-q27.1. In 1993, the molecular defect was shown to be due to abnormalities in a transmembrane TNF-related activation protein expressed on the surface of activated T cells (Allen et al., 1993; Aruffo et al., 1993). This protein is the physiological ligand for the CD40 molecule expressed on the surface of B cells. Thus it was shown that a crucial interaction between B and T lymphocytes via the CD40-CD40 ligand interaction is essential for the B cell to undergo immunoglobulin isotype switching from IgM to IgG, A and E. The specific mechanism for the cellular defects seen in HIGM1 has not as yet been elucidated but it is assumed that the CD40/CD40 ligand interaction may also have important consequences in the T cell. As with XLA,

autosomal recessive pedigrees exhibiting similar phenotypes to HIGM1 also exist and proteins downstream of the CD40 interaction which are coded for on autosomes must be strong candidates.

1.6.3 Wiskott-Aldrich syndrome (WAS)

WAS is another of the classic X-linked primary immunodeficiency syndromes which has recently been shown arise from a defect in intracellular haematopoietic cell signalling. The clinical manifestations of WAS are a combination of microthrombocytopaenia, eczema, combined immunodeficiency and a predisposition to lymphoreticular malignancy. The defective gene was identified using a positional cloning method (Derry et al., 1994). Carrier females exhibit non-random X-inactivation in all haematopoietic lineages suggesting that the WAS protein (WASp) is required for the normal development of all haematopoietic compartments. WASp has been shown to interact with a number of intracellular signalling molecules in *in vitro* and *in vivo* studies (Rivero et al., 1995; Cory et al., 1996; Symons et al., 1996). In addition, WASp co-localises with the actin cytoskeleton, and may therefore play a crucial role in the maintenance of intracellular architecture or in the formation of structures required for cell motility. Such a fundamental role for WASp may explain the extensive variability of the disease phenotype.

1.6.4 T cell receptor development and signalling abnormalities

Signalling events through the T cell receptor are essential for the normal development and function of T lymphocytes. The T cell and B cell receptor show many similarities both in the pattern of their structure and pathways of activation. Briefly, the T cell receptor (TCR) consists of a heterodimer of immunoglobulin like molecules which contain constant and variable regions expressing enormous diversity. The TCR associates with the CD3 complex which consists of a number of transmembrane molecules denoted CD3γ, δ, ε and ζ which are essential for cytoplasmic signal transduction. The immunoglobulin like molecules recognise antigen which is presented to the TCR as peptides within the context of MHC class I or II molecules which are also recognised by the co-receptors CD8 or CD4 respectively. Recognition of antigen in this way results in the activation of the TCR/CD3 complex which in turn leads to the activation of tyrosine kinases (Weiss

and Littman, 1994). The resultant kinase activation cascade has many parallels with BCR signalling (summarised in Fig 1.5).

Development of T lymphocytes proceeds in the thymus through an ordered series of tightly controlled differentiation events (reviewed in Cheng and Chan, 1997). Central to this process is the expression of TCR components and assembly into functional complexes. Similar to the BCR this involves a DNA recombination process mediated by the RAG-1 and RAG-2 proteins. The earliest precursors lack TCR, CD4 and CD8 expression ($CD4^-/CD8^-/TCR^-$). The assembly and expression of the pre-TCR allows differentiation of double negative thymocytes ($CD4^-/CD8^-/TCR^+$). Signalling events now mediated through the pre-TCR induce expression of both CD4 and CD8 co-receptors giving rise to double positive thymocytes ($CD4^+/CD8^+/TCR^+$). These cells now start to express the mature TCR and undergo both postive and negative selection processes in the thymus leading to differentiation into either $CD4^+$ or $CD8^+$ single positive T lymphocytes.

1.6.4.1 Tyrosine kinases in TCR signalling

The Src family of tyrosine kinases, especially Lck and the ZAP-70/Syk family of kinases are crucial elements in TCR mediated signalling and development. Lck is localised to the plasma cell membrane through its myristoylation signal and can bind to the TCR or the CD4/CD8 co-receptors (Rudd et al., 1989; Rudd, 1990). Antigen stimulation of the TCR and its co-receptors results in the tyrosine phosphorylation of Lck. The co-engagement of CD4 or CD8 molecules to MHC molecules together with the TCR may co-localise Lck to the TCR and allows Lck mediated tyrosine phosphorylation of the ITAMs of the cytoplasmic tails of the CD3 complex (Straus and Weiss, 1992). Phosphorylation of the two ITAMs of the ζ chain was originally thought to result in recruitment of ZAP (ζ-associated protein)-70 to the TCR via its tandem SH2 domains and result in its activation (Chan et al., 1992; Wange et al., 1993). However, more recent evidence suggests that the TCR ζ chain is constitutively phosphorylated and associated with ZAP-70 in quiescent cells (van Oers et al., 1993). This association is not sufficient however to activate downstream events.

Fig 1.6 Proximal events following TCR signalling. *In a similar pattern of events to that following BCR stimulation, engagement of the TCR by antigen results in the clustering of the TCR/CD3 complex and associated Src kinases (inc. Lck and Fyn). ITAM residues in the cytoplasmic tail of the CD3 chains are phosphorylated and thus recruit Src kinases by SH2 domain binding. Subsequent ITAM phosphorylation on the ζ chain enhances binding of ZAP-70 via its two SH2 and domains and result in its activation.*

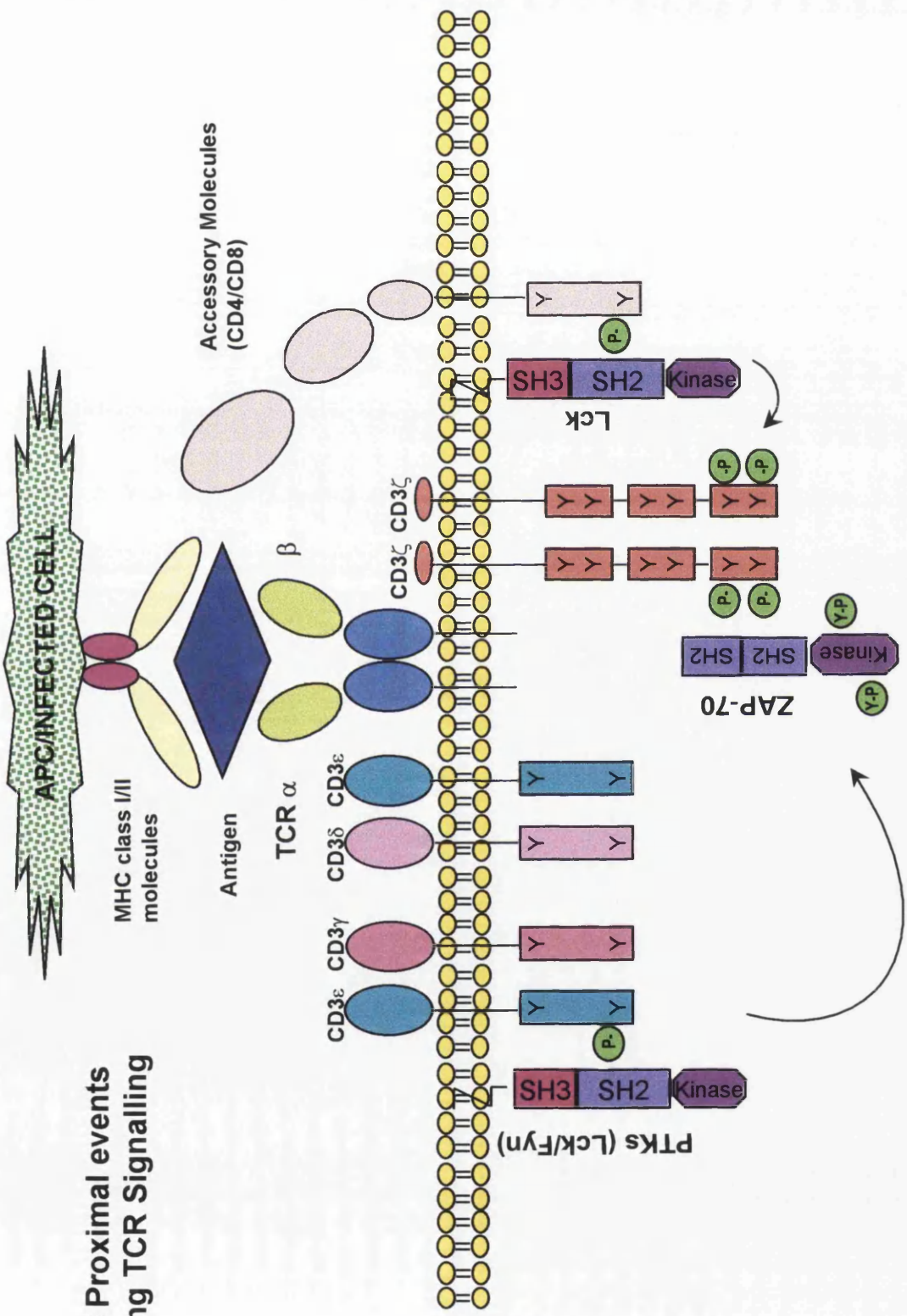


Fig. 1.6 Proximal events following TCR Signalling

It may be therefore that TCR activation results in Lck tyrosine phosphorylation and recruitment to the TCR where it is necessary for phosphorylation of both CD3 chain ITAMS and of already bound ZAP-70 and it is this phosphorylation of ZAP-70 that results in activation of downstream events (van Oers et al., 1996a).

1.6.5 CD3 complex abnormalities

The components of two elements of this signalling cascade have now been shown to result in human disease. The integrity of the CD3 complex has been shown to be essential for normal cellular function. Mutations have been found in both the CD3-ε and CD3-γ chain of the complex (Alarcon et al., 1988; Soudais et al., 1993). Both resulted clinically in a picture of combined immunodeficiency with defective T cell activation and low expression of the CD3 complex. Subsequent sequencing of the respective CD3 proteins showed both point mutations and deletions.

1.6.6 SCID due to defects in ZAP-70

A selective form of severe combined immunodeficiency arising from abnormalities in ZAP-70 function has also been described. A patient was initially described with a clinical phenotype of cell mediated immunodeficiency in whom flow cytometric analysis of peripheral blood mononuclear cells revealed a lack of $CD8^+$ cells but normal numbers of $CD4^+$ cells (Roifman et al., 1989). Analysis of the thymus revealed a normal distribution of $CD4^+$ and $CD8^+$ cells in the thymic cortex but no $CD8^+$ in the thymic medulla . At a later stage, the peripheral mononuclear cells were analysed for ZAP-70 protein expression by immunoblotting and found to be deficient (Molina et al., 1992; Chan et al., 1994). Subsequent mutation analysis revealed an intronic point mutation resulting in a splicing defect leading to an unstable protein. This natural human model demonstrates the crucial role of ZAP-70 in the positive selection of $CD8^+$ thymocytes from the double positive stage in the thymus. In contrast, the murine ZAP-$70^{-/-}$ model has a significantly different phenotype with a block in development at the double positive stage with no peripheral $CD4^+$ or $CD8^+$ cells.

1.6.7 Abnormalities arising from defects in Lck and Itk

The model of TCR activation proposed above indicates a central role for Lck in this system. This is supported by evidence from murine knockouts where $Lck^{-/-}$ mice

show profound thymic atrophy and a dramatic reduction in double positive cells (Molina et al., 1992). There is a lack of single positive cells and very few mature T cells are detected in the periphery. It is also evident that other Src family kinases such as Fyn, which can associate with the TCR cannot compensate for the absence of Lck. Given this crucial role, it is surprising that human immunodeficiencies arising from abnormalities in Lck have not as yet been described, but it may be that such is its importance, the defect is lethal *in utero*.

In addition to the molecules described above, T cell activation and development also involves the tyrosine kinase Itk. This protein is structurally homologous to Btk and is preferentially expressed in T lymphocytes. Given the parallels that exist in B and T cell antigen receptor signal transduction, it has been suggested that Itk may play an analogous role to Btk in T cell development. Genetic studies on mice have demonstrated that $Itk^{-/-}$ mice have reduced numbers of mature thymocytes and lymph node T cells as well as a reduced $CD4^+/CD8^+$ ratio (Liao and Littman, 1995). There was also decreased proliferative responses to allogeneic MHC stimulation and to TCR cross linking. These responses are not as dramatic as might be expected but given the less severe phenotype of the *xid* mouse and $Btk^{-/-}$ knockout mice, abnormalities in Itk may have a more significant effect in humans.

1.6.8 Interleukin receptor mediated signalling abnormalities

The most common form of severe combined immunodeficiency has a characteristic immunophenotype that has normal numbers of B lymphocytes but is completely lacking in T lymphocytes and natural killer cells (denoted T-B+NK- SCID). This is most commonly inherited in an X-linked manner (SCID-X1) and the incidence is approximately 40% of all forms of SCID (Buckley et al., 1997). It has also been recognised that an indistinguishable phenotype exists in female patients and in families displaying an autosomal recessive pedigree. Over the past 3 years the molecular basis of both forms of the condition have been elucidated and have been shown to arise from defects in signalling through the interleukin receptors.

1.6.8.1 Mutations in the common γ chain (γ_c) result in SCID-X1

The SCID-X1 locus was mapped to the Xq12-13.1 locus. It was then found that the γ chain of the IL-2 receptor mapped to the same region and that mutations in the γ chain were found in SCID-X1 patients (Noguchi et al., 1993b). Although initially thought to be only an essential component of the high affinity IL-2 receptor (IL-2R) in association with the α and β chains, the γ chain was later shown to be also an integral part of the IL-4, IL7, IL-9 and IL-15 receptors (Kondo et al., 1993; Noguchi et al., 1993a; Giri et al., 1994; Kimura et al., 1995). In each case the common γ chain (γ_c as it is denoted) was responsible for augmenting the affinity of the receptor for the cytokine and also for the transduction of the signal via its cytoplasmic tail. The multiple roles of the γ_c in cytokine signal transduction may explain the severity of the resulting phenotype in SCID-X1.

1.6.8.2 Mutations in JAK3 result in autosomal recessive T-B+NK- SCID

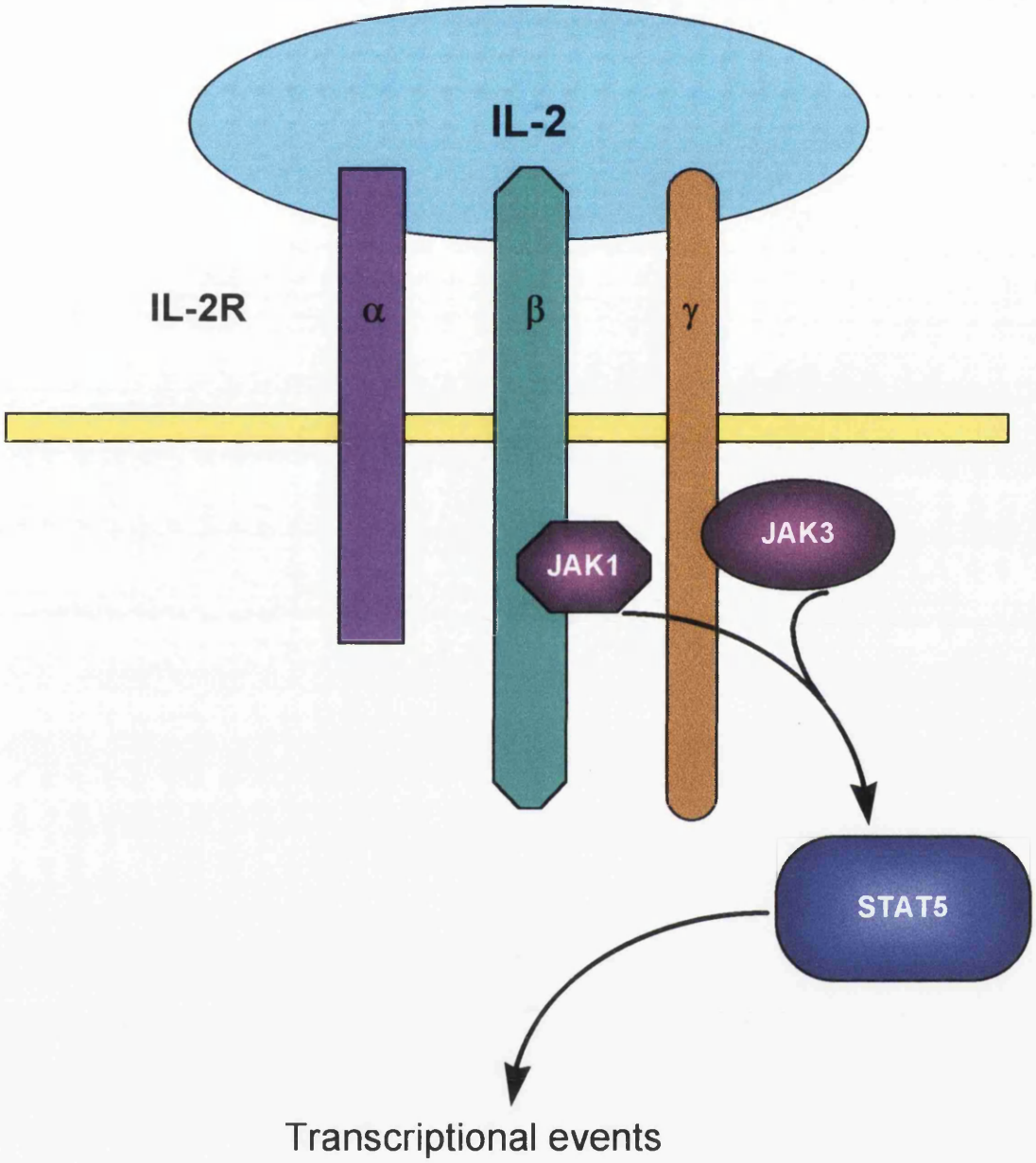
The delineation of the pathway of signal transduction through the γ_c receptor was instrumental in elucidating the defective gene in autosomal recessive T-B+NK- SCID. In IL-2 mediated signalling, binding of the cytokine to the receptor results in heterodimerisation of the cytoplasmic regions of the IL-2Rβ and IL-2Rγ chains and results in the recruitment and activation of another family of phophotyrosine kinases, the Janus kinases (JAKs). In this case, JAK1 associates with IL-2Rβ and JAK3 with the IL-2Rγ_c. Activation of JAK1 and JAK3 occurs by cross phosphorylation and this results in phosphorylation of STAT proteins (Signal Transducers and Activators of Transcription) which when phosphorylated dimerise and translocate to the nucleus to induce gene expression (reviewed by Ihle, 1996). This signal transduction pathway is summarised in Fig. 1.7.

The essential role of Jak 3 in this pathway and association of JAK3 with γ_c led to the hypothesis that it may be defective in the autosomal recessive forms of SCID desbcribed above. Analysis of patients confirmed that this was indeed the case (Macchi et al., 1995; Russell et al., 1995). In B cell lines derived from these patients it has also been shown that there is lack of STAT5 phosphorylation in response to IL-2 stimulation (Oakes et al., 1996).

1.6.8.3 Defects in IL-2Rα also result in cellular immunodeficiency

In addition to defects in the signalling pathway involving γ_c, it would be reasonable to suspect that defects in the other cytokine receptor components would have a deleterious effect. Recently a patient exhibiting a profound cellular immunodeficiency with reduced numbers of CD4+ cells and abnormal proliferative responses to mitogenic stimuli has been described (Sharfe et al., 1997). Surface staining of EBV immortalised B cells from the patient showed absent expression of the IL-2Rα chain but normal expression of other IL-2R components. Subsequent sequence analysis revealed a 4bp deletion in the IL-2Rα gene.

Fig. 1.7 Signalling through the IL2 receptor complex



The activation of the IL-2R by IL-2 binding recruits JAK1 and JAK3 to the β and γ chain respectively. The two JAK molecules phosphorylate each other and in doing so are themselves activated. JAK3 then phosphorylates STAT5. Phosphorylated STAT5 molecules dimerise and are then able to translocate into the nucleus, thereby initiating transcriptional events.

1.7 Management of primary immunodeficiencies

At present the therapeutic options for the management of many of the primary immunodeficiencies are limited. Children with pure humoral deficiencies can be treated with immunoglobulin replacement therapy but for those with combined immunodeficiencies antibiotic prophylaxis and aggressive treatment of ongoing infection are the mainstays of treatment. The only curative therapy available at present is allogeneic bone marrow transplantation (BMT). This has a highly successful outcome in SCID, if a matched sibling donor is available (Fischer et al., 1990), but for approximately 30% of children this is not an option. Success rates of BMT using a nongenotypically identical marrow graft, whether it be from an unrelated donor or from a haploidentical parent, are considerably poorer with the above European study reporting an overall survival of only 53% for all types of SCID. Morbidity and mortality in mismatched transplantation has been to a large extent due to graft rejection and graft versus host disease. A number of factors including genotypic typing of bone marrow and the use of unrelated donors, improved cytotoxic agents for recipient myeloablation and the use of donor T lymphocyte depletion have improved the prognosis but a significant risk still remains. An alternative strategy which may eventually overcome the limitations of allogeneic bone marrow transplantation is somatic gene therapy.

1.8 Somatic gene therapy

The recent advances in the identification of the molecular basis of inherited and acquired disease have led to the development of novel strategies for their treatment based on gene transfer. Somatic gene therapy can thus be defined as the deliberate transfer of genetic material into somatic cells in order to treat or modify disease. Although initially proposed as a curative procedure for classically inherited disease, the application of gene therapy now extends to a wide variety of both acquired and inherited conditions. Indeed, of the numerous clinical trials of gene therapy active at the start of 1996, two thirds were for cancer (Sikora, 1996). However, the primary immunodefciencies have played an important role in the development of this new technology. Although rare, they are in many ways ideally suited to its application and have become model conditions on which to design and test gene therapy protocols.

1.8.1 Gene therapy for the primary immunodeficiencies

In the initial development of protocols, a number of criteria were established for diseases to become amenable for gene therapy. These are summarised in Table 1.1 below. Only monogenic life threatening conditions were initially considered so that the potential risk of serious side effects was ethically acceptable. The gene defect, sequence and basis of its function had to be known. The size of the gene was also an important consideration since the viral vector constructs initially developed were constrained by the amount of genetic material that could be accommodated. Conditions in which the gene defect affected only one specific organ system were also more attractive since gene delivery could be restricted to that particular system. Regulation of the targeted gene was another important factor as methods for regulating the expression of inserted constructs were and are still not particularly sophisticated, and thus a simply regulated gene was deemed a more attractive candidate. Furthermore, it was advantageous to target diseases where subnormal levels of affected gene expression could give rise to a normal clinical phenotype due to the relative inefficiency of gene transfer.

Table 1.1

Criteria for a disease suitable for gene therapy
Life threatening disease
Molecular basis characterised
Function of gene product known
Efficient gene transfer to target organ/tissue possible
Precise regulation of gene product not required
Selective advantage for corrected cells
Alternative treatments suboptimal or not available

Given these guidelines, the primary immunodeficiencies presented themselves as attractive candidates. Many of the genes had been identified and in a number of cases, the expression and function of the gene had been determined. Importantly, since the immunological defect could be corrected by bone marrow transplantation, it was only necessary to introduce a correct copy of the defective gene into bone marrow cells. This target tissue was also easily accessible and readily manipulated *ex vivo* thereby facilitating gene transfer.

1.8.2 Development of gene therapy for primary immunodeficiencies

The development and optimisation of gene therapy for the primary immunodeficiencies has evolved through a natural succession of *in vitro* and *in vivo* models. In the initial stages of development, SCID due to adenosine deaminase deficiency (ADA-SCID) was identified as a candidate disorder for treatment by gene therapy. Therefore, a number of early preclinical studies used ADA gene transfer to assess the feasibility of this new technology and ultimately ADA-SCID became the first human disease in which clinical trials of somatic gene therapy were conducted. The discussion below briefly outlines the pathogenesis of ADA-SCID and details the progression from preclinical gene transfer studies in a variety of models to the first clinical trials of somatic gene therapy for human disease.

1.8.3 Adenosine deaminase deficiency

ADA is an enzyme that is expressed in all body tissues and plays an essential role in the purine salvage pathway, catalysing the irreversible deamination of adenosine to inosine and 2' deoxyinosine. Its deficiency results in the accumulation of the products of adenosine degradation, characterised by elevated erythrocyte deoxyadenosine triphosphate (dATP), and plasma and urinary deoxyadenosine (dAdo) (Hirschhorn, 1993) and also in the reduction in levels of S-adenosylhomocysteine hydrolase (SAHH). The mechanism of the immunodeficiency is not clearly understood, but a block in T lymphocyte differentiation in the thymus may be central to its pathogenesis (Benveniste et al., 1995).

1.8.3.1 Clinical manifestations of ADA-SCID

The condition was first described in 1972 by Giblett and colleagues in Seattle (Giblett et al., 1972). There is considerable heterogeneity in the clinical severity of the disease, ranging from very severely affected infants presenting in the first few months of life through to chronically affected adults presenting later in life (Morgan et al., 1987; Shovlin et al., 1994). The biochemical and immunological abnormalities generally correlate with the degree of immunodeficiency.

1.8.3.2 Therapeutic Options for ADA-SCID

A number of therapeutic options are available for the treatment of ADA deficiency. The treatment of choice is genotypically matched bone marrow transplantation (BMT) as previously discussed (see section 1.5.5). Alternative treatment strategies have been attempted where a matched related donor is unavailable. PEG-ADA, a bovine ADA preparation conjugated to polyethylene glycol (Hershfield et al., 1987) has been used as exogenous enzyme replacement therapy. In the majority of patients there has been a marked improvement in biochemical abnormalities followed by an improvement in both absolute lymphocyte numbers and in their functional responses to mitogenic stimuli (Hershfield et al., 1993; Hershfield, 1995) although in some cases reconstitution is suboptimal. In the clinical trials of gene therapy so far undertaken all the children treated have also received PEG-ADA therapy. This has caused a considerable amount of difficulty in assessing the effect of gene therapy since any clinical improvement may be as a result of concurrent PEG-ADA therapy.

1.8.4 Gene transfer into haematopoietic stem cells

The optimal target cell population for gene transfer in haematopoietic conditions is the pluripotent haematopoietic stem cell (PHSC). The defining characteristic of this cell is the capacity for self-renewal and retention of multilineage differentiation potential. Thus, successful gene transfer into true human PHSCs would theoretically allow the expression of the transduced gene in all haematopoietic lineages for the lifetime of the individual. This goal has been difficult to achieve for a number of reasons. The PHSC population is difficult to define phenotypically and thus difficult to isolate. The CD34 antigen has frequently been used as a marker of PHSC and repopulating experiments in animal models and humans suggest that this population has multilineage reconstitution potential at least in the short term. However, it is likely that this is a heterogeneous and hierarchical primitive cell population within which true PHSCs reside. This problem is further compounded by the lack of adequate assays of PHSC activity. *In vitro* assays have been essentially dependent upon colony forming ability of progenitor cells in semi-solid culture media although the advent of long term bone marrow culture has permitted the investigation of more primitive cells. Only more recent *in vivo* models involving engraftment of bone marrow progenitors into recipient animals have allowed transduction of PHSCs to be adequately assessed (Larochelle et al., 1996).

In addition to these problems, stem cells are a naturally quiescent cell population. In the steady state, the majority of stem cells are dormant in the cell cycle (G_o) and only a few cells enter a proliferative stage at any one time (Hao et al., 1996). Since active cell division is an absolute prerequisite for retroviral infection, this has considerably limited initial PHSC stem cell transduction efficiency.

1.8.4.1 Gene transfer into murine PHSC

Initial studies focused on the transfer of selectable marker genes into murine PHSC's using retroviral vectors. Successful transfer and long term *in vivo* expression of β-globin, HPRT and neomycin genes after transduction of murine stem cells was initially shown (Miller et al., 1984; Eglitis et al., 1985; Dzierzak and Mulligan, 1988). Following this, a number of groups were able to demonstrate successful retroviral mediated transfer of the human ADA gene into murine PHSCs and long term *in vivo* expression of the transduced gene (Lim et al., 1989; Osborne et al., 1990; van Beusechem et al., 1990; Wilson et al., 1990). Lim et al. (1989) transduced pre-stimulated whole murine marrow with a human ADA encoding retroviral vector prior to transplantation into irradiated recipients. Expression of human ADA was seen in all haematopoietic lineages of primary recipients 4 months after transplantation. Human ADA expression was also shown 6 weeks later in splenic colonies and in the peripheral blood of secondary recipients, thus demonstrating the stability of transfer and expression in early progenitor cells. In similar studies, retroviral mediated transduction resulted in the expression of human ADA enzyme in lymphoid, myeloid and erythroid lineages 30 days after transplantation. Multi-lineage ADA expression was also seen in secondary and tertiary recipients (van Beusechem et al., 1990).

1.8.4.2 Gene transfer into large animal HSC

The success achieved with transduction of murine HSCs has not been reproduced in studies performed in non human primates and large mammals. Gene transfer efficiency on primate long term *in vivo* repopulating stem cells is at least one or two orders of magnitutude lower than that observed in murine studies (reviewed by van Beusechem and Valerio, 1996). Similarly poor results were observed in canine and feline models (Lothrop, Jr. et al., 1991; Carter et al., 1992). This may be a result of

decreased efficiency of transduction of primate marrow by retroviruses, due to less well defined co culture conditions or the lack of experience with large animal bone marrow transplantation and engraftment. It may also be due to a lower density of receptors for retroviruses in these species.

1.8.4.3 Gene transfer into human HSC

The ultimate target for gene transfer not only for ADA deficiency but for other conditions curable by BMT is the human haematopoietic stem cell. Initially, a number of studies demonstrated transfer of genes (including ADA) into human haematopoietic cells capable of colony formation up to two weeks after gene transfer (Hock and Miller, 1986; Bender et al., 1988; Fink et al., 1990). Although these experiments were able to show the feasibility of retroviral mediated transfer into progenitor populations, these early systems were not able to evaluate transfer into more primitive progenitor cells.

The development of long term bone marrow culture (LTBMC) systems offered the possibility of evaluating gene transfer into more primitive cells. In this system, haematopoiesis can be routinely maintained for 2-3 months and the clonal cell responsible for this, the long term culture initiating cell (LTCIC) is a more primitive cell than the progenitor cell detected by colony assays (Sutherland et al., 1989). The successful use of cells from LTBMC for autologous bone marrow transplantation (Chang et al., 1989) supports the view that the LTCIC may be functionally related to the primitive stem cells. Using this system several studies demonstrated high frequency retroviral mediated transfer of selectable marker genes into LTCICs (Hughes et al., 1989; Cournoyer et al., 1991). These experiments also allowed the evaluation of growth factors leading to more efficient cell transduction (Cournoyer et al., 1991). Proviral integration into LTCICs was shown to increase if viral supernatant was added to bone marrow cells maintained on a human bone marrow derived stromal layer (Moore et al., 1992) suggesting that stromal support may increase stem cell proliferation and improve retroviral transfer. Despite the efficiency of transfer achieved in these studies, their relevance was unclear since the relationship between these progenitor cells and the *in vivo* human repopulating stem cell is not established.

The engraftment of normal human haematopoietic cells into immune-deficient mice (Lapidot et al., 1992) provided a model by which to assay gene transfer into primitive human cells capable of multilineage haemopoiesis *in vivo*. In contrast to the high level of gene transfer seen in LTCICs and CFCs, transduction of the SCID mouse repopulating cell (SRC) was shown to be much lower (Larochelle et al., 1996). These observations were a more accurate reflection of the results of gene marking studies in clinical trials (Brenner, 1996), supporting the notion that retroviral mediated transduction of true human PHSCs is highly inefficient.

As an alternative source of PHSCs, efforts have been directed at transducing progenitor cells derived from human umbilical cord blood (HUCB). HUCB is rich in the $CD34^+$ progenitor cell population and its repopulating capacity is demonstrated by *in vitro* assays but also more convincingly by successful cord blood transplantations for a variety haematopoietic diseases (Gluckman et al., 1989; Vowels et al., 1993; Wagner et al., 1995). Retroviral gene transfer studies have shown that HUCB progenitors and selected HUCB $CD34^+$ cells can be efficiently transduced at frequencies similar to or greater than bone marrow derived cells (Moritz et al., 1993; Lu et al., 1994; Williams and Moritz, 1994). These initial studies led to one group optimising their transfer protocol for a clinical trial of ADA gene transfer into cord blood $CD34^+$ cells (Hanley et al., 1994; Kohn et al., 1995).

1.8.5 Gene transfer into T lymphocytes

The difficulty associated with achieving successful retroviral mediated gene transfer into human PHSCs meant that certain groups targeted alternative cells for the correction of ADA-SCID. The most obvious of these was the T lymphocyte since this is the cell population primarily affected by the deficiency of ADA. Transduced primary T cells cultured from ADA patients were shown to grow for a significantly longer period of time in comparison with non-transduced cells and continued to express the ADA gene during this time. This suggested that the expression of transduced ADA provided a survival advantage to these cells in a culture environment. Further evidence for the benefit of transducing ADA- T lymphocytes came from animal studies (Ferrari et al., 1992). Peripheral blood lymphocytes from ADA deficient patients were transduced with a retroviral vector containing the human ADA gene and injected into immunodeficient BNX mice. Only ADA vector

transduced cells were able to show long term survival in recipient animals. The expression of the transduced ADA gene also resulted in human immunoglobulin production and the development of antigen specific T cells. These experiments suggested that the expression of vector derived human ADA in the T lymphocytes of ADA- patients would confer prolonged survival upon these cells even in an ADA normal environment. Furthermore, the results of the latter experiment suggested that T cell modification could reconstitute a certain degree of immune function.

1.8.6 Clinical trials of gene therapy

The success of the T cell directed ADA transfer led to the first clinical trials of somatic gene therapy for human disease. These were initiated in 1990 at the National Institutes of Health (NIH) in the USA (Blaese and Culver, 1992; Blaese et al., 1995). Since then 4 further trials involving a total of 12 ADA-SCID patients have been conducted worldwide with varying degrees of success. Three of these trials have targeted ADA gene transfer into haematopoietic progenitor cells. The results of these trials and those of other clinical trials for primary immunodeficiency are further discussed in Chapter 8.

1.9 Gene therapy for XLA

Although XLA is not life threatening in the majority of cases and it is likely that Btk gene expression is precisely regulated during the B lymphocyte maturation process, certain characteristics make the disease attractive for gene transfer studies. While the use of immunoglobulin substitution therapy is clinically effective, regular blood product administration is expensive and may in the long term be considered unsafe due to the danger of viral contamination. Moreover, the predisposition to enteroviral infection suggests that a significant immunological deficit remains. Successful gene therapy appears more feasible if one considers that female carriers of XLA exhibit non random X inactivation specifically in the B cell lineage suggesting that there may be a selective proliferative advantage to genetically corrected cells *in vivo*. Furthermore, the *xid* mouse presents a natural animal model in which to test such hypotheses.

The role of Btk in B cell development and the exact mechanism of the maturation defect remains unanswered and thus the transfer of the Btk gene to early B cell

progenitors may help to address this question. Gene transfer experiments would also be useful tools for studying the interaction of Btk with other proteins *in vivo*.At the start of this study, there had been no documentation of gene transfer experiments for XLA.

1.10 Aims of the study

The aims of this thesis were initially to study the molecular pathogenesis of XLA. Using our cohort of XLA patients, we investigated the defects in the Btk gene by mutation analysis. In addition the expression of the mutant protein in affected individuals was studied. The objective of these studies was not only to identify the underlying defect but also attempt to correlate this with the clinical phenotype. As a result of the observations made, analysis of protein expression was used as a diagnostic tool.

Further work was aimed at investigating the function of the Btk protein. This was done by means of retroviral mediated gene transfer of Btk in two different cell systems. The initial system used transfection of Btk into a fibroblast cell line and attempted to study the interaction of Btk with candidate ligands. In a alternative system Btk was introduced into an EBV immortalised B cell line from an XLA patient in order to reconstitute Btk function.

2

Materials and Methods

2.1 Reagents

All reagents were from Sigma Chemical Company Ltd. unless otherwise stated. Recipes for solutions are given in section 2.13. Radioactive isotopes [^{32}P]dCTP, [^{32}P]dATP, [^{35}S]dATP and [^{35}S] Trans label were obtained from ICN Biomedical.

2.2 Nucleic acid preparation and extraction

2.2.1 Preparation of genomic DNA

Cultured cells or white cells from whole blood isolated by lysis of red blood cells were washed in standard PBS solution. To 2-5 x 10^7 cells was added 25mls 0.1% (v/v) NP-40 solution (Calbiochem) and the pellet centrifuged at 15 000*g* at 4°C for 20 minutes. The supernatant was discarded and 3ml nuclei lysis buffer added to the pellet and resuspended. To this was added 200µl 10% (w/v) SDS and 600µl proteinase K solution and the mixture incubated at 60°C for 1.5 –2 hrs. To this was added 1ml of saturated ammonium acetate and the solution shaken vigorously and allowed to stand prior to centrifugation for 20 mins at 15 000*g*. The supernatant was then removed into a separate tube and the DNA precipitated by the addition of 2 vols absolute ethanol. The DNA was removed with a glass spool, dried and dissolved in TE.

2.2.2 Preparation of RNA from blood

Mononuclear cells were separated from blood collected in EDTA coated tubes by Ficoll (Pharmacia) separation. RNA was then prepared from the mononuclear layer using acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and stored in isopropanol at –70°c. Before use, the sample was centrifuged at 12 000g for 15 minutes and the RNA pellet washed in 70% (v/v) ethanol.

2.2.3 Preparation of RNA from tissue culture supernatant

Viral RNA was isolated from tissue culture supernatants by addition of 50µl of 10 x RNA extraction buffer to 0.5ml of supernatant in an Eppendorf tube, which was incubated at 37^{0}C for 2-3 hours. Viral RNA and carrier was recovered from the aqueous phase by phenol extraction and ethanol precipitation as described below.

2.2.4 cDNA synthesis

RNA prepared from a mononuclear cell layer was resuspended in 18µl of diethyl pyrocarbonate (DEPC) treated water and heated to 70°c for 5 minutes. The sample was chilled on ice and the following were added in order: 1µl RNAguard (Pharmacia), 2.5µl 0.1M dithiotheitrol (DTT) (Life Technologies), 10 µl 0.1mg/ml oligo d(T) 12-18 (Pharmacia), 5µl 10mM dNTPs (Pharmacia), 2.5µl 2mg/ml BSA (NBL) and 2ml reverse transcriptase (M-MLV, 200U/µl, BRL). The cDNA was synthesised by incubation at 42°C for 90 minutes, the enzyme inactivated at 65°C for 5 minutes and the sample then stored at –20°C.

2.2.5 Phenol extraction

Aqueous solutions containing nucleic acid, but contaminated with protein, were de-proteinised by extraction with phenol. For extraction of plasmid and genomic DNA, an equal volume of 0.5M Tris-HCl (pH8.0) saturated phenol:chloroform:isoamyl alcohol (24:24:1), was thoroughly mixed with the solution containing nucleic acid, and centrifuged to separate organic and aqueous layers. DNA (and RNA) was precipitated from the aqueous phase. For heavily contaminated solutions, the extraction was repeated.

2.2.6 Precipitation of nucleic acid

Except where indicated, genomic and plasmid DNA were precipitated with 2-2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate pH5.5 at a temperature of 0 to -20^0C. RNA was precipitated with 2.5 volumes of ethanol in the presence of 0.1 volumes of 3M sodium acetate pH5.5. Oligonucleotides were precipitated with 2.5 volumes of ethanol in the presence of 0.1 volumes of 5M sodium chloride. Precipitation of RNA and oligonucleotides was completed overnight at -20^0C.

2.2.7 Quantitation of nucleic acid

Nucleic acid was quantitated using a Philips PU8620 spectrophotometer. An adsorption of 1 OD_{260nm} was taken to equal a concentration of 50μg/ml double stranded DNA, 40μg/ml single stranded DNA or RNA and 33μg/ml oligonucleotides.

2.3 Amplification of plasmid DNA in bacteria

2.3.1 Preparation and transformation of competent cells

Competent bacterial cells (XL1 Blue strains, Stratagene or DH5α cells) were prepared by a variation of Hanahan's transformation method (Sambrook et al. 1989). Cells were grown overnight in LB at 37°C with agitation. A 5ml innoculum of the overnight culture was grown in 130ml of the same media and agitated at 37°C to a density defined by an absorbance at 600nm of 0.3-0.5. The culture was centrifuged for 5 minutes at 6000*g* (4°C). Cells were resuspended in ice cold transformation buffer (TFB) at one third of the original volume, and incubated on ice for 15 minutes before being pelleted as before, and resuspended in ice cold TFB at 0.08 of the original volume. The cells were again left on ice for 5-10 minutes. Freshly prepared cells were mixed with 15% glycerol, and frozen at -70^0C for later use. For transformation, competent cells were thawed on ice, and incubated with plasmid DNA for 20 minutes on ice, before heat shock at 42^0C in a pre-warmed water bath for 90 seconds. SOC medium (0.8ml) at room temperature was added to the mix, and incubated with gentle shaking at 37^0C for approximately 1 hour. Bacteria were concentrated by centrifugation, resuspended in 200μl of the same media, and spread on 1.5% LB-agar plates

(containing 50μg/ml ampicillin unless otherwise stated). Plates were incubated overnight at 37^{0}C to allow for growth of resistant colonies.

2.3.2 Amplification and recovery of recombinant plasmid DNA

Bacterial colonies from agar plates were innoculated into 10ml of media (LB or TB) containing antibiotics to which the recombinant plasmid conferred resistance (usually 50μg/ml ampicillin) and incubated at 37°C with agitation for 12-36 hours. For bulk cultures, 5ml of culture was used to innoculate an additional 100-250ml of the same media, which was incubated as above. Plasmid DNA was recovered by a modified alkaline lysis method. For large scale recovery, nucleic acid was recoverered by alkaline lysis and precipitation with isopropanol. Resuspended plasmid DNA was further purified by precipitation of high molecular weight RNA with 5M LiCl, and digestion of low molecular weight RNA with RNAase (20μg/ml). DNA and contaminating protein was precipitated with an equal volume of ice-cold polyethylene glycol (1.6M NaCl, 13% (w/v) PEG 8000). Plasmid DNA was finally deproteinised by extraction with phenol:chloroform:iosoamyl alcohol, and precipitated from the aqueous phase with salt and ethanol. For small scale preparations, nucleic acid was prepared from alkaline-lysed bacteria by elution from a silica-based resin ('Magic Minipreps', Promega), according to manufacturers instructions.

2.4 Nucleic acid analysis

2.4.1 Polymerase chain reaction (PCR)

2.4.1.1 PCR primers

PCR primers were designed to be approximately 20 nucleotides long with equal proportions of A+T to G+C with a G or C at the 3' end. Primers were synthesised by Mr. P. Rutland, Dept. of Clinical Genetics, Institute of Child Health (ICH), London, at the Imperial Cancer Research Fund (ICRF) London or by Genosys Ltd. UK. The approximate annealing temperature of the primers was calculated according to the equation $T^o = 2n(A+T) + 4n(C+G)$ where n equals number of residues in the primer sequence. The sequences of primers used in this study are listed in sections 2.14.

2.4.1.2 PCR reaction conditions

All PCR amplifications used approximately 50-500ng template DNA, 200μM dNTPs (Pharmacia), 1.5mM $MgCl_2$, buffer and enzyme according to the manufacturer's instructions (Promega and Bioline) and 50pMol of each primer (forward and reverse) in a final volume of 50 μl unless otherwise stated. The reaction mix was overlaid with mineral oil in order to prevent evaporation during the temperature cycling. A control reaction containing all the components except template DNA was always included to check for any contamination. Reactions were carried out by denaturing initially for 3 mins at 94°C, followed by 30 seconds at the calculated annealing temperature, 30 seconds (for products under 750bp) or 1 minute (for larger products) at 72°C and 30 seconds denaturing at 94°C for 30 cycles. A final annealing step was then followed by 10 minutes at 72°C to ensure that all the products had elongated. Reactions were temperature cycled using a Hybaid thermal cycler.

2.4.1.3 Electrophoresis of DNA

PCR products and other DNA products were checked for amplification and product size on 1% agarose (International Biotechnologies Inc.) gels with 500ng/ml ethidium bromide markers using 1X TAE buffer, with 1kb ladder (Life Technologies) as size markers. Gels for Southern blot analysis and preparative gels were prepared at the appropriate concentration and run using the required voltage and time for the separation of the DNA fragments under study with 1kb ladder (Life Technologies) as size markers. Samples were loaded into wells as required using 6 x DNA loading buffer. Gels were photographed on a UV transilluminator. Polyacrylamide gel electrophoresis for SSCP analysis and DNA sequencing is described separately in sections 2.4.2.2 and 2.4.3.2.

2.4.1.4 Isolation of DNA fragments from from agarose gels

In order to isolate a DNA fragment, the sample was first electrophoresed through a 1% (w/v) agarose gel or a 0.8% (w/v) agarose gel with 500ng/ml ethidium bromide in 1 x TAE. The required DNA fragment was then excised from the gel under UV light and then purified away from the agarose using Genclean II (Bio 101) kit. The DNA was then quantitated using spectrophotometry as described in section 2.2.4.

2.4.2 Single strand conformation polymorphism (SSCP) analysis

2.4.2.1 PCR conditions

For SSCP analysis, the Btk cDNA was PCR amplified in seven overlapping sections and digested into small fragments as detailed in Table 2.1. The PCR reaction was performed using 200μM dGTP, dATP, and dGTP and 20μM dCTP (Pharmacia) with 1μCi [^{32}P]dCTP (ICN Flow), 1.5mM MgCl2, 50pMol of each primer (forward and reverse), buffer and enzyme according to manufacturer's instructions (Bioline) and 1μl cDNA in a final volume of 25ml. The PCR was carried out using conditions outlined previously in section 2.4.1.2. Primer sequences are listed in section 2.14. The PCR products were digested by the addition of 10 units of enzyme to the PCR mix and incubation at 37°C overnight. The amplification products were checked on 1% agarose gels and then diluted between two-fold and twenty-fold in 0.1% SDS/10mM EDTA as required to allow even loading on polyacrylamide gels.

2.4.2.2 Non-denaturing polyacrylamide gels

2μl of diluted sample and 2μl of loading buffer were denatured at 95°C for 3 minutes, chilled on ice and loaded onto a 6% polyacrylamide (Accugel, National Diagnostics) non-denaturing gel. Samples were run at 4°C on gels containing 10% glycerol in 1 x TBE or 0.5 x TBE at 30 Watts for 4 hours. Gels were dried and exposed to autoradiograph film (Kodak XAR-5) at room temperature for 13-14 days. Any differences in mobility between patient and control was scored as positive.

Table 2.1 PCR primers and restriction digest sites for SSCP analysis

Reaction	Primers[1]	Position in cDNA[2]	Product size (bp)	Enzyme	Digest site[3]	Fragment sizes
A	A11F E3R	1829-2132	304	*Rsa* I	2023	195 119
B	BS5(F) 11R	1587-1870	284	*Hae* III	1754	168 116
C	AS5(F) 10R	1294-1690	397	*Eco* RI	1453	160 237
D	849F 7R	964-1336	373	*Ava* II	1107	144 229
E	NTSH3F 5R	619-1019	401	*Hae* III	793	175 226
F	A1F 2R	319-664	346	*Hae* III	459	141 205
G	Start F Ex1R	76-379	304			304

[1]F and R indicate forward and reverse primers respectively

[2]Nucleotide position as numbered in Vetrie et al 1993

[3]5' cut site of enzyme

2.4.3 DNA sequencing

2.4.3.1 Sequencing using the 'Sequenase' kit version 2.0 (USB)

Template DNA for sequencing was produced by using PCR amplification of cDNA with one of each pair of primers biotinylated using standard PCR conditions (section 2.4.1.2) with an annealing temperature of 57°C. Single stranded template was separated using M-280 Streptavidin Dynabeads (Dynal) according to the manufacturer's instructions. Sequencing was carried out using the 'Sequenase' kit version 2.0 as detailed in USB protocols.

2.4.3.2 Sequencing gels

Sequencing reactions were run on 6% (v/v) polyacrylamide (Accugel 40, National Diagnostics) 8M urea denaturing gels in 1 x TBE, using wedged spacers and BRL sequencing equipment according to the manufacturers' instructions. Gels were dried on a vacuum drier and exposed to autoradiographic film (Kodak XAR-5) for 1-14 days at room temperature.

2.4.4 Southern blotting

2.4.4.1 Blotting of gels

Agarose gels for Southern blot analysis were incubated in 240mM HCl for 20 minutes to break large DNA fragments by acid depurination and then in denaturing solution for 60 minutes, changing the solution once. The gels were inverted and DNA transferred onto Hybond N+ membrane (Amersham) through a high salt gradient overnight. Membranes were washed thoroughly in 2 x SSC before hybridisation or storage between acid–free tissue paper.

2.4.4.2 Radiolabelling of probes

Probes were radiolabelled by the random priming method (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) as follows: 30-50ng of double stranded DNA in a volume of 30µl was heat denatured at 98°C for 5 minutes, mixed with 10µl OLB, 2µl BSA (10mg/ml), 50µCi [^{32}P]-dCTP (ICN Flow) and 2U Klenow DNA polymerase (NBL) and incubated at room temperature for 3 hours.

2.4.4.3 Removal of unincorporated [^{32}P]-dCTP

A 1ml syringe was plugged with polymer wool, filled with Sephadex G50, packed by centrifugation for 3 minutes at 300*g* and the column washed with 200µl 2 x SSC. The probe labelling reaction was made up to a final volume of 200µl with 2 x SSC, loaded onto the column and centrifuged as before. The activity of the recovered probe was measured by counting 2µl using a Bioscan QC 2000 β counter.

2.4.4.4 Preparation of membranes

Membranes were wetted in 2 x SSC before being rolled into glass hybridisation bottles (Hybaid) with mesh (Hybaid) interleaved

2.4.4.5 Pre-hybridisation of membranes

Probes were denatured at 98°C for 5 minutes and added to 10ml of hybridisation solution at 106dpm/ml. Hybridisation solution was added to membranes and hybridisation was performed at 65°C for 16 hours in a Hybaid oven.

2.4.4.6 Hybridisation of membranes

Membranes were washed three times in 3 x SSC/0.1% SDS at room temperature for 20 minutes and then in solutions with 2 x SSC, 1 x SSC or 0.5 x SSC all with 0.5% SDS, dependent on the probe used at 65°C for 30 minutes. Membranes were then wrapped in plastic film (Saranwrap).

2.4.4.7 Autoradiography

Membranes were exposed to X-ray film (XAR-5, Kodak) at –70°C with two intensifying screens (Lightening Plus, Cronex, Dupont) for between 1 and 14 days. Films were developed on a Fugi RGII film processor.

2.4.4.8 Stripping of membranes

Membranes were stripped of annealed probe by incubating in 2mM EDTA/1mM Tris-HCl (pH8.0)/0.1% SDS at 98°C until the solution had cooled to room temperature. The stripping procedure was checked by exposure to X-ray film as before.

2.5 Enzymatic DNA modification

2.5.1 Restriction enzyme digestion of PCR products

The PCR product was initially precipitated with 2.5 volumes ethanol and 0.1vol 3M NaOAc at –20°C. After centrifugation at 12 000*g* the pellet was washed in 1 x buffer as supplied by the manufacturer. Restriction enzyme digestion of DNA was carried out following the enzyme manufacturers's instructions (NBL or Life technologies) in 1 x supplied restriction enzyme buffer and an excess of enzyme (5-10μg DNA).

2.5.2 Restriction enzyme digestion of genomic and plasmid DNA

Digestion of DNA was carried out in accordance with the enzyme manufacturer's instructions, in 0.5-2 x universal buffer (Stratagene) and an excess of enzyme (5-10 U/μg DNA). For digestion of genomic samples (except for reactions requiring very low salt conditions), 0.5mg/ml BSA and spermidine at a final concentration of 4mM were added to the reaction mix.

2.5.3 DNA ligation

Ligation of DNA fragments was carried out at 4^0C overnight, using T4 DNA ligase and buffer (containing ATP) at concentrations reccomended by the manufacturer. For cloning into plasmids, where possible, non-complementary ends were used to enhance efficiency and determine orientation of the insert. Otherwise, to prevent intramolecular ligation, the vector molecule was pre-treated with calf intestinal alkaline phosphatase (Promega, 1unit per 100pmol of ends) to remove 5'-phosphate groups (37^0C for 30-60 minutes under specified buffer conditions). Where necessary, phosphatase reactions were terminated by phenol extraction, or gel purification. For blunt end ligation, a vector to insert molar ratio of 3 was used. Reaction products were used directly for transformation of bacteria, and successful ligation determined by restriction digestion of purified plasmid DNA.

2.5.4 Blunting recessed ends

5' overhangs were flushed either with Klenow fragment of DNA-polymerase I or T4-polymerase in the presence of an excess of dNTPs (0.2mM), under recommended conditions. For 3' overhangs, in the presence of an excess of dNTPs, the 3' exonuclease activity of T4 polymerase was used to resect the overhang. Universal buffer (Stratagene) was used for all reactions at recommended concentrations.

2.6 Protein preparation and analysis

2.6.1 Antibodies

Table 2.2 Antibodies used: source and type

Specificity	Name/Cat#	Source	Host	Type
Btk	H360B	ICH, London(Katz et al. 1994)	rabbit	serum
Btk	8-E5	NIMR, London	mouse	monoclonal IgG
PKC-β1	sc-209	Santa Cruz Biotechnology (SCB)	rabbit	serum
PLC-γ1	sc-426	SCB	rabbit	serum
PI3 kinase, p85	sc-423	SCB	rabbit	serum
Phosphotyrosine	4G10	Upstate Biotechnology Inc.	mouse	monoclonal IgG
rabbit IgG	A-4914	Sigma	goat	HRP conjugate
Mouse IgG	A-9044	Sigma	rabbit	HRP conjugate

2.6.2 Subjects studied and diagnostic criteria

Thirteen of the fourteen patients studied for protein expression were evaluated and treated at Great Ormond Street Hospital for Sick Children NHS Trust. Local ethical permission was obtained for investigation of these patients. In one patient, studies were performed on an EBV immortalised B cell line. This cell line was a kind gift from Dr. R. Hendriks, Dept of Immunohaematology, University Hospital, Leiden, The Netherlands.

For the protein study in Chapter 4 and for the mutation analysis study performed in Chapter 3 patients were classified into classical and atypical (leaky) phenotypes. In the classical phenotype there was less than 1% B lymphocytes in the peripheral circulation and less than 1g/l of circulating IgG. In the atypical or leaky phenotype tehre was greater the 1% B cells and greater than 1g/l IgG in the peripheral circulation.

2.6.3 Preparation of cell lysates

Mononuclear cells were separated from peripheral blood by density dependent centrifugation using Ficoll (Pharmacia Biotech.). EBV immortalised B cells from Patient 12 were grown in RPMI 1640 (Gibco Life Technologies) supplemented with 10% FCS, 2mM L-glutamine (Gibco Life Technologies), 50u/ml penicillin and 50μg/ml streptomycin (Sigma Chemical Co.) at 37^0C under 5% CO_2. Cultured cells were trypsinised as described in section 2.7.1. and washed in PBS. Cells (0.5-1.0 x 10^7) were washed twice in PBS and lysed in 0.5ml of NP-40 lysis buffer at 4^0C for 10 min. Lysates were clarified using centrifugation and used for western blot analysis or immunoprecipitation with specific antibody

2.6.4 SDS-PAGE analysis

Resolving gels contained 8% (w/v) acrylamide (Protogel, National Diagnostics) in 375mM Tris-HCl, pH 8.8, 0.1% (w/v)SDS. Stacking gels were run on top of 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.

Minigels (8 x 5 cm): precipitates from the equivalent of 10^6-10^7 cells were loaded per lane. Whole cell lysates from the equivalent of 10^5 cells were loaded per lane. Samples were electrophoresed across 100-180V.

Large gels (12 x 17cm): precipitates from the equivalent of 10^7 cells were loaded per lane. Whole cell lysates from the equivalent of 5 x 10^5 cells were loaded per lane. Samples were electrophoresed across 40-50V.

2.6.5 Coomassie brilliant blue (CBB) staining

250mg/L CBB was dissolved in an aqueous solution containing 40% methanol (v/v), 10% acetic acid (v/v) solution. Gels were stained for 30min before destaining with an aqueous solution containing 10% methanol (v/v), 5% acetic acid (v/v). Gels were vacuum dried.

2.6.6 Protein transfer to nitrocellulose filters

Gels were incubated with transfer buffer for 5min (minigels) or 20min (large gels) and transferred onto Electran nitrocellulose membranes (British Drug House) using a semi-dry blotter (Bio-Rad.) according to the manufacturers' instructions. As a general rule, minigels were transferred at 20 min at 12V and large gels for 50 min at 18V.

2.6.7 Immunoblotting

The membranes were blocked with 5% (w/v) non fat milk/PBS for 1 hour at room temperature and then washed briefly in PBS with 0.05% Tween (PBS-T). Membranes were then incubated with the primary antibody of choice at the appropriate dilution in PBS-T with 3% (w/v) non-fat milk. Filters were washed 3x in PBS-T prior to incubation with horse radish peroxidase (HRP)-conjugated secondary antibody in PBS-T with 3% non-fat milk at room temperature for 45mins to 1 hour. A 1/1000 dilution of goat anti-rabbit IgG HRP was used for detection of primary rabbit polyclonal antiserum and a 1/1000 dilution of rabbit anti-mouse IgG HRP was used for detection of primary mouse monoclonal antibody. Filters were then washed again 5x in PBS-T Antigen/antibody complexes were visualised using the enhanced chemoluminescence detection system (Amersham). Reactive species were visualised by exposure to X-ray film for between 5s to 15mins.

2.6.8 Stripping of nitrocellulose filters

Filters were stripped for reblotting by incubating the nitrocellulose filter in 100ml of 100mM 2-β-mercaptoethanol (Sigma Chemical Co.), 62.5mM Tris-HCl (pH 6.7) and 2% SDS at 50^0C for 30min and then washing in PBS.

2.6.9 Immunoprecipitation

All steps were carried out at 4°C. Clarified whole cell lysates were incubated with 5µl of appropriate antiserum for 1 hour.10µl of Gammabind beads (Pharmacia Biotech.) was added and incubated for a further hour. Gammabind beads with attatched antibody-protein complexes were recovered by centrifugation and then washed three times in ice cold lysis buffer. Antiserum immunoprecipitates were analysed by immunoblotting as described above.

2.6.10 In vitro kinase assay

Gammabind beads washed in lysis buffer were further washed once in PBS, twice in 0.5M LiCl/20mM Tris-HCl, pH 8.0, and once in kinase buffer (10mM $MnCl_2$/10mM $MgCl_2$). Pelleted beads were then resuspended in 30ml kinase buffer with 5-10µCi [^{32}P]-γATP and incubated for 20min at room temperature. SDS sample buffer was added to the reaction mixture and the samples boiled before SDS-PAGE analysis. Incorporated phosphate was visualised by autoradiography of dried gels.

2.7 Tissue culture

2.7.1 Culture of cell lines

Unless otherwise indicated, all suspension cell lines were grown in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) FCS (Globepharm), 2mM L-glutamine (Life Technologies), 100iu/ml penicillin, and 100µl/ml streptomycin (Life Technologies) at 37°C under 5% (v/v) CO_2. Adherent cell lines were grown in DMEM (Life Technologies) supplemented as above at 37°C under 10% (v/v) CO_2. Trypsin-EDTA (Gibco) was used to loosen adherent cells.

2.7.2 Long term storage of cells

Cells were frozen at $2x10^7$/ml in growth media with 20% (v/v) FCS and 10% (v/v) DMSO by first cooling slowly to -70°C in a polystyrene box overnight and transferring

to liquid nitrogen for long term storage. To thaw, cells were warmed quickly at 37°C, washed and pelleted by centrifugation and resuspended in the appropriate medium.

2.8 Cell transfection

2.8.1 Calcium phosphate transfection

6-16 hours before transfection, adherent cells were trypsinised and replated at a density of $5x10^5$-$5x10^6$ per 9cm tissue culture dish (depending on cell type). For transfection, 10-30μg of plasmid DNA was mixed with sterile ddH2O to a volume of 437.5μl and mixed with 62.5μl of sterile 2M $CaCl_2$. This was added dropwise to 0.5mls of sterile 2 x HBSS (280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES (sodium salt), pH7.05+/–0.05) through which a constant stream of air was bubbled. The mixture was allowed to stand for 20-30 minutes, then applied dropwise to the cell culture medium, and incubated at 37^0C for 4 hours to overnight. Formation of a fine precipitate was noted by light microscopy. Cells were then washed three times with PBS and fed with fresh medium.. For modified calcium phosphate transfection (Chen and Okayama, 1987), 10-30μg of plasmid DNA was mixed with 0.5ml of 0.25M $CaCl_2$, and added to 0.5ml of 2 x BBS (2 x BES-buffered saline, containing 50mM BES pH 6.95, 280mM NaCl, 1.5mM Na_2HPO_4). This was incubated at room temperature for 20 minutes, added dropwise directly to the cell culture medium (DMEM supplemented with 10% FCS (v/v) and antibiotics, and incubated at 37^0C at 2-4% CO_2 overnight. The next day, cells were refed with fresh medium and incubated under normal conditions.

2.8.2 Liposome mediated transfection

Liposomal transfections were carried out according to manufacturers instructions (Lipofectin, Gibco). Briefly, equal volumes of serum and antibiotic-free medium (Optimem, Gibco) were mixed with DNA or liposomes at an optimised ratio of between 1:2 and 1:10 respectively. The combined mixture was incubated for 20 minutes at room temperature, and added directly to the cells (adherent or non-adherent) for a period of 4 hours to overnight. Cells were refed with complete medium after transfection.

2.9 Production and titration of retroviruses

2.9.1 Production of replication defective retrovirus

Retrovirus producer cell lines were created by calcium phosphate transfection of subconfluent (30-50%) packaging cell lines (GP+envAm12, ψ-2) with 10μg of vector plasmid per 9cm plate. After 48 hours, cells were harvested and replated at 1-20% density in fresh medium to which puromycin was added (2μg/ml). After 14-21 days, colonies were isolated by ring cloning, and passaged from 6 well plates, eventually to 9cm dishes. Cells were then grown in the absence of drug selection. When confluent, cells from each clone were refed with fresh medium for 12-24 hours, after which time the medium was harvested and frozen at -70^0C, and cells frozen in liquid N_2. For a polyclonal producer cell line, the cloning steps were ommitted.

2.9.2 Titration of replication defective retrovirus

To titre virus stocks by expression of drug resistance, frozen supernatants (1-100μl) were thawed and used in dilution to infect 10^5 3T3 cells freshly plated in 6 well plates, in the presence of polybrene (2-4μg/ml). After 48 hours, cells were split into fresh medium containing puromycin (2μg/ml), at dilutions of 1:10 to 1:1000. Titre was estimated by growth of resistant colonies. High titre clones were thawed and expanded in selection as before for use in further experiments.

2.10 Cell transduction

2.10.1 Transduction of cells in suspension

EBV-LCLs B cells, UD108 cell line, were grown in culture medium, pelleted, washed in serum free medium and resuspended in medium to a concentration of 5 x 10^5cells/ml. Producer cells were grown in six well plates to 50-70% confluence and the DMEM medium removed. The UD108 cells were then overlaid onto the producer cell line in 2-4μg/ml of polybrene for 48hrs of cocultivation at 37°C and 5%CO_2. After this time UD108 cells were removed and resuspended in fresh medium. Cells were then passaged regularly (every 2-4hrs) in order to remove any adherent producer fibroblasts. After cells had been allowed to expand, puromycin was added to the medium in increasing concentrations to allow selection of successfully transduced cells.

2.10.2 Transduction of adherent cells

Transduction of the adherent fibroblast cell line NIH3T3 was carried out by repeated infection with viral supernatant. Medium from dividing producers was harvested and passed through a 0.45μ filter (Millipore). NIH3T3 cells were grown to 50-70% confluence and repeated 1ml aliquots of freshly harvested supernatant was added to the flask at regular (4-6hrly) intervals with 2-4μg/ml of polybrene. After 48 hours, cells were grown to confluence and then split 1:5 into 9cm flasks. Puromycin at a concentration of 1μg/ml was initially added to growing cells and then increased to 2μg/ml. Cells were then subsequently grown in puromycin selection.

2.11 Measurement of of β-galactosidase expression

2.11.1 X-gal staining of fixed cells

Transduced cells were removed from the producer cell layer, washed and resuspended in fresh medium. Cells were then fixed onto glass slides by cytospin. Cells were then fixed for 15 minutes in 0.5% glutaraldehyde, and washed twice with PBS. Sufficient X-Gal solution (2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% (v/v) NP40, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 0.1% X-gal was added just before use) was added to cover cells, which were incubated for 1-4 hours at 37^{0}C. Cells were washed, and staining visualised by light microscopy.

2.11.2 Measurement of β-galactosidase activity in cell extracts

β-galactosidase was detected in cell extracts by colourimetric assay for enzyme activity (Promega) according to the manufacturer's instructions.

2.12 FACS staining of cells

Approximately 10^6 cells were used for staining with first step antibodies at a concentration of ~1μg/ml in PBS, with 0.05% BSA, 0.02% sodium azide for 30 minutes at 4°C. Cells were washed twice in the same buffer and then incubated with fluorescent isothiocyanate (FITC) conjugated anti-goat $F(ab)_2$ fragments. Samples were analysed by flow cytometry using a FACSCalibur.

2.13 Buffers

Double distilled water (ddH_2O) was used to prepare all solutions. Solutions were autoclaved at 121°C for 20 minutes unless otherwise stated.

Ampicillin stock 50mg/ml in water, filter sterilised, working concentration 50μg/ml.

BBS (2x) 50mM BES, pH 6.95, 280mM NaCl, 1.5mM Na_2HPO_4.

Denaturing solution

1.5M NaCl, 0.5M NaOH.

Denhardts solution (100x)

20g Ficoll 400 (Pharmacia), 20g polyvinylpyrrol-idone, 20g BSA (Fraction V), water to 1 litre. Sterilised by filtering and stored at –20°C.

DEPC-treated water

DEPC added to 0.1% (v/v), incubated overnight at room temperature and autoclaved.

DNA loading buffer (6x)

1.5g Ficoll in 10ml water, bromophenol blue, xylene cyanol.

DNA lysis buffer 10mM Tris-HCl pH8.0, 5mM EDTA, 0.5% (w/v) SDS, 150μg/ml proteinase K added just before use.

HBSS (2x) 280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES (sodium salt), pH7.05+/–0.05.

Hybridisation solution

('Starks') 1 x Denhardts, 150μg/ml sonicated salmon sperm DNA (denatured at 98°C for 5 minutes), 5 x SSC, 0.5% SDS, 50% deionised formamide, 20mM phosphate buffer.

Immunoblotting blocking buffer

PBS-T with 5% (w/v) non-milk fat (Marvel)

Kinase buffer 10mM $MgCl_2$, 10mM $MnCl_2$

LB (Luria-Bertani) bacterial growth medium

10g Bactotryptone (Difco), 5g bacto yeast extract (Difco), 10g NaCl, water to 1 litre. 15g/litre bacto-agar (Difco) added for LB agar plates.

NP-40 lysis buffer 1% (v/v) NP40 (Calbiochem), 20Mm Tris-HCl, pH 8.0, 130mM NaCl, 10mM NaF, 1mM DTT, 20μM leupeptin, 1% aprotinin, 100 μM Na_3VO_4, 1mM PMSF

PBS Phosphate buffered saline tablets (Oxoid) were dissolved 1/100ml water

PBS-T PBS with 0.05% (v/v) Tween-20

Protein transfer buffer

48mM Tris-HCl, 39mM glycine (Calbiochem), 20% (v/v) methanol (BDH) pH 9.2

SDS protein sample reducing loading buffer (2x)

The following were added to 40ml of ddH_2O: 1.52g Tris base, 20ml glycerol, 2g SDS, 2ml 2-mercaptoethanol, 1mg bromophenol blue (BDH). 1M HCl was added until the pH was 6.8 and the solution made up to 100ml with ddH_2O.

Sephadex G50 10g Sephadex G50 (Pharmacia) was incubated in 100ml 2 x SSC at 65°C for 3 hours, replacing the supernatant with new SSC during the incubation. Stored at 4°C.

SOB medium 20g bactotryptone (Difco), 5g yeast extract (Difco) and 0.5g NaCl dissolved in 1 litre of water/25mM KCl/10mM $MgCl_2$, pH 7.0

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

SSC (20x) 3M NaCl, 0.3M sodium citrate.

SSCP/Sequencing loading buffer

95% formamide, 20mM EDTA, 005% bromophenol blue and 0.05% xylene cyanol (BDH)

Terrific broth 12g bactotryptone, 24g bacto-yeast extract, 4mls glycerol added to 900 mls H_2O, sterilised by autoclaving. When cool, 100 mls of autoclaved 0.17M KH_2PO_4, 0.72M K_2HPO_4 was added.

TAE (50x) 0.2M Tris base, 1M glacial acetic acid (BDH), 50mM EDTA, pH8.0.

TBE (10x) 0.9M Tris base, 0.9M Boric acid, 1mM EDTA, pH 8.0.

TD buffer 8g NaCl, 0.38g KCl, 0.1g Na_2HPO_4, Tris HCl, pH 7.8.

TE (pH8.0) 10mM Tris-HCl, pH8.0, 1mM EDTA.

Transfer buffer 48mM Tris-HCl, pH9.2, 39mM glycine, 20% methanol (BDH).

X-Gal 20mg/ml in dimethylformamide, stored at -20°C.

X-Gal solution 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 0.1% X-Gal added just before use.

2.14 PCR primers

2.14.1 Btk gene primers

Table 2.3 Primer sequences used for Btk gene PCR and sequencing

Name	Sequence	5' position	Comments
Start F	AGCTACCTGCATTAAGTCAG	76	Bio[1]
A1F	TGTTTGAAACAGTGGTTCC	319	
3F	AGAAGAGGTGAAGAGTCCAG	373	
NTSH3F	AATTCGGATCCATGGGCTGCCAAATTTTGGAG	619	BamHI
849F	ATGTATGAGTGGTATTCCAAACAC	964	
EX6F	CGTCATTATGTTGTGTGTTCCAC	1126	
AS5(F)	GCAGGCCTGGGATACGGATC	1294	Vetrie et al 1993c
BS5(F)	CCTGAGGGAGATGCGCCAC	1587	Vetrie et al 1993c
A11F	CGGAAGTCCTGATGTTATAGC	2829	
EX1R	CTCTTCTCGGAATCTGTCTTTC	379	
4R	TTTGAGCTGGTGAATCCACC	519	
2R	CAGGTTTTAAGCTTCCATTC	664	
SH3NTR	AATCAGAATTTTCCTTTTTCAGCTCACTTGTGG	787	EcoRI[2]
5R	TTTAGCAGTTGCTCAGCCTG	1019	Bio[1]
847R	GAGACACTGGATATTTGAGCCTGG	1267	
7R	GGTCCTTTGGATCAATTTCC	1336	
A8R	TCATCTTCAGACATGGAGCC	1454	
10R	GGTGAAGGAACTGCTTTGAC	1690	
A3R	GGAAATTTGGAGCCTACTGAG	1811	Bio[1]
11R	TGTCAGATTTGCTGCTGAAC	1870	
E3R	CAAGAAGCTTATTGGCGAGC	2132	
ERB2	ATTGAGTGGGAGCACAAAGG	2224	Bio[1]

[1]Biotinylated at the 5' end

[2]Enzyme site incorporated into the 5' end of the primer sequence

2.14.2 Retroviral primers

Table 2.4 Primer sequences used for PCR retroviral construct

Name	Sequence[1]	Position
MLVF1	CGCAACCCTGGGAGACGTCC	31-50
MLVB5	CGTCTCCTACCAGAACCACATATCC	140-164

[1]from Gerard et al. 1996

3

Btk mutation analysis in XLA

3.1 Introduction

Following the identification of Btk as the gene defective in XLA, a number of groups embarked upon a plan of investigation to look at the pathogenesis of XLA at the molecular level. In most cases the initial studies focused upon the detection of mutations in affected individuals. The relative ease of access to patient material, the documentation of methods for gene mutation analysis and the benefits of results to both patients and investigators alike, make mutation analysis a natural starting point for these investigations.

3.1.1 Importance of mutation analysis

The analysis of mutations in individuals with XLA is important for a number of reasons. A significant proportion of patients with the clinical and laboratory findings of XLA have no family history of the disorder. In these individuals this may be as a result of a sporadic mutation in Btk. Detection of a mutation in Btk confirms the diagnosis of XLA. More recently it has been shown that mutations in other molecules involved in B cell signalling and differentiation can also lead to a clinical phenotype very similar to XLA (Yel et al., 1996; Minegishi et al., 1998), thereby underlining the importance of mutation analysis in making a molecular diagnosis. The analysis of mutations also allows for improved carrier detection and prenatal diagnosis in affected families. Although other methods are available for genetic counselling, the identification of a specific mutation in the relevant gene is the most reliable.

Individuals with XLA can be regarded as natural 'knockouts' of the Btk gene. The identification and characterisation of mutations in such individuals may help in understanding the function and role of Btk. Missense mutations and other small in frame mutations may also help to define specific residues and domains that are critical for Btk function. Furthermore by studying a large number, of individuals including those with 'leaky' or atypical phenotypes, it may be possible to correlate the genetic defect with the clinical phenotype. It has also been suggested that as new approaches to therapy for XLA are developed, the choice of appropriate treatment may be dependent upon the nature of the specific mutation in the patient (Conley et al., 1994b). Certain mutations may have a dominant negative effect and would therefore be less amenable to gene transfer therapy than those with a null mutation in Btk.

3.1.2 Previous Btk mutation analysis

At the beginning of this study only very few mutations in Btk had been documented. In the original report of Btk as the gene defective in XLA, Vetrie et al. (1993) found deletions of genomic DNA in the XLA locus in five families with an XLA pedigree. Additionally they found altered *Taq* 1 digest restriction patterns in two patients with XLA by Southern blot analysis. These alterations were shown to be co-inherited with XLA in the affected family. Further analysis of the two patients identified single amino acid substitutions in the kinase domain of Btk. In patient A there was a G→A substitution at position 1706 leading to Arg 525→Gln and in patient B an A→G substitution at position 1420 resulting in Lys 430→Glu. Both mutations were postulated to eliminate kinase activity of the mutant protein product.

3.1.3 Mutation analysis

A study of mutation analysis in XLA was undertaken. At the start of this study the genomic structure of Btk was not known and therefore genomic DNA could not be used for analysis. However the expression of Btk in cells of the myelomonocytic lineage allowed the isolation of messenger RNA from peripheral mononuclear cells in patients. The study was therefore performed on Btk cDNA.

cDNA from 14 patients with a suspected diagnosis of XLA was obtained. In twelve of the fourteen cases, the samples were received from other Institutions. Eight patient samples were from the Hospital for Sick Children, Toronto, Canada, three from Northwick Park Hospital, London, U.K., and one, an EBV immortalised B cell line (EBV-LCL), from the Schering-Plough Centre de Recherche, Dardilly, France.

The definitive way to detect mutations would be to sequence the entire coding region of the gene. However, with a large gene and numerous samples, a method to screen the gene for mutations is more appropriate. The mutation can be localised to a small region of the gene, which can then be directly sequenced to define the mutation. The screening method chosen for this study was single stranded conformation polymorphism (SSCP) analysis (Orita et al., 1989).

3.1.4 Use of SSCP analysis

Since Orita et al. (1989) first reported the use of SSCP analysis to detect mutations in DNA, it has become the most widely used of the scanning technologies. SSCP works on the principle that single stranded DNA under denaturing conditions, will form a secondary structure which is sequence dependent. Analysis of denatured double stranded DNA on polyacrylamide non-denaturing gels allows the single strands to migrate, not only according to their size but according to their sequence dependent secondary structure. Single strands can be visualised by radioactive labelling or alternative detection methods. A single base change in a fragment up to 400bp can alter the secondary structure resulting in a change in the pattern of migration. This is detected as a shift in one or both of the single strands in comparison to control samples and thus localises the sequence alteration to that region of the gene. This area must then be sequenced to define the exact nucleotide alteration.

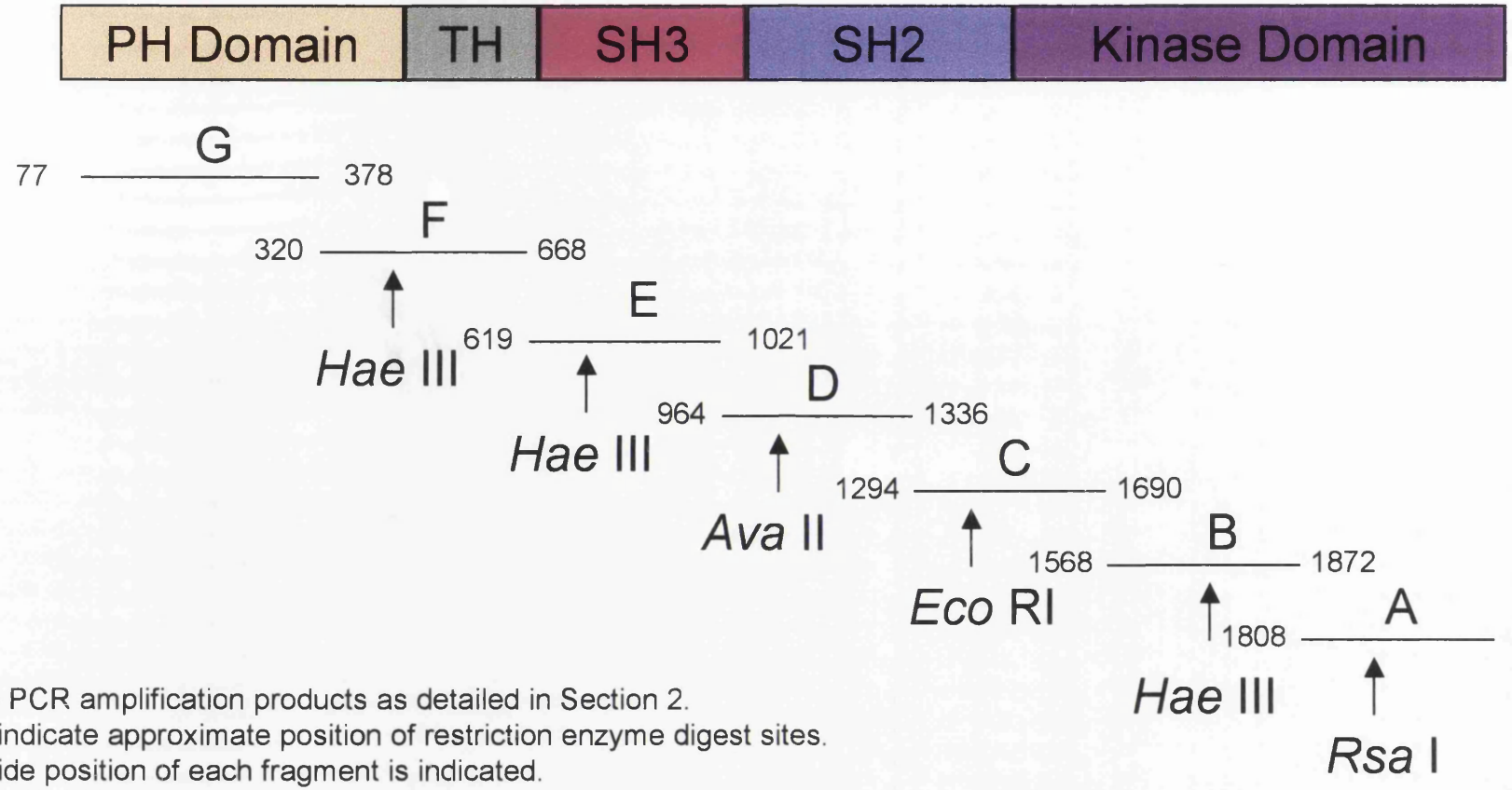
Experience with SSCP suggests that there is a mutation pick up rate of ~ 80-90% in any particular gene (Fan et al., 1993; Sheffield et al., 1993). As the technique relies on the formation of secondary structures sensitive to sequence changes, it does not identify 100% of mutations and this should be borne in mind when undertaking studies of this type. The sensitivity of the technique depends upon a number of factors. The size of the fragment under analysis is crucial, with 150 bp having been reported as the optimum size (Sheffield et al., 1993). Increasing size of fragment

generally gives a lower sensitivity but size limitations can be overcome by restriction enzyme digestion of the amplification product prior to electrophoresis. As the technique is dependent upon the conformations formed by single strands in the gel, it is sensitive to the physical environment of the gel. Thus the temperature, buffer concentration, cross-linking of acrylamide and concentration of acrylamide have all been shown to affect the sensitivity of this technique (Liu and Sommer, 1994; Ravnik et al., 1994). Prior use of the technique in this laboratory suggested that room temperature gels with 5% glycerol and 0.5 x TBE with additional runs at 4°C and 1 x TBE provided a good level of sensitivity.

3.1.5 SSCP analysis of Btk

The analysis of mutations in Btk had already been initiated in this laboratory prior to the start of this particular study. Thus the primers for amplification of fragments in Btk had been designed and the conditions for their use determined. These are described in Bradley et al. (1994). In summary the ~2kb coding region of Btk cDNA was amplified into seven fragments using seven pairs of overlapping primers (see Fig. 3.1). Each fragment was then digested by restriction enzymes into two smaller fragments which were of a size that would optimise SSCP analysis. Restriction sites were also designed such that there was a greater than 50 bp difference in length between the two fragments so that the single strands could be unambiguously separated on the gel (see Table 2.1 in Materials and Methods). Samples from each amplification were run at room temperature and at 4°C to maximise the sensitivity of the technique. Undenatured and denatured double stranded DNA from a normal control was run on every gel to allow identification of the normal single strands. Any difference in mobility between patient and control samples was scored as positive. In all cases, if a band shift was found, all the other reactions across the DNA were performed and found to be normal, indicating that there was only one mutation in each patient.

Fig 3.1 Diagrammatic Representation of SSCP analysis for the *BTK* gene



A-G are PCR amplification products as detailed in Section 2.
Arrows indicate approximate position of restriction enzyme digest sites.
Nucleotide position of each fragment is indicated.

3.2 Mutation analysis in Btk

3.2.1 Mutations detected

cDNA analysis of Patient UD showed a band shift in SSCP reaction G. This amplification reaction was not normally digested prior to being run. The initial band shift detected was unclear and therefore the PCR product of reaction G was digested with *Hha* I at nucleotide 215. A clear band shift was seen in the 3' fragment. Sequence analysis of this region showed a missense point mutation A→C at nucleotide 229 (Fig 3.2). This results in a Thr33→Pro amino acid substitution. EBV-LCLs were obtained from this patient (the UD 108 cell line) and it was possible to show by western blot analysis that this mutation results in the loss of Btk protein (Fig 3.2). More sensitive protein analysis by immunoprecipitation of Btk protein with anti-Btk antisera and autophosphorylation assay did not show the presence of any Btk protein (see chapter 7).

Three patients JT, JJP and BD all showed band shifts in both the SSCP reactions G and F (band shift in reaction F shown in Fig 3.3a). This narrowed the site of the mutation to the overlapping region between the two PCR reactions (nucleotides 339-357). The band shifts in all three were of exactly the same pattern and the mutation in all three individuals was shown to be the insertion of an A into a run of seven A's in the PH domain (n 341-n347) (Fig 3.3b). This mutation would result in the Btk protein being translated out of frame from residue 72 onwards, with premature termination at residue 84.

The families of these individuals were not known to be related to one another. Further evidence for this came from the study of an SSCP polymorphism in PCR reaction A. Patients BD and JJP exhibit this band shift whereas patient JT does not (Fig 3.4). Although this evidence cannot confirm that all three families are unrelated, it does nevertheless confirm the presence of at least two different pedigrees.

Patient KK showed a significant lower band shift in the larger 3' fragment of PCR reaction E (Fig 3.5a). Sequence analysis of this region showed a large deletion. A 63 bp fragment corresponding to the entire coding region of exon 9 was shown to be deleted (Fig 3.5b) leaving the rest of the coding region of the Btk cDNA in frame.

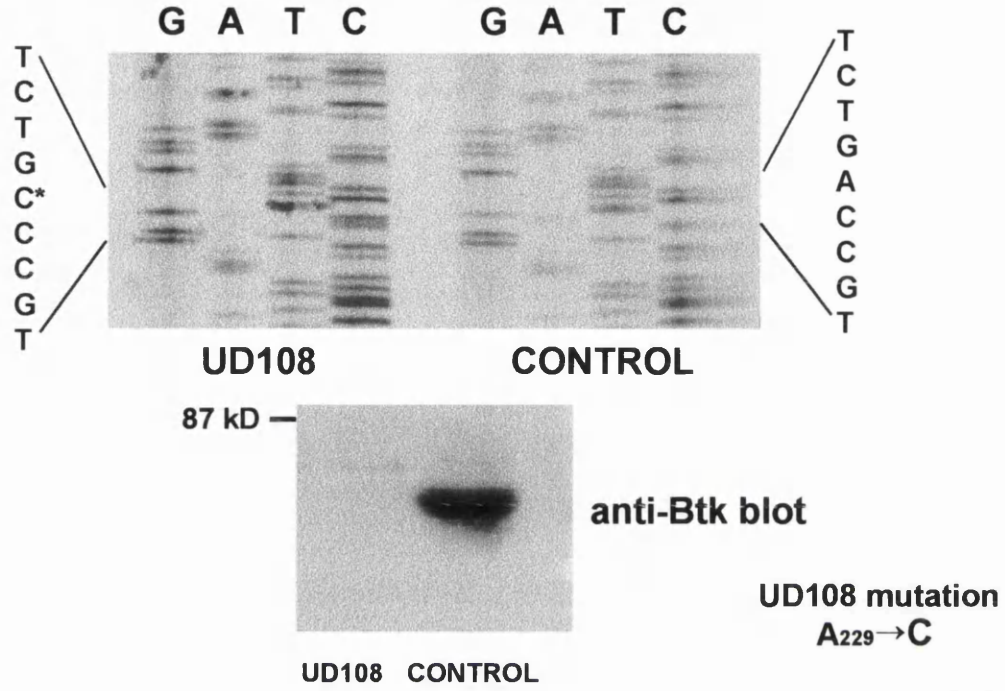


Fig. 3.2 Mutation detection in patient UD108. *Patient UD108 showed a band shift in SSCP reaction G. Sequence analysis of this region showed a missense substitution at nucleotide 229. Western blot analysis of EBV-LCLs from this patient immunoblotting with anti Btk antisera shows absence of Btk protein expression.*

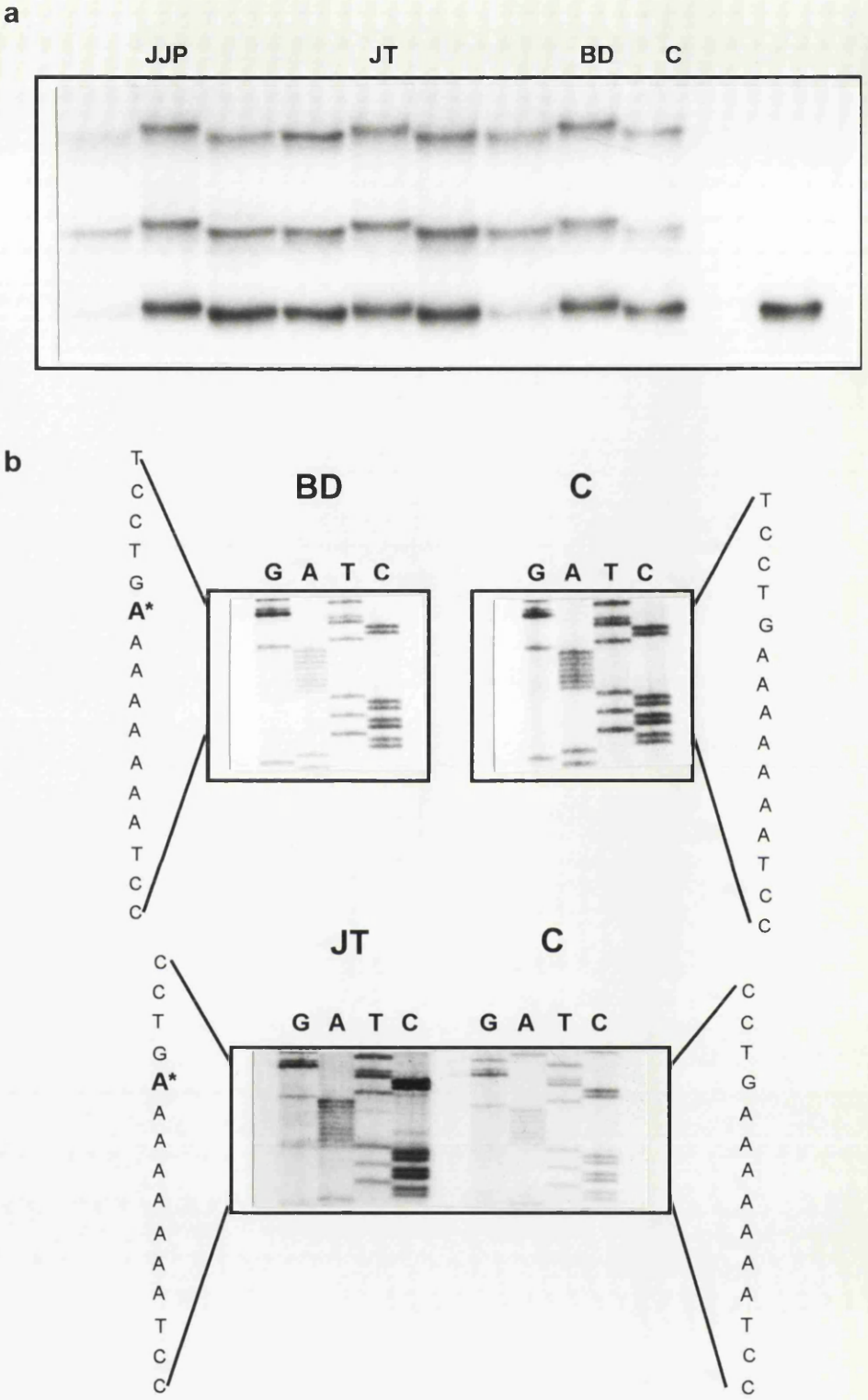


Fig. 3.3 Detection of mutation in patients JJP, JT and BD. a) *All three patients show an upper band shift in SSCP reaction F in comparison to a normal control (C) and other patient samples.* **b)** *Sequencing of this region in patients BD and JT shows an insertion of an A in a run of seven As at nucleotides 341-347. A similar change was found in patient JJP (not shown).*

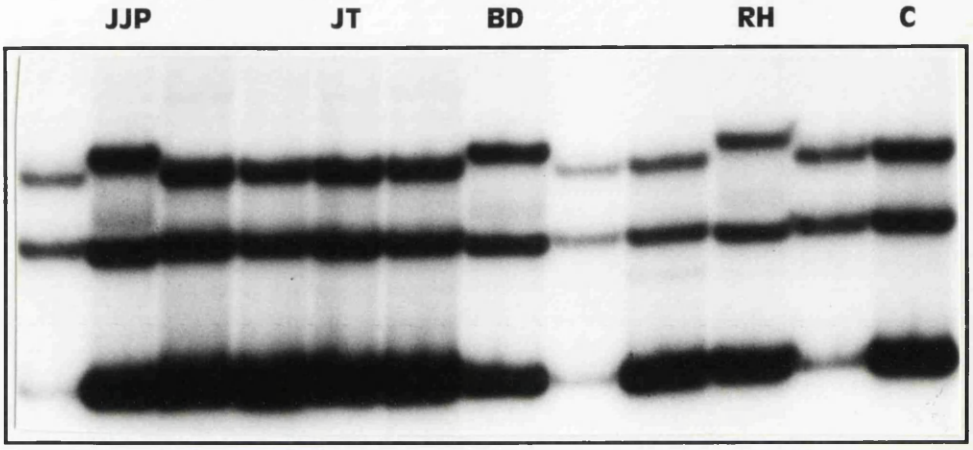


Fig 3.4 Analysis of SSCP polymorphism in Btk gene. *An SSCP polymorphism seen as an upper band shift in reaction A is present in* Btk *as a result of a TGT→TGC change at codon 633 (Bradley et al. 1995). Patients BD and JJP exhibit this band shift whereas patient JT , other patients and control sample (C) do not. Another patient, RH, also shows this polymoprhism*

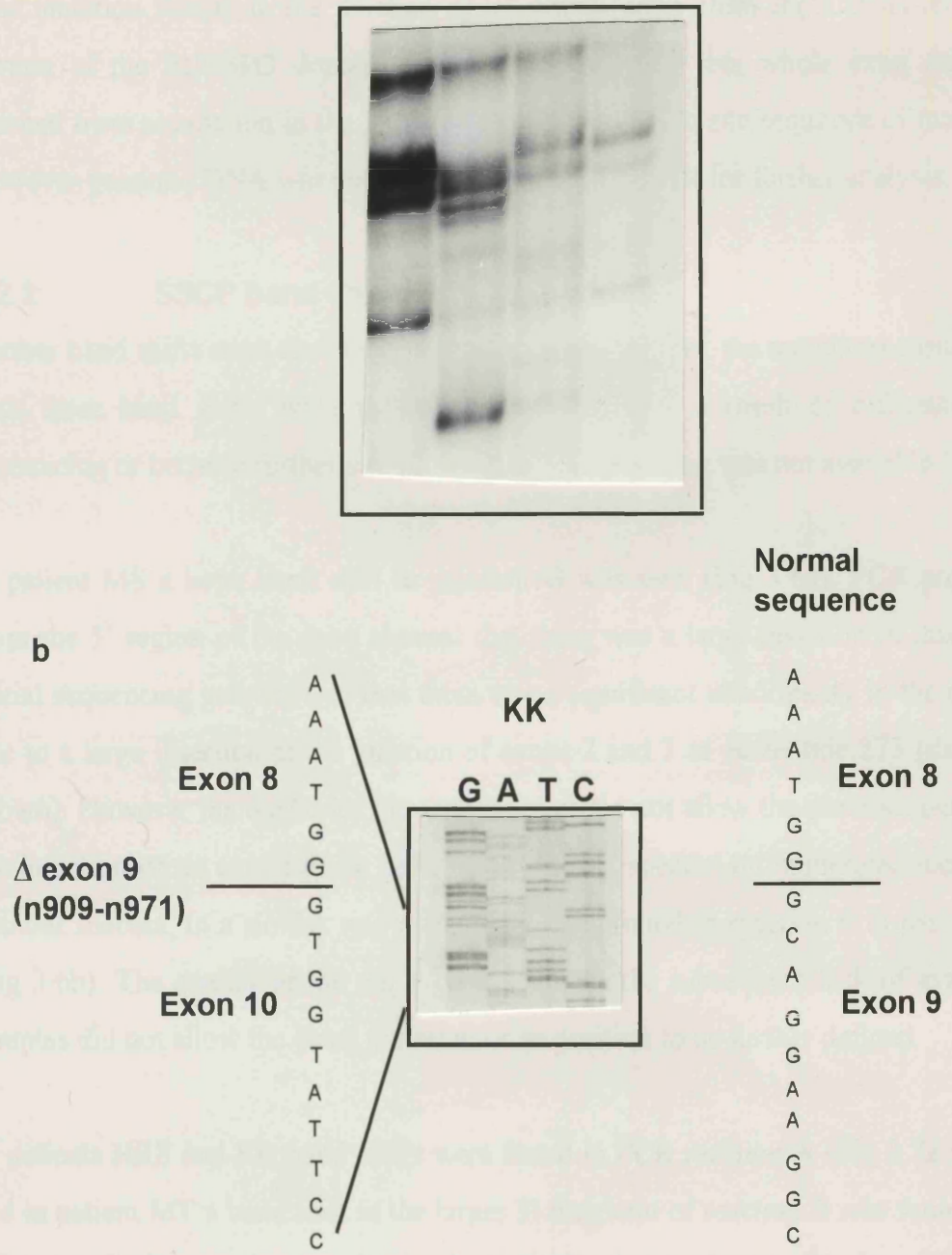


Fig. 3.5 Deletion of exon 9 in patient KK. a) *Patient KK shows a lower band shift in SSCP reaction E in comparison with a control sample (C) and other patients.* **b)** *Sequence analysis showed the deletion of 63 nucleotides which corresponds to the entire coding region of exon 9.*

This mutation results in the deletion of 21 amino acids from the COOH-terminal portion of the Btk SH3 domain. It is most likely that this whole exon deletion resulted from a mutation in the 5' splice donor recognition site sequence of intron 9. However genomic DNA was not available from this patient for further analysis.

3.2.2 SSCP band shifts

Further band shifts were found on SSCP analysis. However, the mutations associated with these band shifts were not determined either as a result of difficulties in sequencing or because further sample cDNA for sequencing was not available.

In patient MS a large band shift in reaction G was seen (Fig 3.6a). PCR products from the 5' region of the gene showed that there was a large insertion in this area. Initial sequencing gels showed that there was a significant abnormality in the cDNA due to a large insertion at the junction of exons 2 and 3 at nucleotide 273 (data not shown). However the quality of the sequence gel did not allow the exact sequence of the insertion or its extent to be defined. This was repeated on numerous occasions without success. In a similar way a band shift was found in reaction F in patient ID (Fig 3.6b). The quality of the sequencing gels and the subsequent lack of available samples did not allow the exact mutation or its position to be further defined.

In patients HRS and RB band shifts were found in PCR reaction A (Fig 3.7a and b) and in patient MT a band shift in the larger 5' fragment of reaction B was found (Fig 3.7c). In all these patients, PCR products were of normal size, thereby giving no indication of whether there was a significant deletion or insertion mutation. Sequencing of the appropriate section of the gene in these patients was attempted on numerous occasions without any success. The lack of available cDNA samples eventually led to further attempts being abandoned.

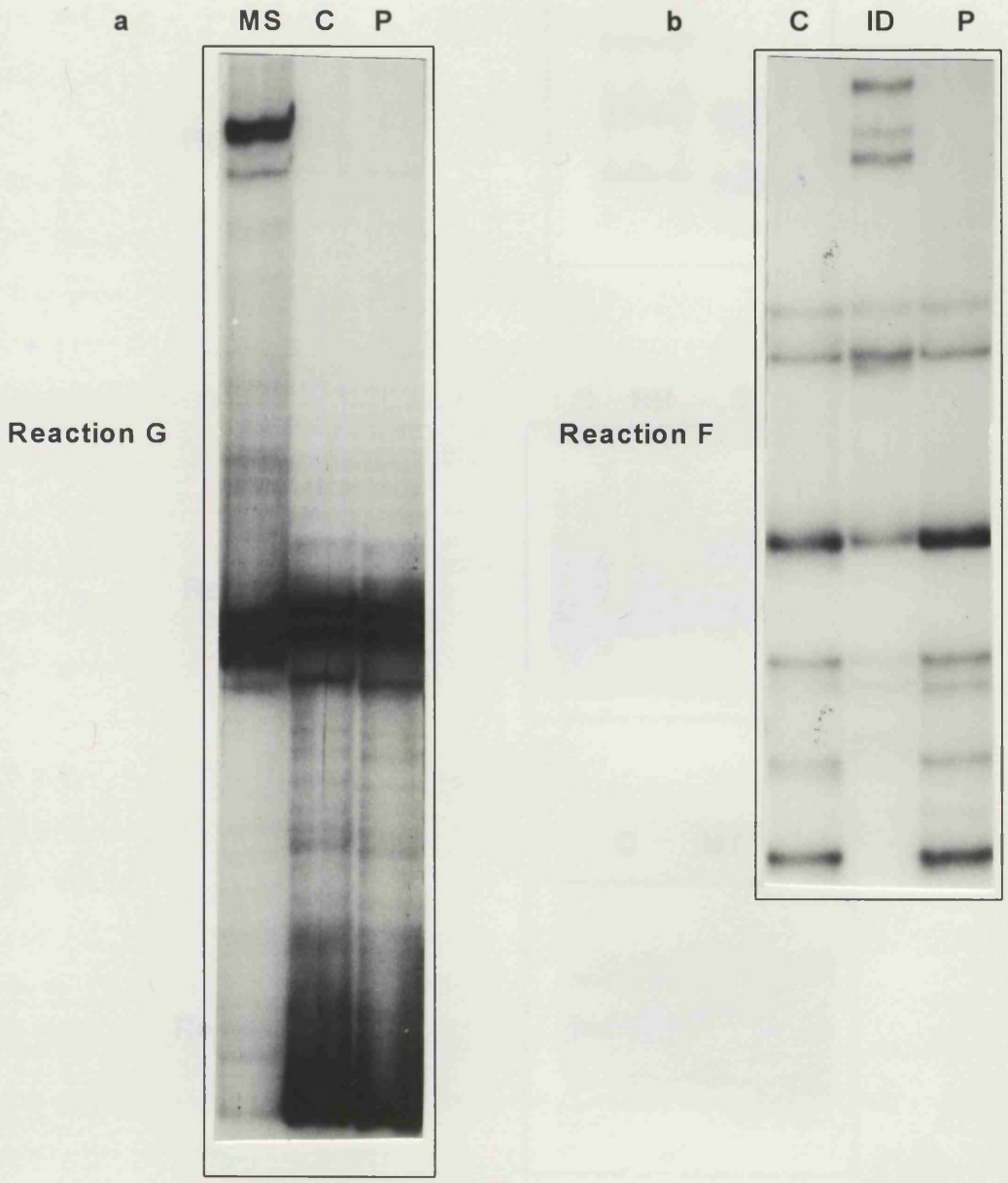


Fig. 3.6 SSCP band shifts in reactions F and G. a) *Patient MS shows a large band shift in reaction in comparison to a control sample (C) and another patient (P)* **b)** *Patient ID shows a large upper band shift in SSCP reaction F.*

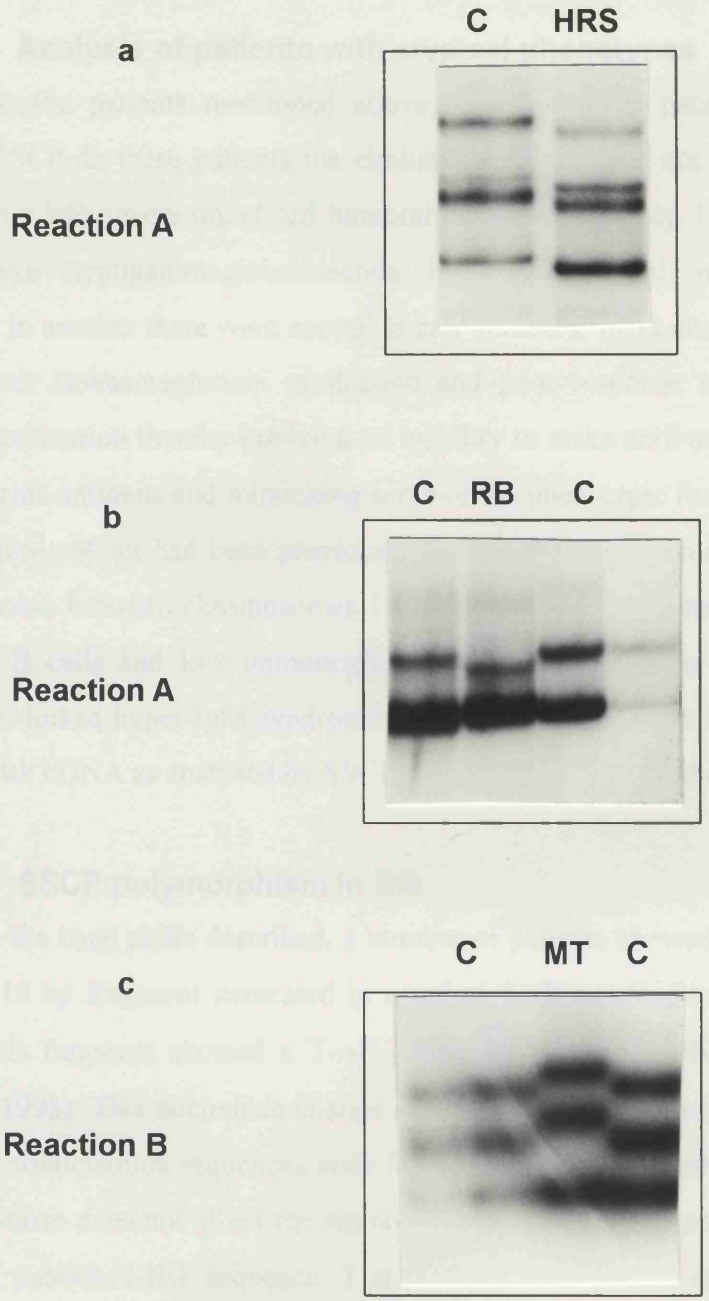


Fig. 3.7 SSCP band shifts in reactions A and B. **a)** *Patient HRS shows a lower band shift in SSCP reaction A in comparison with a control sample (C)* **b)** *Similarly Patient RB shows a lower band shift in the same reaction* **c)** *Patient MT shows an upper band shift in SSCP reaction B in comparison with control samples.*

3.2.3 Analysis of patients with atypical phenotypes

In addition to the patients mentioned above, a further four patients were also analysed by SSCP. In these patients the clinical phenotype was not consistent with XLA but with a less severe undefined humoral immunodeficiency. One patient was found to have hypogammaglobulinaemia but with normal numbers of B lymphocytes. In another there were normal B cell numbers, IgG2 and IgG4 subclass deficiency, poor isohaemaglutinin production and poor response to Haemophilus influenza B vaccination thereby implying an inability to make antibodies in response to polysaccharide antigens and mimicking some of the phenotypic features of the *xid* mouse. The third patient had been previously shown to have Down's syndrome due to a centric fusion between chromosomes 14 and 21 but also exhibited low numbers of peripheral B cells and low immunoglobulin production. In the final patient a diagnosis of X-linked hyper IgM syndrome was subsequently made. In this group of patients, the Btk cDNA as analysed by SSCP was normal (data not shown).

3.2.4 SSCP polymorphism in Btk

In addition to the band shifts described, a number of patients showed a band shift in the smaller 119 bp fragment generated in reaction A (Fig 3.4). Previous sequence analysis of this fragment showed a T→C change at nucleotide 2031 (L. Bradley, Ph.D. Thesis 1995). This nucleotide change is found in codon 633 and changes TGT to TGC. Both trinucleotide sequences code for the amino acid cysteine and therefore this polymorphism does not affect the amino acid sequence of the protein. The rarer allele has the published Btk sequence, T at position 2031 (Vetrie et al., 1993) and was found 14 of 58 patients studied (L. Bradley, Ph.D. Thesis 1995). The more common allelle has C at position 2031 and was found in the remaining 44 patients. The predicted heterozygosity for this polymorphism is 36%, though the figure is lower in this study. The nucleotide change does not destroy or create any restriction fragment enzyme site.

3.3 Discussion

3.3.1 Efficiency of Btk SSCP analysis

In this study, fourteen patients with a suspected clinical diagnosis of XLA were investigated for mutations in Btk cDNA by SSCP analysis. The clinical phenotype, SSCP band shifts and results of mutation analysis are summarised in Table 3.1. Ten band shifts were found and subsequent sequence analysis yielded five mutations. Assuming all band shifts found were associated with mutations, this gives a detection efficiency for SSCP analysis of ~ 71% which is slightly lower than the figures quoted in other studies (Michaud et al., 1992; Sheffield et al., 1993; Kauppinen et al., 1995), although with the small number of patients analysed this is not a significant finding. In four patients, no band shifts were found on SSCP analysis. These patients may have alterations in untranslated regions or control elements. Alternatively, their diagnoses of XLA may be incorrect and the clinical phenotype may have arisen as a result of abnormalities in other genes controlling B cell development and antibody production.

3.3.2 PH domain mutation in Btk

The mutation found in patient UD results in the substitution of proline for threonine at residue 33 in the PH domain of Btk. The same mutation has also been described in a severely affected XLA patient in another report of Btk mutations (Zhu et al., 1994b). The structural effect of this mutation on the PH domain of Btk has been studied using a nuclear magnetic resonance (NMR) three dimensional model of the PH domain (Vihinen et al., 1995). On the basis of its homology to other PH domains, the Btk PH domain was found to have a similar folding pattern, consisting of a β-barrel made up of seven anti-parallel β strands (A-G), and a C-terminal α-helix (Timm et al., 1994; Zhang et al., 1995). Residue 33 lies on a tight turn between strands B and C. The substitution of proline for threonine at this point is predicted to weaken the interaction between the two strands and thus disrupt the secondary structure of the domain. Alternatively, it has been suggested that the amino acid substitution might disturb the interaction of the PH domain with other domains of Btk itself or with other molecules. However, analysis of Btk protein by western blot analysis with anti-Btk antisera on an EBV-LCLs derived from patient UD shows complete absence of protein expression (Fig 3.2).

Table 3.1 Summary of Mutation Analysis in XLA patients

Patient	XLA phenotype	Band Shift	Exon	Mutation	Nucleotide change	Protein	Comments
KK	not known	E	Deletion exon 9	Deletion 63bp	909-971	Q260-21 aa	21 amino acid deletion in SH3
JT	severe	G and F	3	Insertion A	341	N72 +	Frameshift in PH
JJP	severe	G and F	3	Insertion A	341	N72 +	Frameshift in PH
BD	severe	G and F	3	Insertion A	341	N72 +	Frameshift in PH
UD	severe	G	2	A→C	229	T33P	Substitution in PH
HRS	leaky	A		Mutation not sequenced			
RB	severe	A		Mutation not sequenced			
MT	severe	B		Mutation not sequenced			
ID	severe	F		Mutation not sequenced			
MS	severe	G	2/3	Insertion at exon junction			
RH	severe	No SSCP band shift					
PF	severe	No SSCP band shift					
PN	severe	No SSCP band shift					
DL	severe	No SSCP band shift					

It is most likely that alteration of the secondary structure results in instability of the resultant protein and this, rather than abnormalities of binding, leads to XLA. Further discussion of protein expression in XLA follows in chapter 4.

3.3.2.1 PH domain mutation in the *xid* mouse

A number of missense mutations have now been found in the PH domain of Btk. Of interest are mutations found at residue Arg 28. This conserved residue is mutated to cysteine in the *xid* mouse. However, in humans only arginine to histidine substitutions have been found and the phenotype arising from the human mutation is far more severe than the murine *xid* phenotype (de Weers et al., 1994). The R28 residue is surrounded by charged residues (R13, Q16, K17, K18, K26, E41 and K53) which form a highly charged patch on the surface of the molecule. It has been proposed that this cluster of basic residues form a binding site at the lip of the β-barrel close to R28 (Vihinen et al., 1995). Therefore, mutations at this site may cause electrostatic changes that disrupt the binding of the Btk PH domain to its putative ligands.

3.3.3 Insertional mutation in Btk

Three patients had the same mutation in Btk. An extra A was inserted into a run of seven As at nucleotides 341-347. The frameshift caused by this mutation results in a premature termination codon at nucleotides 383-385. In addition to these 3 patients, another XLA patient analysed in this laboratory was found to have the same mutation (Gaspar et al., 1995). By means of an SSCP polymorphism it was possible to show that these patients came from at least two different pedigrees and thus this site appears to be a hotspot for mutagenesis in Btk.

3.3.3.1 Insertional Mutagenesis

Studies of insertional and deletional mutagenesis causing human disease have shown that both processes are non-random and the location and nature of the event are highly dependent upon the surrounding DNA sequence (Cooper and Krawczak, 1991; Krawczak and Cooper, 1991). The majority of insertions can be explained by either direct repeats or runs of single bases promoting slipped mispairing or by inverted repeats and symmetrical elements facilitating the formation of secondary

structure intermediates. Both mechanisms are associated with abnormalities in DNA replication and are similar to those involved in deletional mutagenesis.

Of all the types of insertional mutations, insertion of a single base into a run of identical bases is the most common (Cooper and Krawczak, 1991). This type of insertion is thought to occur due to misincorporation of an extra base by DNA polymerase into the primer strand as a result of slipped mispairing at the replication fork. The extra base then becomes fixed as a result of further base pairing downstream. If the misalignment occurs on the template strand, the looping out of the extra base is excised by DNA repair mechanisms resulting in a single base deletion (Fig 3.8).

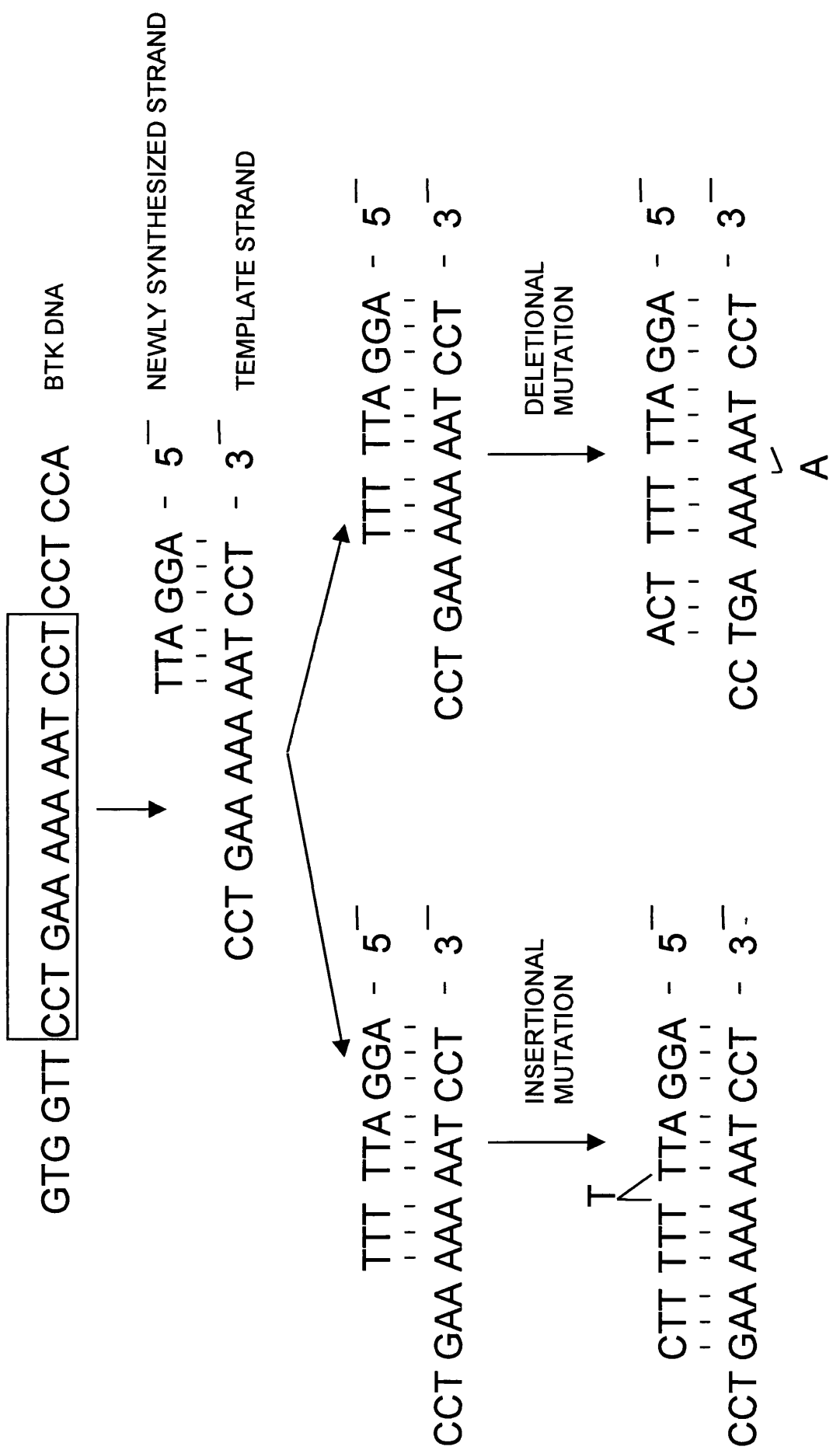
3.3.4. Splice site mutation in the SH3 domain of Btk

Patient KK was shown to have a complete deletion of exon 9 of Btk. Mutations resulting in the deletion of a whole exon are most likely to arise from an abnormality of mRNA splicing and more specifically from a disruption of the intron splice recognition site. A similar mutation to that in patient KK has been described elsewhere (Zhu et al., 1994a). In that study an EBV-LCL was available and genomic DNA was analysed for mutations in intron 9 (previously designated intron 8) of Btk. A g→a mutation was found at the 5' splice donor site which would result in an exon skipping mutation.

3.3.4.1 Mechanism of splice site mutations

The primary trancript from most vertebrate genes contains sequence from the whole length of the gene and encompasses both exon and intron sequences. This transcript then undergoes a sequential series of transesterification reactions in which the intronic sequences are snipped out and discarded and the exon segments spliced together to create the mature messenger RNA transcript. The splicing reactions take place within a multi-component complex called the spliceosome, which consists of small nuclear RNA (snRNA) molecules and protein splicing factors (Lamond, 1993).

Fig 3.8. Mechanism of Replication Slippage in N-Terminal domain of Btk



The process involves recognition by snRNAs of the 5' splice donor, the 3' splice acceptor site and the branch site located 20-40 base pairs upstream of the 3' splice site. Cleavage at the 5' splice site is followed by nucleolytic attack by the terminal G of the 5' splice site at the invariant A of the branch site creating a lariat shaped structure. This is followed by cleavage at the 3' splice site junction leading to release of the intron sequence and splicing together of exon RNA sequences.

Splice recognition sites virtually all follow the GT-AG rule: introns start with GT and end with AG. However these dinucleotides themelves are insufficient to signal the presence of an intron and so consensus sequences have been derived for the nucleotides adjacent to the GT and AG dinucleotides. A point mutation at the 5' or 3' splice recognition site can lead to an exon/intron boundary no longer being recognised and therefore exclusion of an exon from the mature transcript. Alternatively, cryptic splice sites may be activated within an intron resulting in a partial exon deletion or inclusion of intronic sequences into the final mRNA transcript.

3.3.5 SH3 domain mutations in Btk

In the report by Zhu et al. (1994) of the whole exon 9 deletion in the SH3 domain of Btk, the expression and function of the resultant protein was analysed. Western blot analysis using anti-Btk antisera showed the presence of a truncated Btk protein and an *in vitro* kinase assay showed that the autokinase activity of the protein product was preserved. The mutant protein was also shown to be capable of phosphorylating the exogenous substrate enolase. It appears, therefore, that the clinical phenotype in this patient occurs as a result of a functional abnormality in the Btk SH3 domain. However, exactly how this mutation causes its effect remains unclear.

NMR modelling studies of domains of the Src tyrosine kinases shows that the SH3 domain forms a compact β barrel composed of two β sheets, each sheet being formed by two or three anti-parallel β strands. At the interface of the two sheets is a hydrophobic core formed by highly conserved aromatic residues which constitute the ligand binding site of the SH3 domain. Sequence variations in SH3 domains result in variable loops being formed around the ligand binding site which may confer ligand

specificity on individual SH3 domains. Modelling of the Btk SH3 domain by comparison with the SH3 domain of Fyn shows that the exon 9 deletion mutation results in the loss of 21 amino acids, most of which are highly conserved, from the COOH-terminal of the domain. This is predicted to remove two of the five β strands, one from each β sheet, forming the β barrel and is also predicted to remove three residues which reside within the ligand binding site of the Src family SH3 domains (Zhu et al., 1994a). Thus, the mutation described in patient KK may result in aberrant folding of the mutant Btk SH3 domain and in abnormal binding to putative ligands.

3.3.6 Spectrum of mutations in Btk

Mutations in Btk from over 400 unrelated families have been identified to date. An international study group has been set up to facilitate analysis of Btk mutations and a database of mutations has been established (BTKbase) (Vihinen et al., 1995; Vihinen et al., 1998). Mutations span the length of the gene and are distributed almost uniformly throughout the domains, the exceptions being the TH domain and the upper lobe of the kinase domain. There is considerable heterogeneity in the types of mutation found but missense mutations are the most common, comprising 40% of all mutations.

Mutation analysis in Btk has enabled the achievement of a number of the original aims of this study. Unambiguous molecular diagnosis can be assigned to individuals with XLA, especially those with atypical or less severe phenotypes. Arguably its most important clinical role is in carrier detection and antenatal diagnosis and this has been illustrated by a number of studies (Lovering et al., 1994; Hagemann et al., 1995; Schuster et al., 1996). As described in sections 3.3.2.1 and 3.3.5, modelling studies and the comparison with structurally homologous proteins have been used to show how specific mutations can alter residues and domains of Btk important for normal function. However in one respect, the use of mutation analysis has been unsuccessful. Despite the number of mutations found and the large volume of clinical information collected, it is still not possible to make a correlation between the clinical phenotype and the genetic defect (Holinski-Feder et al., 1998). In some cases, individuals within the same family have been shown to have the same

mutation but very different clinical phenotypes (Kornfeld et al., 1994). It is becoming increasingly likely that, although mutations in Btk are responsible for XLA, lack of Btk alone may not be responsible for the clinical phenotype. This may be dependent on the interplay between a number of different molecules. Evidence to support this assumption comes from the analysis of protein expression in chapter 4. It also remains to be seen whether extrinsic factors such as early in fective episodes can also determine the final clinical outcome.

4

Analysis of Btk expression and activity in XLA

4.1 Introduction

Mutation analysis in Btk has identified a large number of defects distributed throughout the gene. These mutations, together with the knowledge acquired from the study of other protein kinases, can be used to predict how the function of the Btk protein may be disrupted. However, until the protein from patients can be characterised, these functional effects remain only predictions. The lack of correlation between mutations and the clinical phenotype also suggests that genotype analysis alone is not enough to predict the clinical outcome. Therefore, the study of mutant proteins may be important in understanding the molecular pathogenesis of XLA.

Although many groups have studied mutations in Btk which result in XLA, very few have followed the analysis of mutations with a study of the mutant protein expression and function. In many of the reports, it has been postulated how mutations in Btk, especially those involving single amino acid substitutions and premature stop codons resulting in truncated proteins, may affect the functional activity and binding properties of the protein (Conley et al., 1994a; Zhu et al., 1994b; Jin et al., 1995). However in most of these reports, no study of protein expression or function has been carried out. The case for the analysis of mutant Btk protein becomes stonger, since a number of studies have now attempted to model Btk protein domains incorporating known mutations in an attempt to understand how the mutation may

affect the 3D structure of the protein (Vihinen et al., 1994a; Vihinen et al., 1994b). In these studies also, there has been no prior assessment of the expression and function of the mutant protein.

Where studies have been performed this has consisted of assessment of protein expression by western blot analysis and of the ability of Btk to autophosphorylate itself by an *in vitro* autokinase assay. At present, unlike in some other immunodeficiencies, there is not any assay for physiological function of Btk. In the case of chronic granulomatous disease, defective expression of any of the four NADPH-oxidase components results in the inability to form a functional NADPH-oxidase complex which is unable to produce the oxidative burst required for bacterial killing. This inability can be measured by a chemiluminescence assay and thus provides a physiological assessment of protein function (Porter et al., 1992). However in XLA, little is known of the role of Btk in B cell signalling pathways and it is not known to which substrates Btk binds and which proteins it phosphorylates. Until such information becomes available, autophosphorylation activity remains the best measurement of Btk enzymatic activity.

Although no formal study of Btk protein expression and function in XLA patients has been performed, a few reports of protein expression or activity exist. In the initial report by Tsukada et al. (1993) documenting the isolation of a cytoplasmic tyrosine kinase, then designated Bpk, as the likely XLA gene, they found absence of autokinase activity of this protein in 4 of 5 EBV immortalised B cell lines from XLA patients. In the one XLA line in which there was detectable protein expression, there was considerably diminished autokinase activity. These results correlated with reduced expression of Btk mRNA but no mutation analysis was performed. A single case report on an atypical XLA patient described a mutation (Y361→C) in the SH2 domain of Btk (Saffran et al., 1994). The effect of the mutation was complete absence of protein expression rather than a functional defect in the SH2 domain thereby stressing the importance of protein analysis prior to making assumptions regarding the functional effect of mutations. In contrast, another case report documented a mutation similar to that previously described in patient KK in chapter 3. The effect of the deletion of 21 amino acids in the SH3 domain was the expression of a smaller mutant protein which retained its catalytic activity (Zhu et al., 1994a).

The deleterious effect of the mutation could therefore be ascribed to a functional defect in the SH3 domain. In this study it was also shown that the mutant Btk was capable of transphosphorylating the exogenous substrate enolase. More recently, a three dimensional model of the Btk kinase domain has been used to interpret the structural basis for disease in eight independent missense mutations in the kinase domain of patients with XLA (Vihinen et al., 1994b). The mutations ranged from residue Lys 430 to Gly 613. The consequences of only three of these mutations (Arg 525→Gln; Arg 562→Pro; Ala 582→Val) on protein expression and activity was studied but in all other cases it was assumed that there would be normal amounts of protein expressed.

With the wealth of mutation data available and the relative ease of access to XLA patient material at this Institution, a formal study was carried out. The aim of the study was to analyse protein expression and autophosphorylation activity in patients with a diagnosis of XLA in an attempt to characterise the relationship between a particular mutation, the resultant Btk protein and the clinical phenotype.

In order to carry out the study, an antibody to Btk was required. This was generated in this laboratory against a GST fusion protein containing residues 163-218 in the N-terminal domain of Btk. The polyclonal antiserum had been previously shown to identify Btk protein by western blot analysis of whole cell lysates and also to purify Btk by immunoprecipitation (Genevier et al., 1994; Hinshelwood et al., 1995). Immunoprecipitation also allowed *in vitro* kinase assays to be carried out to assess Btk enzymatic activity. One of the difficulties in studying Btk protein expression has been the choice of material on which to carry out studies. Most of the studies have been performed on EBV immortalised B cell lines (EBV-LCLs) from patients. However, such lines are notoriously difficult to obtain from XLA patients due to the lack of circulating B cells. Inevitably those that are obtained are from very early cells in the B lymphoid lineage. Studies on primary cells such as a peripheral mononuclear cells (PMC) or polymorphonuclear cells have been rarely performed presumably due to the difficulty in obtaining fresh patient material or to technical difficulties in performing the analysis. The studies described below were carried out on whole cell lysates of peripheral mononuclear cell layers from all patients, except one from whom an EBV-LCL was available. PMCs were extracted from fresh whole blood

samples or a sample previously stored in liquid nitrogen was thawed and used. The specificity of the antibody and the previous experience in this laboratory with protein analysis allowed this method to be used.

4.2 Results

4.2.1 Patient Selection

A total of fourteen patients were selected for analysis. For twelve of these patients, the molecular diagnosis of XLA had been determined. Of these twelve patients ten, P1-P10, had a severe phenotype with less than <1% peripheral B cells and less than 1g/l of IgG production while two, P11 and P12, had an atypical or less severe phenotype. In a further two individuals, P13 and P14, a clinical diagnosis of XLA had been made on the basis of a typical phenotype, but no mutation data was available. Ten of the mutations have been reported in previous publications (Vetrie et al., 1993; Bradley et al., 1994; Gaspar et al., 1995). The other two were characterised in a similar manner initially by single stranded conformation polymorphism (SSCP) analysis and subsequently by direct sequence analysis.

The mutation data is summarised in Table 4.1. Mutations are spread throughout the gene. There were four missense mutations including one in the start codon, four nonsense mutations, two mutations resulting in a frameshift and one insertion and one deletion which did not lead to a frameshift in the coding region.

4.2.2 Control Samples

Btk expression in haematopoietic cells is restricted to the B cell lineage (except plasma cells) and to the myelomonocytic lineage (Genevier et al., 1994; Smith et al., 1994). T lymphocytes have been shown not to express Btk. One problem of studying Btk protein expression in peripheral mononuclear cells was that reduction or absence of Btk in patient samples might be due to the lack of cells expressing the protein rather than an abnormality in protein expression *per se*. In order to address this problem two control samples were used, the first (C1) being from a healthy donor. The second (C2) was a PMC from a girl with an 'XLA-like' phenotype, agammaglobulinaemia and no peripheral B cells and who has Klippel-Trenaunay-Weber syndrome in association with a constitutional 22q11:8q34 chromosomal translocation.

Table 4.1. Btk mutations and protein analysis in XLA patients

Patient	Phenotype	Sample	Mutation	Protein change	Protein expression	Auto-kinase activity
P1	severe	PMC	T134→ C (Bradley et al .1994)	M1→T	absent	absent
P2	severe	PMC	T163→ C	L11→ P	absent	absent
P3	severe	PMC	442-2bp a→ g 21bp insertion (Bradley et al .1994)	Q103 7 aa insertion	absent	absent
P4	severe	PMC	T558→ A	Y142→ stop	absent	absent
P5	severe	PMC	del. C752 (Gaspar et al. 1995)	FS	absent	absent
P6	severe	PMC	C895→ T (Bradley et al .1994)	R255→ stop	absent	absent
P7	severe	PMC	del. exon 16-19 (Vetrie et al .1993)	Truncated	absent	absent
P8	severe	PMC	del. 1883-2040 (Gaspar et al. 1995)	FS	absent	absent
P9	severe	PMC	G2038→ T (Bradley et al .1994)	E636→ stop	absent	absent
P10	severe	PMC	G2038→ T (Bradley et al .1994)	E636→ stop	absent	absent
P11	leaky	PMC	C1952→ A (Bradley et al .1994)	A607→D	absent	absent
P12	leaky	EBV-BCL	C994→ 7 (Bradley et al .1994)	R288→W	normal	normal
P13	severe	PMC	Undiagnosed		absent	absent
P14	severe	PMC	Undiagnosed		absent	absent

Abbreviations
del. - deletion
FS - frame shift mutation leading to premature stop codon

She has no abnormality in her Btk gene as determined by SSCP analysis (Linda Bradley, unpublished).

Both controls showed equal amounts of Btk protein expression by western blot analysis using anti-Btk antisera (Fig 4.1) and also autophosphorylation activity of a 77kD protein by *in vitro* kinase assay (Fig 4.2). The results from C2 indicate that despite a lack of peripheral B cells, Btk protein and autokinase activity can be readily detected from a peripheral mononuclear cell layer.

4.2.3 Analysis of patients with severe phenotypes

Ten patients with nine different mutations and all suffering from classical XLA were analysed by western blot analysis of whole cell lysates. The range of mutations are summarised in Table 1 and varied from single base substitutions to whole exon deletions and frameshift mutations resulting in premature stop codon formation. The mutation analysis data would lead one to predict that full length protein in some cases or truncated proteins in others would be detectable. However, in all these patients no Btk protein expression was detectable (Fig. 4.1). To exclude the possibility of non-specific protein breakdown, the nitrocellulose filter was stripped and reblotted with antisera from a more widely expressed protein, protein kinase C (PKC) β1. The resultant blot showed PKCβ1 protein expression in all patient samples (Fig 4.1).

Further investigation by anti-Btk immunoprecipitation of whole cell lysates and subsequent *in vitro* kinase assay did not detect any autophosphorylation activity in these patient samples, although activity of a 77kD protein was clearly detectable in both control samples (Fig 4.2).

4.2.4 Analysis of atypical XLA phenotypes

In order to investigate whether protein analysis could be used to give an indicator of clinical phenotype, we analysed two patients with less severe phenotypes. Patient 11 came from a family with three affected boys and at presentation had 1% B cells and low rather than absent immunoglobulin production (Jones et al., 1996). Patient 12 came from a large pedigree in which there was considerable heterogeneity in the clinical and immunological phenotype.

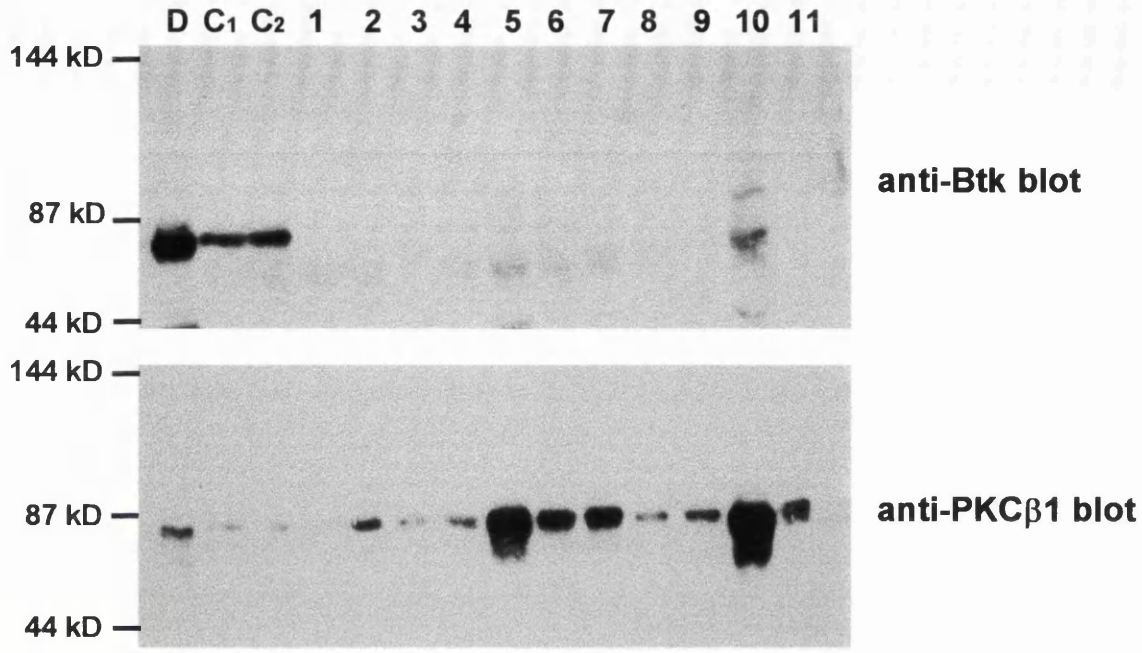


Fig 4.1. Absence of Btk protein expression in XLA. *Lysates of ~10^7 mononuclear cells from individuals with XLA (1-11) were blotted with anti-Btk antisera. Lysates from Daudi cells (D) and two control individuals (C1 and C2) were also analysed. The same filter was stripped and reblotted using anti-PKCβ1 antisera.*

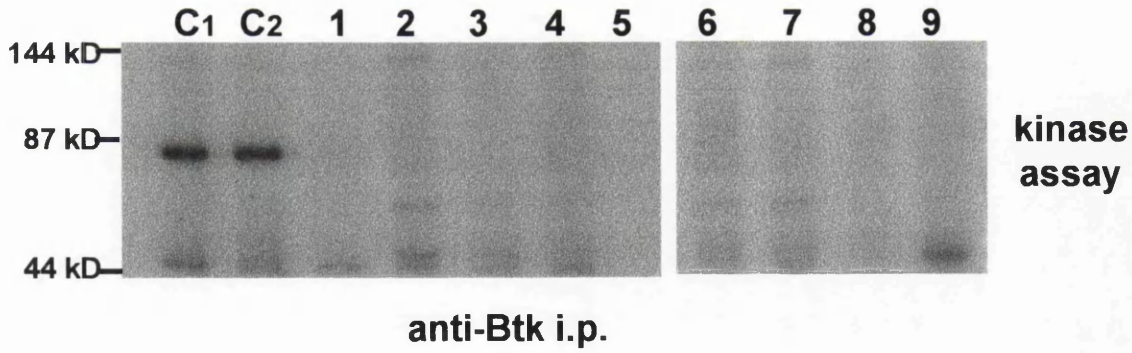


Fig. 4.2. Lack of Bkt autophosphorylation activity in XLA. *Mononuclear cell lysates from XLA patients (1-9) were incubated with anti-Btk serum and the immunoprecipitates washed and subject to in vitro kinase assay. Control samples C1 and C2 were also analysed.*

Our results show that in patient 11 there was no Btk protein detectable by western blot analysis of whole cell lysates or Btk immunoprecipitates and also no autophosphorylation activity of Btk (Fig. 4.3a and 4.3b). In patient 12, however, protein expression and kinase activity were comparable to the control sample. Unequal protein loading and non specific protein degradation were ruled out by stripping the western blot and reprobing with PKCβ1 antisera (Fig 4.3a).

4.2.5 Analysis of previously undiagnosed XLA patients

Two patients with a clinical phenotype of XLA and for whom no mutation data was available were also analysed. Both had less than 1% B cells and no significant immunoglobulin production on presentation. cDNA from Patient 13 had been screened previously by SSCP analysis and no band shift had been found thereby leaving the diagnosis of XLA in doubt. Patient 14 was of a severe phenotype and presented during the period of this study and had not undergone any mutation analysis. In both patients western blot analysis showed complete absence of Btk protein expression (Fig 4.4). Equal loading of protein was shown by performing an anti-PKCβ1 western blot (Fig 4.4) on the same whole cell lysate samples. Patient 13 was further analysed by western blot of Btk immunoprecipitates and by *in vitro* kinase assay. No Btk expression or activity was detectable (data not shown).

4.2.6 Confirmation using alternative Btk antisera

In order to confirm our findings, immunoblotting was carried out on eleven of the fourteen patients studied using an alternative antibody (further cell lysate samples were not available on patients 1, 10 and 14). This antibody was a mouse monoclonal raised against residues 26-175 of mouse Btk. Once again a 77kD band was recognised in the control sample and in patient 12 but not in any of the other patients studied (Fig 4.5).

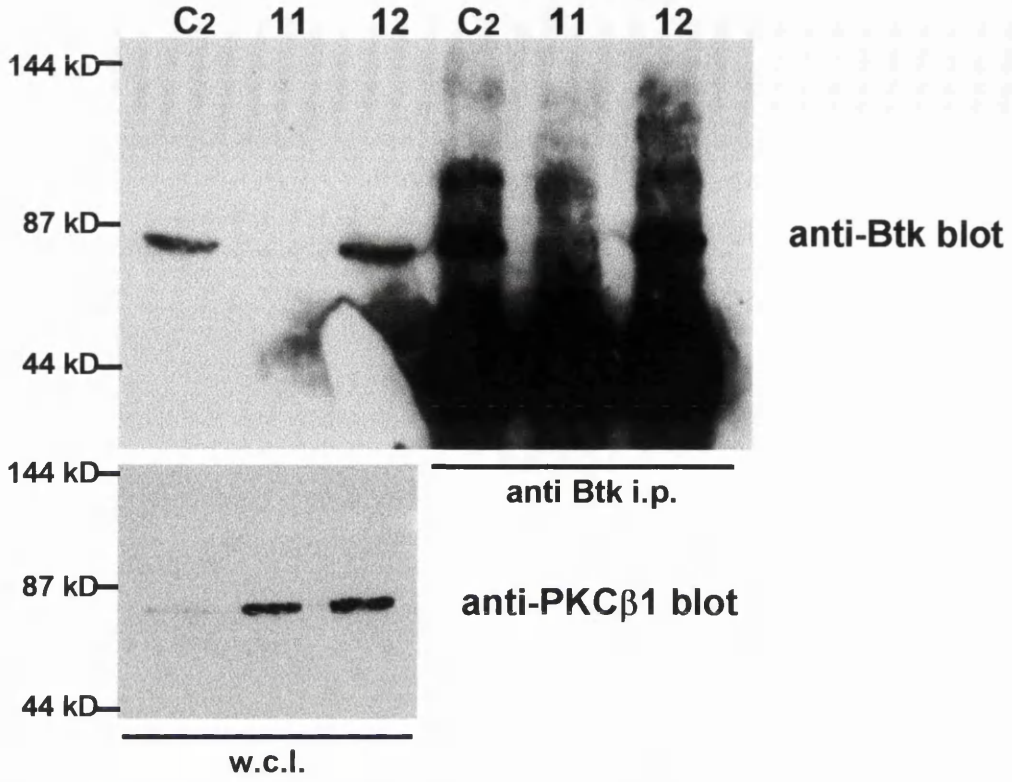


Fig. 4.3a. Different patterns of protein expression in individuals with atypical XLA.. Mononuclear cell lysates and anti-Btk immunoprecipitates of cell lysates from patients with atypical XLA (11,12) were blotted with Btk antisera. Control sample C2 was analysed in a similar manner. The filter was stripped and reblotted with PKCβ1 antisera.

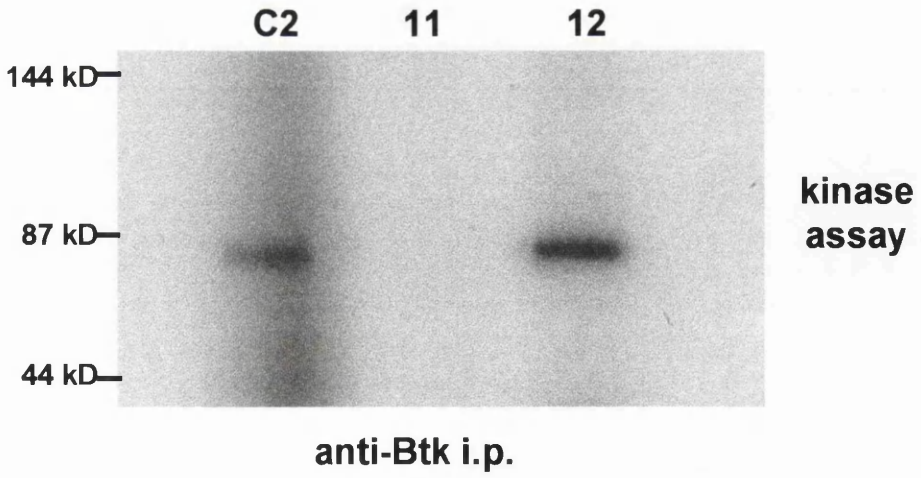


Fig 4.3b. Different patterns of Btk kinase activity in individuals with atypical XLA. *Anti-Btk immunoprecipitates were washed and subject to in vitro kinase assay.*

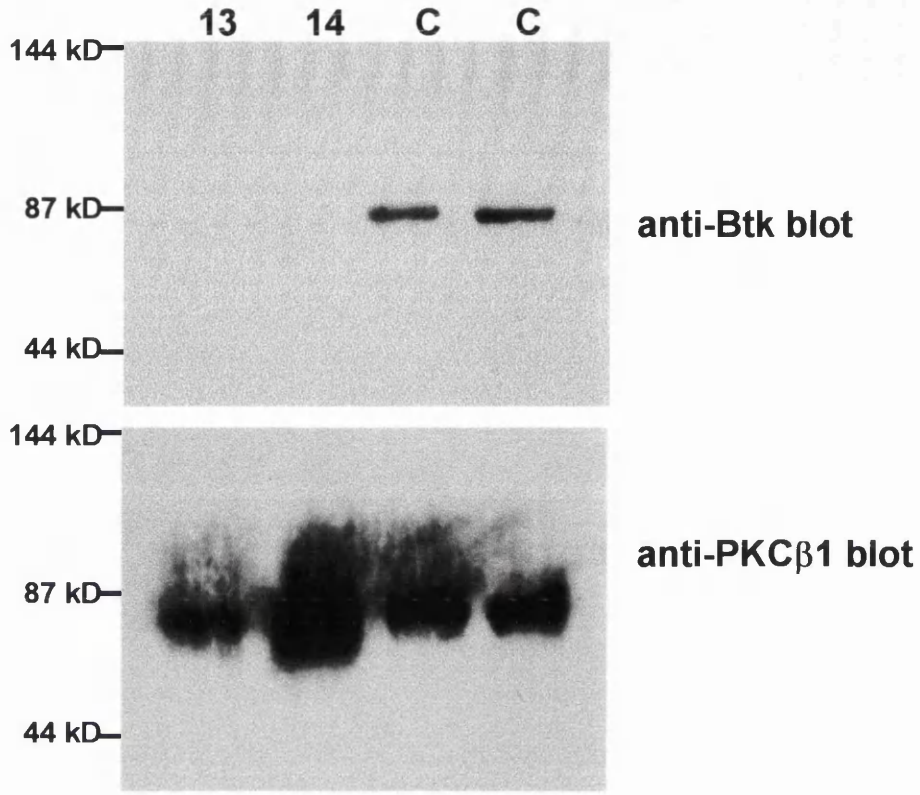


Fig 4.4. Diagnosis of XLA by western blot analysis. *Mononuclear cell lysates from two previously undiagnosed patients with the clinical phenotype of XLA (13,14) and two healthy individuals (C) were blotted with anti-Btk antisera. The same lysates were blotted with anti PKCβ1 antisera.*

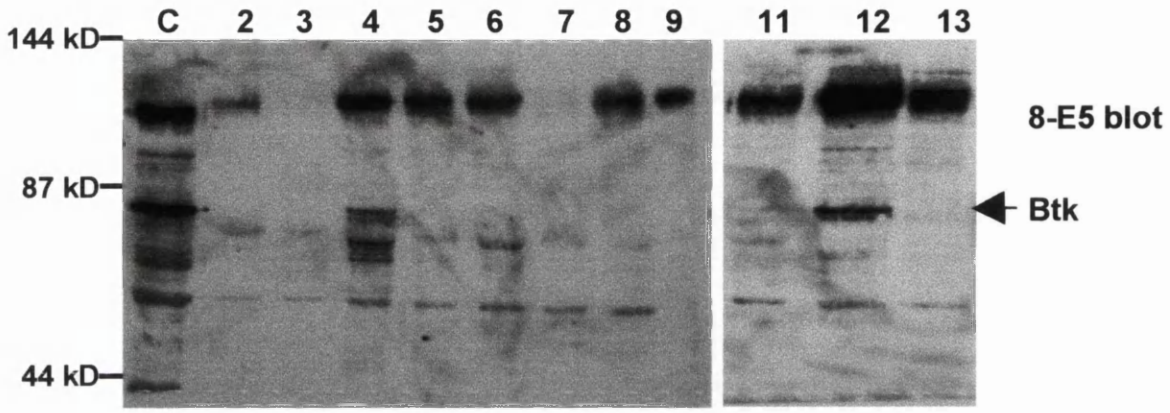


Fig 4.5. Analysis of Btk expression using alternative Btk antisera. *Mononuclear cell lysates from the majority of patients previously analysed were immunoblotted with a mouse monoclonal antibody (8-E5) against Btk. Lack of Btk expression is seen in all patient samples other than P12 and the control sample thereby confirming the findings from previous experiments.*

4.3 Discussion

4.3.1 Protein expression in XLA

For a better understanding of Btk function we have analysed protein expression and autophosphorylation activity in 14 patients with XLA. In all the patients with severe XLA for whom mutations were identified previously, no protein expression or catalytic activity were detectable. This may have been predicted in certain cases where mutations were situated in the N terminal region of the gene as in patient 1 but in the majority of cases one would have predicted expression of truncated proteins. Patient 2 has a missense mutation leading to a L11→P substitution and one would expect a full length protein with a structural defect in the PH domain as a result of the amino acid substitution. In patients 9 and 10 where the protein is truncated at C636, a near full length protein would be predicted, albeit one lacking autokinase activity.

The lack of protein expression may be explained by instability of the mRNA transcript or resultant protein instability. In all patients Btk mRNA was successfully amplified by RT-PCR cDNA (data not shown). Although this was not a quantitative assay, it does indicate the presence of Btk mRNA. The expression of PKCβ1 by western blot analysis of these patient samples would also rule out the possibility of non-specific protein degradation. The results could also be due to either instability of the aberrant protein product or failure of recognition of this aberrant protein by our antisera. This latter explanation may be possible in some cases, but since it was raised against an anti Btk peptide of amino acids 163-218, one would expect recognition of the larger predicted proteins from patients 7-10, if these were present. Similar results obtained using an alternative anti-Btk antibody adds further credence to these findings.

Instability of the resulting protein appears to be the most likely explanation for these results. The high level of mutated proteins being unstable appears surprising but our findings are supported by the other reports mentioned above in which mutated proteins have been analysed. The reports by Tsukada et al. (1993) and Saffron et al. (1993) both show the absence of protein expression in whole cell lysates of EBV transformed B cell lines from XLA patients. These results suggest that the prediction

of aberrant protein structure or modelling mutant Btk protein domains from mutation analysis data may not be appropriate unless it is known that intact protein is indeed expressed.

4.3.2 Correlation of Btk expression with clinical phenotype

The analysis of mutations in Btk has not been able to predict the clinical phenotype of affected individuals perhaps for the reason that so many mutations result in a lack of Btk expression. It was therefore hypothesised that a correlation may exist as a result of protein expression with the more severely affected patients expressing no protein and those less severely affected individuals having some degree of protein expression. The results from the analysis of patients 11 and 12 with atypical phenotypes do not support this idea. In patient 11 the substitution of an alanine residue by aspartic acid in a non-highly conserved region of the kinase domain was originally thought to partially disrupt autokinase activity and thereby lead to the less severe phenotype. However our results show that this mutation results in complete loss of protein expression and autokinase activity. By contrast in patient 12, the substitution of the arginine by tryptophan at residue 288 does not lead to any abnormalities of protein expression or autokinase activity. It is thought that this mutation affects the phosphotyrosine binding pocket of the SH2 domain and may affect the binding of Btk with tyrosine phosphorylated proteins. Thus the analysis of mutant proteins, as with mutation analysis, cannot be used to predict the clinical phenotype.

4.3.3 Compensation of Btk function in B cell development

The findings in patient 11 may have important implications. The complete absence of Btk protein does not necessarily lead to a severe phenotype. Moreover, the presence of mature circulating B lymphocytes and significant production of immunoglobulin suggest that in the absence of Btk, other signalling molecules or pathways may partially compensate for its function. Our results are supported by reports of similar findings in other less severely affected XLA patients (Hashimoto et al., 1996). The concept of redundancy in protein function has a parallel in another human immunodeficiency. Mutations in the T cell receptor (TCR) associated tyrosine kinase, ZAP-70, leads to a cellular immunodeficiency in which there is failure of $CD8^+$ lymphocyte development and abnormality of signalling through the TCR in

$CD4^+$ lymphocytes (Gelfand et al., 1995). However, in the thymocytes of ZAP-70 deficient individuals, upregulation of the structurally homologous tyrosine kinase Syk compensates for the absence of ZAP-70 and can restore signalling through the TCR and allow differentiation and positive selection of $CD4^+$ lymphocytes. Such compensatory mechanisms may exist throughout the immune system and may well explain the findings in Btk deficient patients with residual B lymphocyte development and function.

The clinical phenotype may therefore not be the result of an abnormality in one protein alone but the result of an interplay between a number of components involved in one common functional pathway. In such a model Btk may have an essential, though partially redundant role, whereby the consequences of its lack of function are deleterious but can be compensated for by the function of other components in the pathway.

4.3.4 Protein analysis as a diagnostic indicator of XLA

In earlier studies protein analysis was performed predominantly on EBV immortalised B cell lines from XLA patients. However, due to the lack of peripheral B cells in XLA, such lines are difficult to establish. We have demonstrated from our study of C2 that Btk expression and autokinase activity can be readily detected from a peripheral mononuclear layer despite the lack of B cells. This makes the analysis of protein in suspected individuals a more convenient and rapid assay.

The ability of these assays to make a molecular diagnosis of Btk deficiency is illustrated by the study of patients 13 and 14. Patient 13 had no family history and was thought to be a sporadic case while patient 14 was part of a large pedigree although the genetic defect was unknown. Although in the cases above, one would have strongly suspected Btk deficiency as a result of the clinical phenotype, other cases are not so clear cut. In a recent study (Hashimoto et al., 1996) patients who had been previously labelled as common variable immunodeficiency (CVID) or IgG subclass deficiency were found to have mutations in Btk and found to have abnormalities in Btk protein expression and autokinase activity.

In our study 13 of 14 patients were found to have an abnormality on protein analysis. The report quoted above in which 35 families were studied found that 31 of 35 mutations gave rise to abnormalities of protein expression and activity. Given the high rate of abnormalities in mutated Btk proteins, the ease of obtaining material and the speed and reliability of the assays themselves, it is suggested that protein expression and *in vitro* kinase assay be used as an initial diagnostic screen for XLA. The use of these assays on a wider group of patients such as those previously diagnosed as IgG subclass deficiency and CVID may also allow us to understand more fully the heterogeneity of Btk abnormalities.

5

Generation of Btk Retroviral Vectors

5.1 Introduction

Gene transfer technology has now been used in the study of many genetic conditions both to further understand the molecular pathology of the condition and also to develop therapeutic strategies. The development of *in vitro* and *in vivo* models of gene therapy for XLA would greatly add to the understanding of the nature of Btk and its interactions and could also lead to therapeutic strategies for treatment of the disease.

5.1.1 Retroviral vectors for gene transfer

The transfer of genes into mammalian cells can be mediated by a number of vector systems. The ability of DNA and RNA tumour viruses to cause cellular transformation by the transfer of genetic material first suggested that viral gene delivery systems could be adapted for therapeutic use. Since then retroviral vectors and in particular the type C oncoretroviruses (murine leukaemia viruses (MLV) being the most popular) have been the most widely used and the first clinical trials of somatic gene therapy have used retroviral mediated gene transfer. The study of normal retroviral biology has highlighted many features that make retroviruses attractive vectors for gene therapy (reviewed by Coffin, 1992).

5.1.2 Structure of retroviral particles

In simple retroviruses such as MLV, the retroviral particles (virions) are approximately 100nm in diameter and consist of two identical copies of a plus-sense single stranded genome (approximately 7-10kb in length) complexed with viral-coded proteins *gag* and *pol*. The retroviral genome encodes *gag*, *pro* (protease), *pol* and *env* genes respectively. The *gag-pro-pol* proteins are synthesised from a full length mRNA species whereas the *env* protein is translated from a spliced sub-genomic species. The nucleo-protein complex is encased in a host cell derived lipid bilayer envelope in which is embedded glycoprotein encoded by the *env* gene (Luciw & Leung, 1992).

The single stranded RNA genome consists of an internal region containing viral gene open reading frames and flanking regions which are necessary *in cis* for viral replication. The 5' ends of the virion RNA and viral mRNA are capped and methylated and the 3' ends are polyadenylated, these modifications being mediated by host cell enzymes. At each end of the genome is a short repeated sequence R which mediates the initial intermolecular DNA strand transfer during reverse transcription. At the 5' end, downstream from R and upstream of the minus strand primer binding site (PBS-), is another short sequence U5 which is necessary for the formation of secondary structures to guide efficient initiation of reverse transcription (Aiyar et al., 1992). The PBS- sequence is complementary to 16-19 bases at the 3' end of the specific tRNA molecule. Following PBS- is another untranslated sequence which lies immediately upstream and extends into the *gag* open reading frame (ORF) and which incorporates the packaging signal (ψ). This sequence (approximately 300bp long in MLV) is necessary for packaging of the virion (Adam and Miller, 1988; Dornburg and Temin, 1990). At the 3' end of the gene downstream of *env* is a short purine sequence (polypurine tract, ppt) which initiates synthesis of plus strand DNA (PB+). A unique sequence at the 3' end of the RNA genome, U3, is duplicated in the proviral DNA and contains elements which regulate transcription of the integrated provirus (Speck and Baltimore, 1987).

5.1.3 Normal retroviral lifecycle

The normal lifecycle of retroviruses involves the entry of the retroviral particle into the host cell by receptor mediated endocytosis in the cytosol, conversion of retroviral

RNA to DNA by reverse transcription, the integration of the proviral DNA into the host genome and the expression of viral genes under the control of the retroviral LTRs (long terminal repeats). Fig. 5.1 illustrates this process in detail.

5.1.4 Recombinant retroviral vectors

Gene therapy technology has utilised this normal biological process to create recombinant replication defective retroviral vectors which can integrate heterologous genetic material into the host genome but which cannot replicate and undergo further rounds of integration. In the engineering of recombinant retroviral vectors, nearly all of the viral protein coding sequences are removed and replaced with the gene of choice (Fig 5.2). The packaging signal sequence is left intact to allow efficient packaging of the particle. However, the loss of retroviral coding information renders the vector replication defective and vector virus particles can only be successfully produced by supplying the necessary viral proteins *in trans*. This is achieved by the engineering of an integral component of this system, the packaging cell line. Such cells have been engineered to provide the missing functional and structural retroviral proteins from one or more expression constructs. In this way the introduction of the recombinant vector into the packaging cell line generates recombinant viral particles capable of target cell entry, integration and gene expression but incapable of further replication (Fig 5.3) (Miller, 1990).

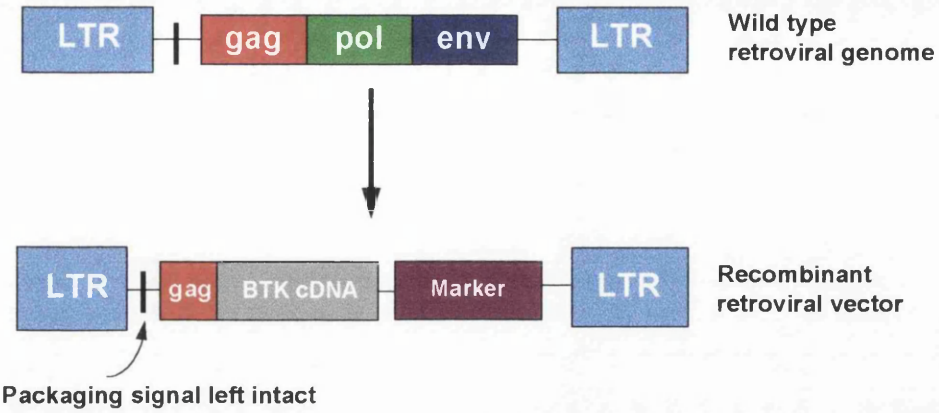
5.1.5 Packaging cell lines

These engineered cell lines contain retrovirus derived constructs that code for the authentic retroviral *gag*, *pol* and *env* proteins. However, the ψ packaging signal has been deleted from the constructs thus preventing the packaging of the RNA transcripts into virion particles. In the early packaging cell systems, the packaging cell carried a retroviral provirus in which the ψ sequence had been deleted. An example of these early systems is the ψ-2 packaging system (Mann et al., 1983) which was used to generate ecotropic virus in the experiments described below. Although these early systems were capable of generating replication defective recombinant virus, only one recombination event between the vector and packaging construct was required to generate replication competent retrovirus.

Fig. 5.1 Life cycle of a typical retrovirus. *Retrovirus virions consist of a core containing two identical single stranded viral RNAs and enzymes (reverse transcriptase and integrase) surrounded by host cell plasma membrane in which are embedded a transmembrane protein (TM) and an external glycosylated surface domain (SU). Viral host range is determined by a specific interaction between the SU domain of env and the cell surface receptor. Following binding, the virus and cell membranes fuse, a process mediated by the TM domain of env. The viral particle is endocytosed and the virion capsid is released into the cytosol. This nucleoprotein complex is the site of reverse transcription in which viral RNA is converted to linear double stranded DNA. A tRNA specifically binds to the primer binding site as a primer and the reaction is performed by viral reverse transcriptase which is present as part of the nucleoprotein complex. During this process sequences located at either end of the viral RNA (U5 and U3) become duplicated and placed at either end of the newly synthesised DNA thereby creating identical LTRs with the structure (U3-R-U5) (see inset). The proviral DNA is then translocated to the nucleus where integration of provirus into host DNA occurs, a process mediated by the viral integrase protein. Following integration, the viral genome is transcribed by the host cell transcription machinery from the viral promoter located in the U3 region of the viral LTR. Transcription results in viral RNA that can be translated into viral proteins gag and pol and env and also genomic RNA that can be packaged in new viral particles. Viral proteins and RNA assemble into new cores that then interact with envelope proteins on the host cell membrane to produce new virions. These then bud out of the infected cell where they undergo the final stages of modification to form fully infectious particles which can then undergo a further round of infection.*
(Fig 5.1. taken from Gunzberg and Salmons (1996))

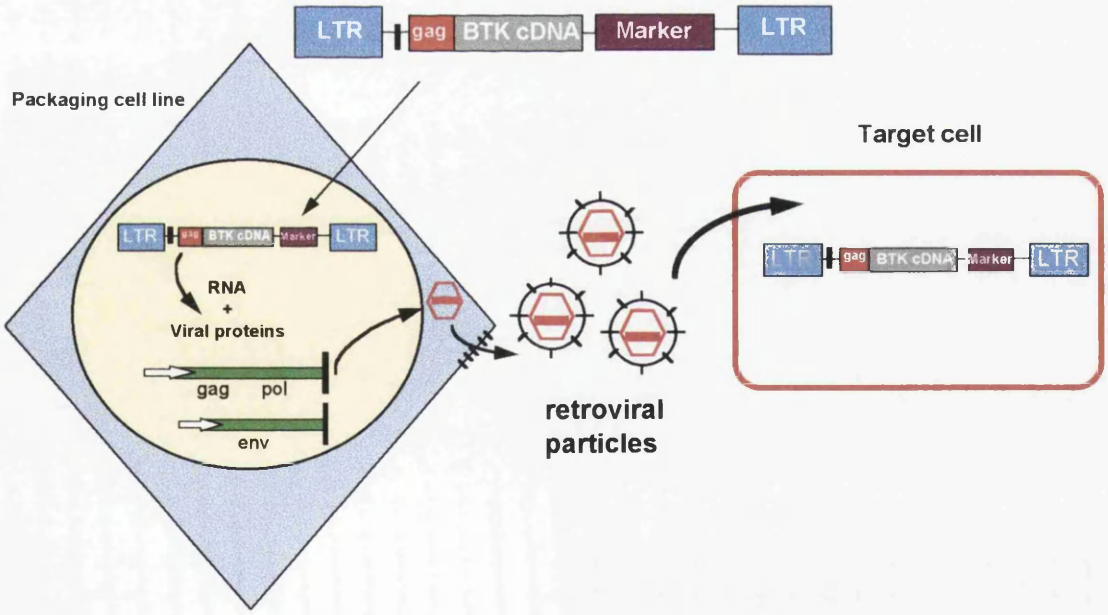
Fig. 5.1 Life cycle of a typical retrovirus

Fig. 5.2 Replication defective recombinant retroviral vectors



The retroviral coding sequences are removed and replaced by the gene of interest and the gene encoding a selectable marker. Part of the viral sequence encoding the packaging signal (ψ+ sequence) which extends into the gag sequence remains allowing efficient packaging of retroviral particle.

Fig. 5.3 Generation of infectious recombinant virus from packaging cell lines



Packaging cell lines provide the structural proteins required for the assembly of infectious viral particles. In the third generation systems (illustrated above), the cell line carries two independent constructs, one expressing gag and pol and the other env. At least three recombinant events are required to generate wild type replication competent virus. After infection of the target cell, further rounds of replication cannot occur due to the absence of helper functions.

To eliminate this possibility, packaging cell systems have evolved through ever more sophisticated designs, such that in the third generation systems, *gag* and *pol* gene products are expressed from one construct and the *env* product from another. In these systems, at least three recombination events are required for the generation of replication competent retrovirus. The most widely used of the third generation amphotropic packaging cell lines and that used in the experiments described below is the GP+envAm12 line producing amphotropic virus (Markowitz et al., 1988).

5.2 Generation of Btk retroviral vectors

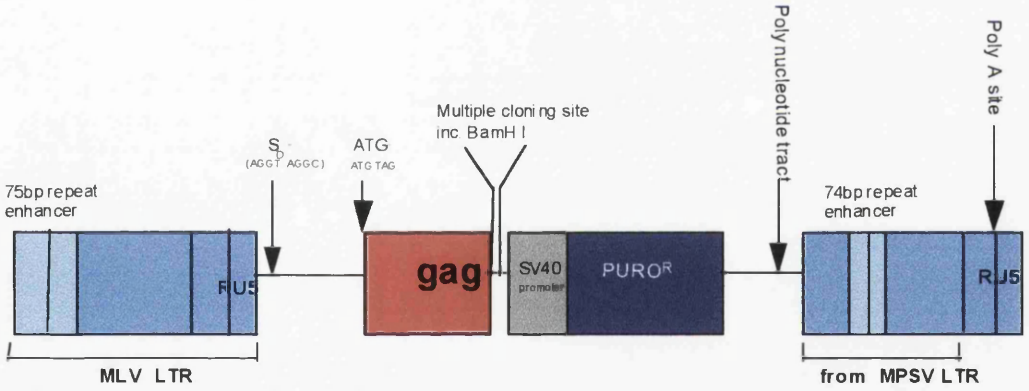
5.2.1 Choice of retroviral construct

The retroviral construct chosen for the experiments was based on the pBabe recombinant vector construct which arose from a series of experiments involving extensive deletion mutatgenesis analysis of a basic Moloney murine leukaemia (MLV) viral construct (Morgenstern and Land, 1990). The resultant retrovirus contained a mutated splice donor sequence (AGG*T*→AGG*C*) and the presence of a *gag* sequence at the 5' end of the virus adjacent to the ψ site with a mutation in the *gag* initiation codon (ATG→TAG). The manipulations performed generated a retrovirus, pBabe, capable of transmission of inserted genes at high titres with expression under the control of the MLV LTRs. In addition to the basic construct, a series of retroviruses containing a variety of selectable markers under the control of a heterologous internal SV40 promoter were also generated. Our experiments were based on the pBabe Puro construct (Morgenstern and Land, 1990), illustrated in Fig. 5.4, which contains the puromycin acetyl transferase gene conferring puromycin resistance.

This pBabe Puro construct was itself modified to allow better gene expression in haematopoietic cells. Although studies have shown that MLV based viruses efficiently infect and express genes in fibroblasts, there is significantly decreased efficiency in haematopoietic cells. MPSV based viruses, however, show more efficient transformation of both fibroblasts and haematopoietic cells (Stocking et al., 1985). Furthermore, using deletion mutation analysis, this capacity has been attributed to the U3 region of the MPSV LTR (Stocking et al., 1986; Laker et al., 1987). Therefore our experiments were conducted using a retroviral construct that

contained the 5' MLV LTR and a hybrid 3' LTR containing the majority of the U3 MPSV sequence with a partial 3' MLV LTR sequence. The 3' LTR is duplicated in the 5' region on provirus formation and thus becomes the promoter utilised in vector gene expession. The 3' enhancer was functionally equivalent to the MPSV LTR and was therefore expected to allow efficient gene expression in haematopoietic cells. This virus was denoted pMB.SP (Fig. 5.4). Between the LTRs are 900bp of MLV sequence encompassing the packaging sequence but with a mutated splice donor site and *gag* start codon as in pBabe Puro. This construct had previously been used in our laboratory for transfer of the gene for X-linked chronic granulomatous disease (X-CGD) into EBV immortalised B cells from X-CGD patients (Porter et al., 1993). Successful X-CGD gene transfer, intgration and expression was achieved with correction of the phenotypic abnormalities. As a result of such convincing evidence for gene expression in haematopoietic cells the pMB.SP construct was used for the Btk transfer experiments.

Fig 5.4 pMB.SP retroviral vector

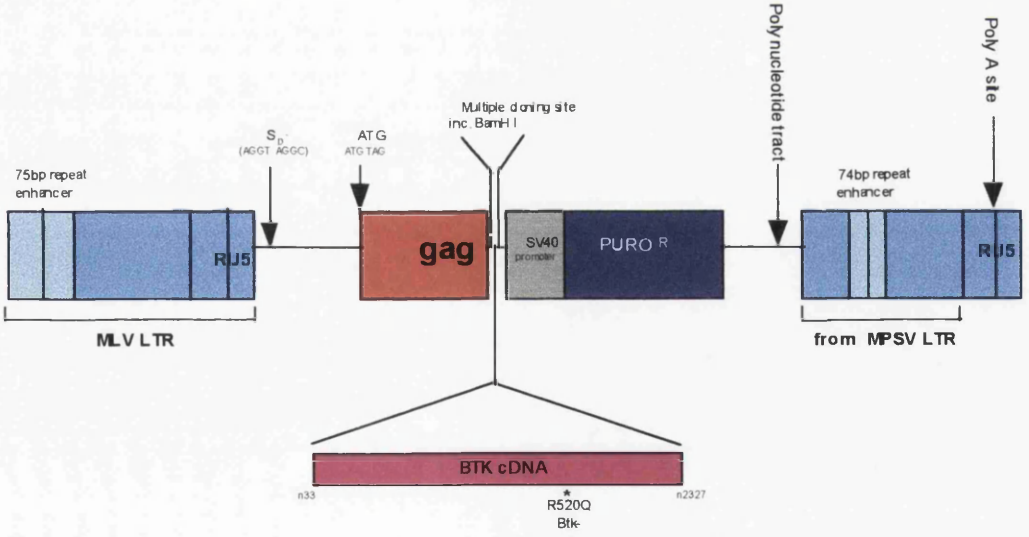


The basic pMB.SP construct demonstrating the 5' MLV LTR and the hybrid 3' MPSV LTR. The vector also contains a mutated splice donor site (S_D) and a mutated gag initiation codon. A multiple cloning site downstream of the gag sequence allows insertion of heterologous genes.

5.2.2 Introduction of Btk into pMB.SP

The full length Btk cDNA was obtained from a plasmid designated 14-6, a cloning Bluescript II KS vector into which the Btk cDNA had previously been introduced. Digestion of this vector with *PvuI* and *AvaII* allowed excision of a Btk cDNA fragment from nucleotide 33 to 2327. This 2.3kb sequence contains the entire Btk protein coding sequence but lacks the mRNA polyadenylation signal. The fragment was purified, blunt end ligated using T4 DNA polymerase buffer and repurified. This blunt ended fragment was then cloned into the *SmaI* site of the cloning vector PUC1813. The *SmaI* site exists in a multiple cloning site and is flanked by *BamHI* restriction sites. *BamHI* digestion of the plasmid resulted in the excision of a 2.3kb Btk fragment flanked by *BamHI* sticky ends which facilitated ligation of the Btk cDNA into the *BamHI* cloning site of the pMB.SP retroviral construct. The resultant retroviral construct containing the Btk cDNA was designated pMBbtk.SP (Fig. 5.5) and verified using restriction digest analysis (Fig. 5.6).

Fig. 5.5 pMBbtk.SP and pMBbtk-.SP retroviral vectors



A retroviral construct encoding wild type Btk was generated by insertion of the wild type Btk sequence into the BamHI cloning site (denoted pMBbtk.SP) A mutation was introduced into the kinase domain of Btk (R520Q) and this mutant sequence introduced into BamHI cloning site of the pMB.SP construct to generate an alternative vector encoding kinase inactive Btk (denoted pMBbtk-.SP).

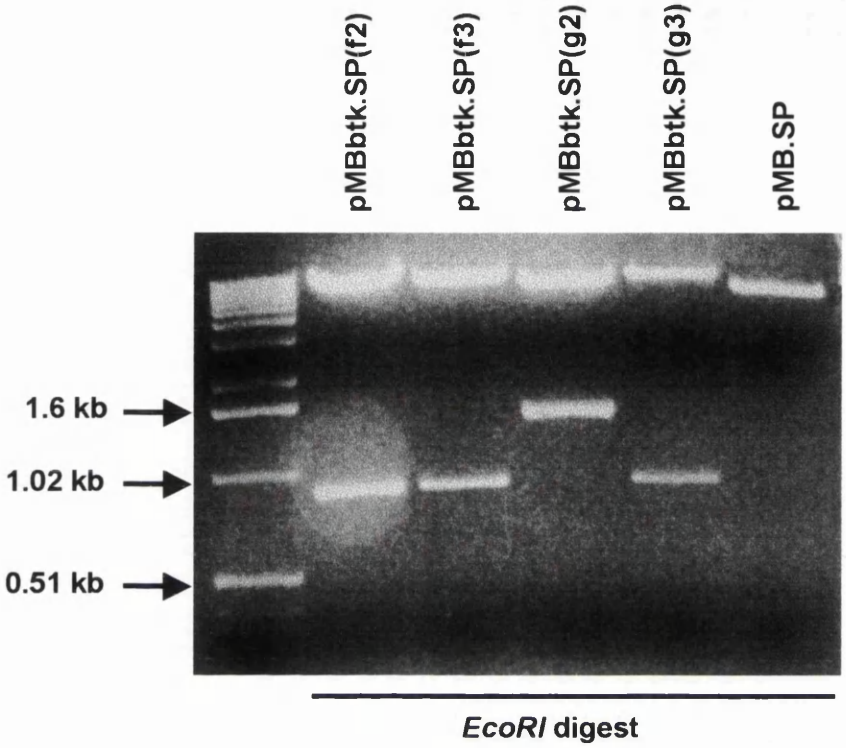


Fig. 5.6 Confirmation of pMBbtk.SP plasmid. *After ligation of the Btk cDNA into the Bam HI site of the pMB.SP vector, individual colonies were screened for cDNA insertion. Plasmid DNA was digested with EcoRI and analysed by agarose gel electropheresis and visualisation using ethidium bromide. Individual clones containing retroviral constructs with Btk cDNA inserted in the correct orientation show the presence of 0.82kb band (f2/f3/g3). Btk cDNA in the reverse orientation (g2) gives a 1.4kb band. The original pMB.SP vector is included as a negative control.*

5.2.3 Generation of a Btk Kinase mutant vector

A mutant Btk vector was also generated in our laboratory to allow greater understanding of the roles of the various domains of Btk in signalling experiments. The most common missense mutation so far identified in Btk is the R520Q substitution in the kinase domain of Btk. Mutation analysis in our laboratory had already identified an individual with this mutation. Therefore Btk cDNA from this patient was obtained and a cDNA fragment spanning the site of the mutation was amplified, isolated and purifed and then ligated into the PMBbtk.SP vector in place of the wild type sequence. The new vector with a Btk sequence containing the R520Q kinase domain mutation was designated pMBbtk-.SP (Fig 5.5) and the sequence was verified by direct sequencing analysis (data not shown).

5.3. Generation of replication defective recombinant retrovirus

5.3.1 Packaging cell line transfection

Having obtained the two retroviral constructs, the next step was the generation of a replication defective retrovirus capable of target cell infection. The plasmids containing the two constructs pMBbtk.SP and pMBbtk-.SP were introduced into the amphotropic packaging cell lines GP+envAm12 by calcium phosphate transfection. In addition the pMBbtk.SP construct was also introduced into the ecotropic ψ-2 packaging cell line using a liposome mediated transfection protocol. Successfully transfected cells were selected by growth in puromycin containing medium. After transfection the cells were diluted at different concentrations prior to plating and growth in puromycin containing medium. In this way, colonies arising from transfected single cells could be grown up and expanded. For each retroviral construct transfection, a number of puromycin resistant packaging cell clones were expanded and assessed for the production of replication defective recombinant retrovirus.

5.3.2 Assessment of retroviral titre

Retroviral titre has been shown to be of great importance in retroviral transduction with high titre producing cell lines capable of greater transfection efficiency (Pages et al., 1995; Soneoka et al., 1995). In order to measure the titre of the retroviruses produced by our transfected packaging cell line clones, 1 ml of viral supernatant was

used to transduce NIH3T3 cells (a murine fibroblast line). By sequentially diluting the transduced fibroblast cells and growing them in puromycin containing medium it was possible to quantitate the titre of retroviral supernatant from the different clones selected. The highest titre packaging cell clone was selected for further experiments. In the case of the pMBbtk.SP virus, 6 GP+am12 packaging cell line clones were selected and assessed for retroviral titre as outlined above. The GP+am12 cell line clone, denoted BtkC2, had the highest titre of 10^4 retroviral colony forming units (cfu)/ml and was used in subsequent experiments. Table 5.1 shows the titres of the various GP+am12 clones selected and assessed. In the case of the pMBbtk-.SP virus a clone, Btk-C23, also with a titre of 10^4 cfu/ml was selected.

Table 5.1 Analysis of retroviral titre from amphotropic clones

CLONE	1:10	1:100	1:1000
F2AM12	1000	5	-
F2C1	10	-	-
F2C2	1000+	50	10
F2C3	50	-	-
F2C4	-	-	-
F2C5	-	-	-
F2C6	50	-	-

In the case of the ecotropic producing packaging cell lines, once again a number of packaging cell line clones shedding pMBbtk.SP were expanded and analysed for retrovirus production. On this occasion, in order to screen a number of ecotropic clones more rapidly, northern dot-blot analysis of viral supernatant was used to determine the clone with the highest level of virus production (Fig 5.7). The ecotropic packaging cell clone ψ2C8 was found to give the strongest signal and was therefore used in subsequent experiments.

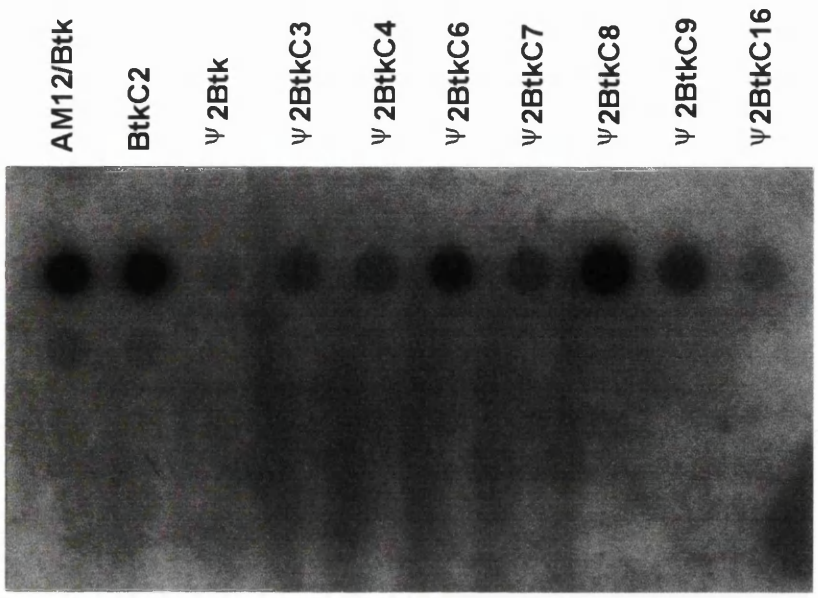


Fig. 5.7 Dot-blot analysis of ψ2 ecotropic Btk vectors to identify the highest titre virus. *Individual ψ2 packaging cell line clones shedding the Btk vector were screened for viral titre by RNA dot-blot analysis. 1ml of supernatant from each clone was taken and RNA prepared. After transfer to nitrocellulose, RNA was hybridised to a radiolabelled probe corresponding to exon 8 of Btk. Of the ψ2 clones, the stongest signal is from ψ2BtkC8. Positive controls from the polyclonal AM12/Btk producer and the Btk.C2 producer (estimated to have a titre of 104 cfu/ml) are shown.*

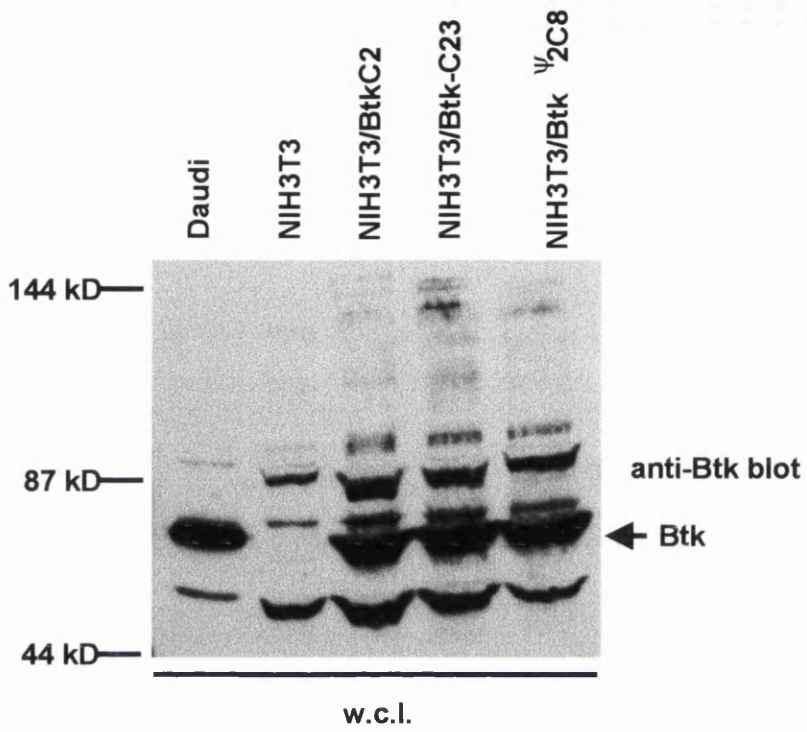


Fig. 5.8 Btk vectors express Btk efficiently in NIH3T3 cells. *Supernatant from amphotropic and ecotropic vectors shedding Btk and Btk- vectors was used to infect NIH3T3 cells. After selection in puromycin, cells were lysed and immunoblotted with H360 anti-Btk antisera. All transfected lines show expression of Btk protein. No Btk is seen in untransfected 3T3 cells.*

5.3.3 Generation of a mock virus

In order to ensure that any changes seen in experiments performed were not as a result merely of the introduction of a retroviral construct, a mock virus was generated. The backbone retroviral construct, pMB.SP, was introduced into the GP+am12 cell line and on this occasion a polyclonal population of puromycin resistant cells were selected and expanded. In similar 3T3 transduction experiments for assessment of retroviral titre (see section 5.3.2), this polyclonal packaging cell line pool, denoted MB.SP, was found to generate 10^4 cfu/ml.

5.3.4 Assessment of Btk expression by recombinant retrovirus

Although packaging cell line clones shedding recombinant retrovirus had been selected by the ability to express the selectable marker puromycin, this method of selection has only tested the expression of the puromycin resistance gene from the internal SV40 promoter. In order to examine whether the retrovirus produced was capable of infection and Btk expression, NIH3T3 cells were infected with supernatant from the selected amphotropic clones, MBbtk.C2, MBbtk-.SPC23 and the ecotropic clone MBbtk.SPψ2C8. Transduced puromycin resistant NIH3T3 cells were then expanded and then analysed for Btk protein expression by immunoblotting whole cell lysates with anti-Btk antisera. Fig 5.8 shows that all the recombinant retroviruses generated are capable of target cell infection and expression of the Btk gene from viral LTRs.

5.4 Discussion

A series of replication defective recombinant retroviruses have been generated. Amphotropic viruses containing wild type Btk and kinase mutant constructs have been produced as well as an amphotropic virus carrying only the puromycin resistance gene. The retroviral titre of the three packaging cell lines shedding these viruses has been assessed as being 10^4 cfu/ml. The BtkC2 and Btk-C23 constructs have been shown to be capable of fibroblast cell transduction and Btk/Btk- expression. In addition an ecotropic packaging cell line shedding wild type Btk has also been generated from the ψ-2 packaging cell line and been shown to be capable of infection and Btk expression. These vectors were used for the experiments outlined in the following two chapters.

6

Candidate Ligands for Btk

6.1 Introduction

The function of Btk in B cell development remains poorly understood. Although Btk has been implicated in a number of signalling pathways, its precise role within any particular signalling cascade has not been defined. Identification of other proteins to which Btk binds may help to position Btk within the intracellular signalling network. A number of strategies, both *in vitro* and *in vivo*, have been used to try and identify binding proteins. Although physical association of the whole Btk protein with other molecules has been difficult to demonstrate, data has been more forthcoming from alternative methods.

6.1.1 GST fusion protein studies with Btk

One such method has been the use of Glutathione Sepharose Transferase (GST) fusion proteins containing the various modular domains of Btk. While the kinase domain of Btk is thought to hold the enzymatic activity of the protein, the other domains are thought to be involved in protein-protein binding interactions Constructing fusion proteins containing the protein binding domains can identify proteins that bind to that particular domain from chosen cell types. Although this is an *in vitro* system, it could nevertheless give initial clues as to possible ligands which can then be tested in a more physiological setting. Studies using GST fusion proteins have been performed with all the various domains of Btk. SH3 domains are known to bind to proteins containing proline rich sequences and this has been demonstrated using a Btk-SH3 GST fusion protein. This construct has been shown to bind to the

proline rich peptides c-cbl and WASP (Wiskott-Aldrich syndrome protein) (Cory et al., 1995; Cory et al., 1996). As yet no *in vivo* data demonstrating a physical association between Btk and these proteins has been reported.

SH2 domains have been shown to contain a binding pocket that allows binding to phosphorylated tyrosine residues (Pawson, 1995). This would be concomitant with the idea that Btk may be involved in a signalling cascade involving other tyrosine kinases. However, Btk-SH2 GST fusion protein studies have not convincingly demonstrated a specific tyrosine phosphorylated protein that might bind to Btk.

6.1.2 Interactions of the Btk-PH domain

PH domains have been found in a number of signalling molecules and it has been suggested that they act as general membrane localization signals, though the binding partners mediating this process may be very diverse. Btk-PH GST fusion protein studies have identified two proteins to which Btk might bind. It has previously been shown that the PH domain of the β-adrenergic receptor kinase (β-ARK) binds to the βγ subunit complex of heteromeric guanine nucleotide binding proteins (G proteins) (Koch et al., 1993). It was also shown that PH domain GST fusion constructs, containing the complete PH domain and adjacent sequences, of other proteins such as Ras-GAP, PLCγ, β-spectrin, IRS-1, and Atk (as Btk was then known) were also able to bind Gβγ *in vitro* (Touhara et al., 1994). In this assay the constructs containing the PLCγ, and Btk PH domains showed the greatest Gβγ binding activity. The Gβγ binding site was localised to the C-terminal region of the PH domain and a few amino acids beyond. A subsequent study using GST fusion constructs containing only subdomains 5 and 6 of the PH domain and its C-terminal flanking sequence was shown to bind Gβγ as efficiently as the complete PH domain itself thus confirming the localisation of the Gβγ binding site (Tsukada et al., 1994). This latter study also provided evidence of an *in vivo* association of the Btk-PH domain with Gβγ subunits using an indirect assay system. COS-7 cells expressing a G-protein coupled receptor stimulated with an appropriate agonist exhibit increased phosphatidyl inositol (PI) hydrolysis with the release of IP, as a result of Gβγ activation of β PLC. Transfection of the Btk-PH domain into these cells considerably reduced the PI hydrolysis which

was interpreted as being due to Btk-PH sequestration of Gβγ and thus decreased β PLC activation.

6.1.2.1 Interaction of the Btk-PH domain with Protein Kinase C

A second possible ligand for the Btk-PH domain was again determined using the GST fusion protein system. Using bone marrow derived mouse mast cell lysates, binding of the GST-Btk-PH domain to several different isoforms of protein kinase C (PKC) was shown (Yao et al., 1994). In the same set of experiments, it was also shown that a PH domain construct carrying the *xid* mouse R28C mutation bound PKC isoforms with a significantly reduced binding capacity.

The same study also reported the first association of full length Btk protein with another protein. Btk in the lysates of unstimulated or IgE/antigen stimulated MCP-5 cells, an immortalised bone marrow derived mouse mast cell line, was immunoprecipitated with an anti-Btk antibody. Immunoblotting of the immunoprecipitates with an anti-PKC monoclonal antibody showed co-immunoprecipitation of PKC with Btk suggesting a physical association between these two proteins *in vivo* in mast cells. In reciprocal experiments, Btk was detected in anti-PKC immunoprecipitates. Furthermore it was shown that PKC can serine phosphorylate Btk and GST Btk-PH and the effect of this phosphorylation was significant downregulation of Btk autophosphorylation activity. No such association and interaction has been shown in human cells.

6.1.3 Protein Kinase C β as a candidate ligand for Btk

The PKC family of serine/threonine kinases have been implicated in a wide variety of signalling pathways. Over ten different isoforms of PKC have been identified, all showing a high degree of structural homology but each with their own patterns of tissue expression and activation (Dekker and Parker, 1994). The family of proteins has been broadly split into 3 groups based on their requirement for Ca^{++} or diacylglycerol (DAG) or both for their catalytic activity. The PKC-α, PKC-β1 -β2 and -γ isoforms are dependent on both Ca^{++} and DAG, while the PKC-δ, -ε, -η, and θ isoforms are DAG dependent but Ca^{++} independent. PKC-ζ and -λ are not activated

by either Ca^{++} or DAG. Although various isoforms of PKC are known to be expressed in lymphocytes very little is known about their physiological role.

Evidence for a crucial role for PKC-β in B lymphocyte development came from the generation of a PKC-β knockout mouse in which the expression of both -β1 and -β2 isoforms was disrupted (Leitges et al., 1996). PKC-$\beta^{-/-}$ were shown to have a number of phenotypic abnormalities. Most striking was the significant reduction in B-1 ($CD23^-$, IgM^+) and B-1a ($CD5^+$, IgM^+) B lymphocyte populations in the peritoneal cavity of PKC-$\beta^{-/-}$ mice in comparison with wild type mice. The pre-B and mature B lymphocyte populations in the bone marrow and the B cell populations in the spleen and lymph nodes were not significantly affected. The levels of IgM and IgG3 were significantly reduced in PKC-$\beta^{-/-}$ mice but the titres of other immunoglobulins were found to be similar to those found in wild type mice. Furthermore PKC-$\beta^{-/-}$ mice were shown to be unable to mount a detectable humoral reponse to T-independent antigens. B cell proliferative responses to IgM stimulation were decreased ten fold in comparison to the responses seen in wild type mice. Some of these phenotypic abnormalities closely resemble the abnormalities seen in *xid* and in Btk knockout mice. This data, taken together with the physical associations between Btk and PKC shown by Yao et al. (1994), suggest a physical and/or functional link between these two molecules. It may be hypothesised that Btk and PKC-β participate in the same signalling pathways in B cell development such that the absence of one or the other results in highly similar B lymphocyte abnormalities.

6.1.4 Alternative candidates for Btk binding

Other candidates were also considered for binding to Btk. The choice for candidate proteins came from the available information on B cell receptor signalling. The temporal sequence of events following B cell stimulation suggests that a number of phosphotyrosine kinases are activated (Saouaf et al., 1994). Studies measuring the timing of PTK activation indicate that Lyn and Blk are activated seconds after BCR stimulation while Btk shows increased autophosphorylation activity 2-5 minutes following stimulation. No studies had showed an association between Btk and other PTKs though it could be postulated that PTKs proximal to the BCR such as Lyn or Blk might interact with Btk in a sequential kinase signalling cascade.

It was also not known what the downstream effect of Btk might be. Again studies suggested that B cell receptor stimulation resulted in the increased activation of certain effector molecules. Phospholipase-γ1 had previously been shown to be activatated following T cell receptor stimulation resulting in the production of second messengers, namely inositol phosphates (and primarily inositol 1,4,5-triphosphate, IP_3) (Mustelin et al., 1990). It was further shown that this effect could be blocked by the inhibition of protein-tyrosine kinase activity, suggesting that PTKs play a critical role in PLC-γ1 stimulation. Studies on B lymphocytes showed that B cell receptor stimulation also resulted in PLC-γ1 activation (Mustelin et al., 1990). However a further report, while confirming the above study, documented that in B lymphocytes PLC-γ2 was the predominant isoform expressed and that it, not PLC-γ1, was primarily stimulated following BCR crosslinking (Coggeshall et al., 1992). Such studies identify phospholipase-γ as a substrate for the action of a putative B cell tyrosine kinase.

Another protein which is tyrosine phosphorylated and activated following BCR stimulation results is phosphatidyl inositol-3 kinase (PI-3 kinase). The PI-3 kinase is a heterodimeric protein consisting of a non-catalytic p85 subunit, catalytic 110-kD subunits and phosphorylated inositol lipids. It catalyses the phosphorylation of PI-4 P and PI-4,5-P2 on the D3 position of the inositol ring to yield PI-3,4-P_2 and PI-3,4,5-P_3. Anti-sIgM crosslinking stimulation of PI-3 kinase activation was seen in murine cells at different stages of maturation and also in the mature human B cell lines, Daudi and Ramos (Gold et al., 1990). Studies have also shown that the p85 subunit of PI-3 kinase is associated with the src-like kinase Lyn (Yamanashi et al., 1992) and furthermore that this association is mediated by the binding of a proline region of the p85 subunit to the SH3 domain of src kinases resulting in an increase in PI-3 kinase activity (Pleiman et al., 1994). This data together with studies from fibroblasts in which PI-3 kinase was shown to be activated by tyrosine phosphorylation (Varticovski et al., 1991) suggests that PI-3 kinase may be associated with Src family tyrosine kinases in B cell receptor signalling.

6.2 Models to study Btk binding

In order to test the above hypotheses, the binding of Btk to PKCβ1, PLC-γ and the p85 subunit of PI-3 kinase was studied in two different models. B cells provide the most physiological setting for this assay and so the mature B lymphoblast cell line, Daudi was used. Cells were stimulated via the BCR receptor for 2 and 5 minutes prior to lysis. Effective tyrosine phosphorylation of a variety of proteins is shown by immunoblotting of whole cell lysates using the antiphosphotyrosine antibody 4G10 (Fig 6.1a). Equal loading of samples can be seen when the lysates are immunoblotted with anti-Btk antibody (Fig. 6.1b). Btk was also immunoprecipitated from stimulated and unstimulated Daudi whole cell lysates (Fig. 6.2) and blotted with the anti-Btk antibody. Non-specific immunoprecipitation can be excluded by the absence of Btk in normal rabbit serum immunoprecipitates (Fig. 6.2.). Btk immunoprecipitates from stimulated and non stimulated cells were used for co-precipitation studies.

A surrogate system was also employed. Using Btk retroviral vectors carrying the Btk wild type and Btk kinase mutant (Btk-), both forms of Btk were introduced into NIH3T3 fibroblasts. The fibroblast cell line was repeatedly infected with retroviral supernatant on successive days in order to over express both forms of Btk in this cell type. By expressing Btk and Btk- at high levels it was hoped that binding to a putative ligand could be demonstrated. Western blot analysis of 3T3 fibroblast whole cell lysates with anti-Btk antibody was performed. While no Btk expression is seen in uninfected cells, relatively high levels of Btk and Btk- expression can be seen in 3T3 transfected cells (Fig 6.3a). 3T3 cell lysates were also immunoprecipitated with anti-Btk antibody and subject to *in vitro* kinase assay. As can be seen from Fig 6.3b, cells infected with the Btk- vector, despite the presence of a mutation in the kinase domain show evidence of autophosphorylation activity of a 77kD protein, although the activity is considerably reduced.

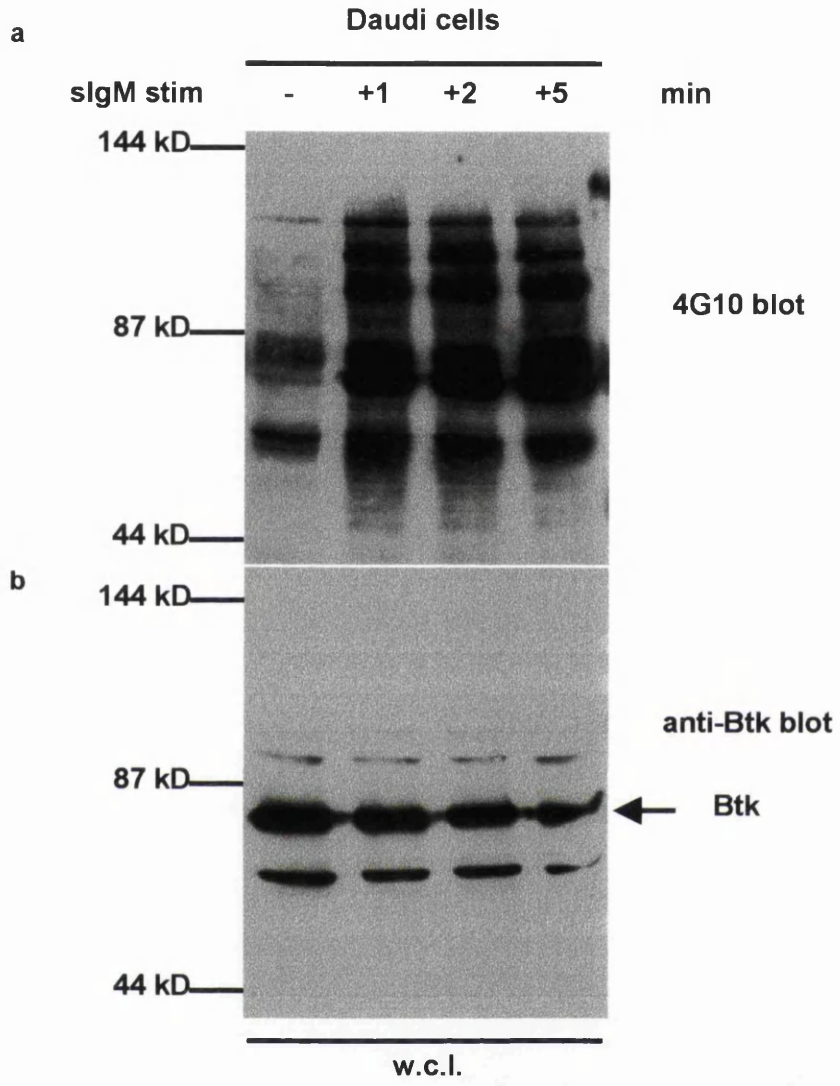


Fig 6.1 Increased tyrosine phosphorylation of Daudi cells on sIgM stimulation. a) *Daudi cells were incubated with cross-linking sIgM antibody for the time periods shown before lysis. Whole cell lysate was immunoblotted with antisera to phosphorylated tyrosine residues, 4G10. Phosphorylation of a number of different proteins is seen within a minute of sIgM activation.* **b)** *Equivalent amounts of lysate were blotted with H360B and similar amounts of Btk are seen in all lanes.*

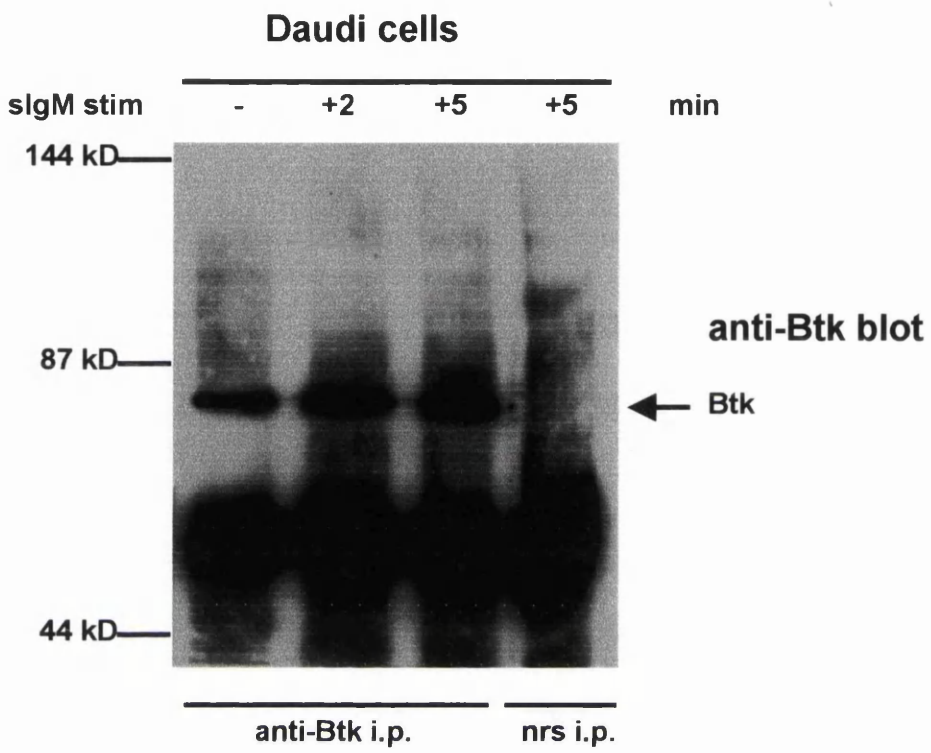


Fig. 6.2 H360B precipitates Btk from Daudi cells. *Cells were stimulated with anti sIgM for the times shown prior to lysis. Cleared supernatant was incubated with H360B antisera before immunoblotting with same antisera. Btk is precipitated in equal amounts from stimulated and unstimulated cells. Normal rabbit serum immunoprecipitation (nrs i.p.) is shown to exclude the possibility of nonspecific immunoprecipitation.*

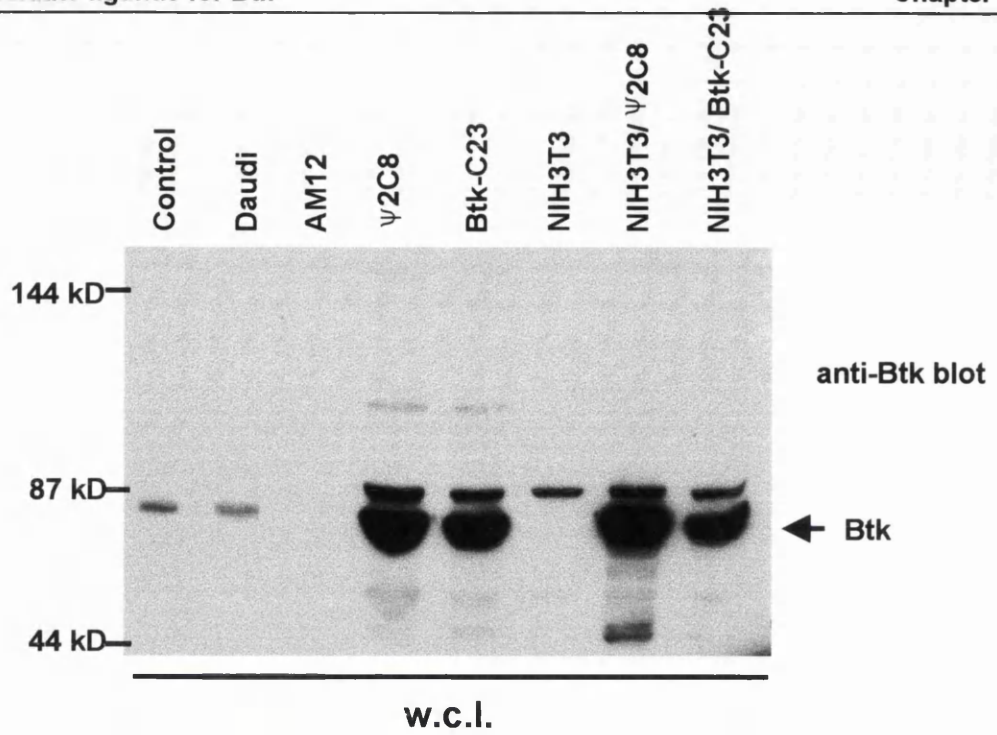


Fig. 6.3a Expression of Btk in NIH3T3 cells. *3T3 fibroblasts were repeatedly infected with supernatant from Btkψ2.C8 and Btk-.C23 producer cell lines. Producer cell lines and infected 3T3 cells were grown to confluence, lysed and immunoblotted with anti-Btk antisera. Btk is shown to be expressed at high levels in transfected cell lines. A Daudi cell lysate is included as a positive control.*

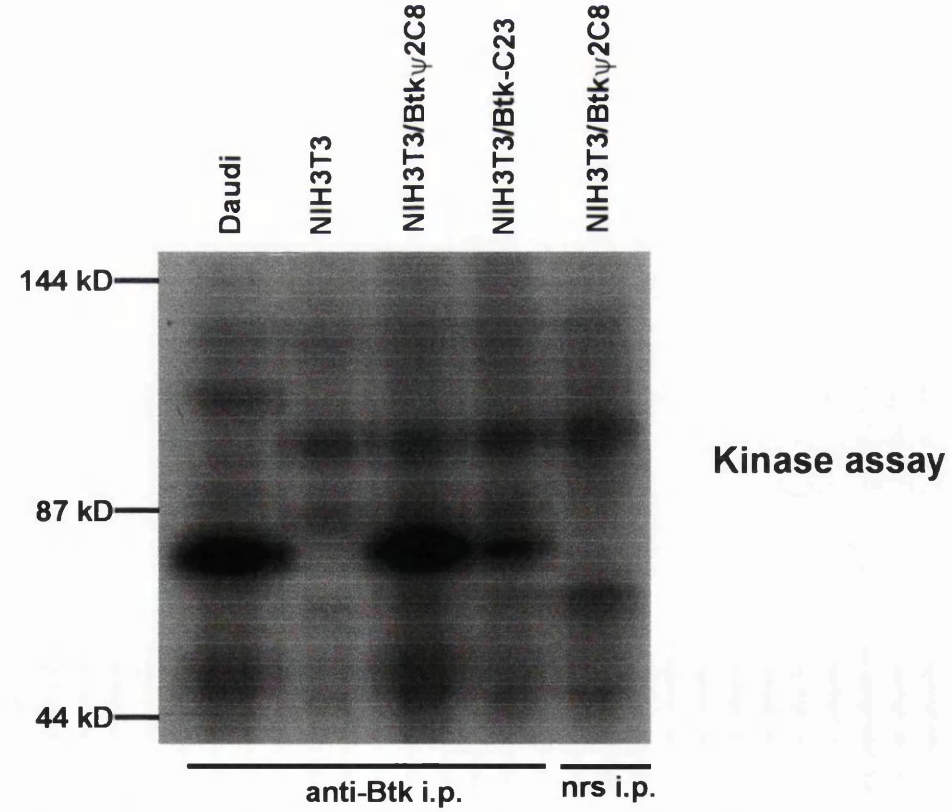


Fig. 6.3b Autophosphorylation activity of Btk in transfected NIH3T3 cells *Transfected and untransfected NIH3T3 cells were lysed, the supernatant cleared and incubated with H360B antisera. The immunoprecipitates were subject to in vitro kinase assay. Wild type transfected cells show autokinase activity and the Btk- transfected cells show considerably reduced activity. nrs i.p. (see previous legend) does not show any non-specific kinase activity.*

6.2.1 Btk/ PKCβ1 co-immunoprecipitation studies

Initial studies focused upon the association of Btk with PKCβ1 in Daudi cells. For each immunoprecipitation ten million cells were taken and either stimulated with sIgM or left unstimulated. Immunopreciptation was then carried out using an anti-Btk antibody or an anti-PKCβ1 antibody or immunoprecipitated with normal rabbit serum to exclude non-specific binding. Immunoprecipitates were then subject to western blot analysis with either anti-Btk antibody or the anti-PKCβ1 antibody. Immunoblotting with anti-Btk shows the presence of Btk in anti-Btk immunoprecipitates but not in anti-PKCβ1 immunoprecipitates (Fig 6.4). Conversely immunoblotting with PKCβ1 demonstrates PKCβ1 in anti-PKCβ1 precipitates but not in anti-Btk immunoprecipitates (data not shown). Analysis of non rabbit serum immunopreciptation samples shows the lack of any non-specific binding. Since no coprecipitation was seen in these initial studies, further experiments were carried out in the 3T3 fibroblast model.

Near confluent plates of stimulated or non-stimulated 3T3 fibroblasts and Btk/Btk-transfected 3T3 fibroblasts were grown overnight in DMEM without additives, washed and then stimulated with recombinant human epidermal growth factor (RhEGF) or left unstimulated (RhEGF has been shown in a number of studies to activate tyrosine kinases in 3T3 fibroblasts (Margolis et al., 1990; Decker et al., 1992)). Cells were incubated at 37^0c for 20 mins prior to lysis. Again, whole cell lysates from stimulated and unstimulated cells were immunoprecipitated with either anti-Btk, anti- PKCβ1 or normal rabbit serum. All immunoprecipitates were then immunoblotted with both sets of anti sera. Blotting the anti-Btk immunopreciptate with anti-Btk shows that Btk has been succesfully immunoprecipitated from the Btk/Btk- transfected 3T3 cells (Fig 6.5). However, immunoblotting the same samples with anti-PKCβ1 does not show any co-immunoprecipitation of PKCβ1 with Btk (Fig 6.5). Similar findings were noted on analysis of anti-PKCβ1 immunoprecipitates. No co-immunoprecipitation of Btk with PKCβ1 was seen on anti-Btk immunoblotting (Fig 6.6).

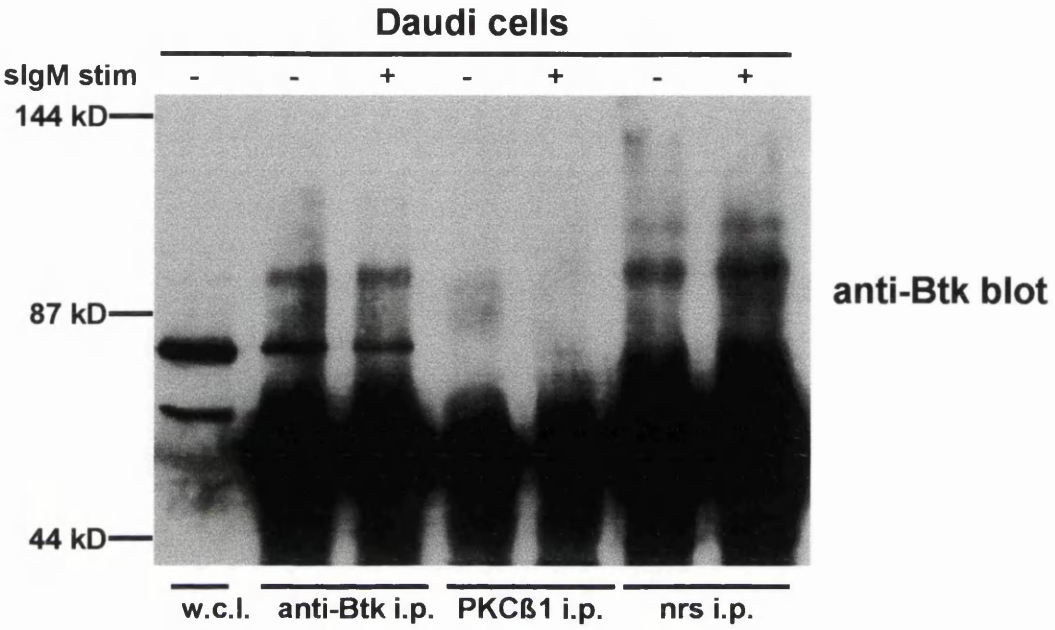


Fig. 6.4 PKCβ1 is not immunoprecipitated with Btk in Daudi cells. *Daudi cells were stimulated with sIgM and lysed. H360B and anti-PKCb1 antisera were used to immunoprecipitate Btk and PKCβ1 respectively from whole cell lysates. Immunoblotting the immunoprecipitates with H360B confirms Btk immunoprecipitation but does not show any evidence of PKCβ1 coprecipitation on blotting with anti-PKCβ1 anti-sera.*

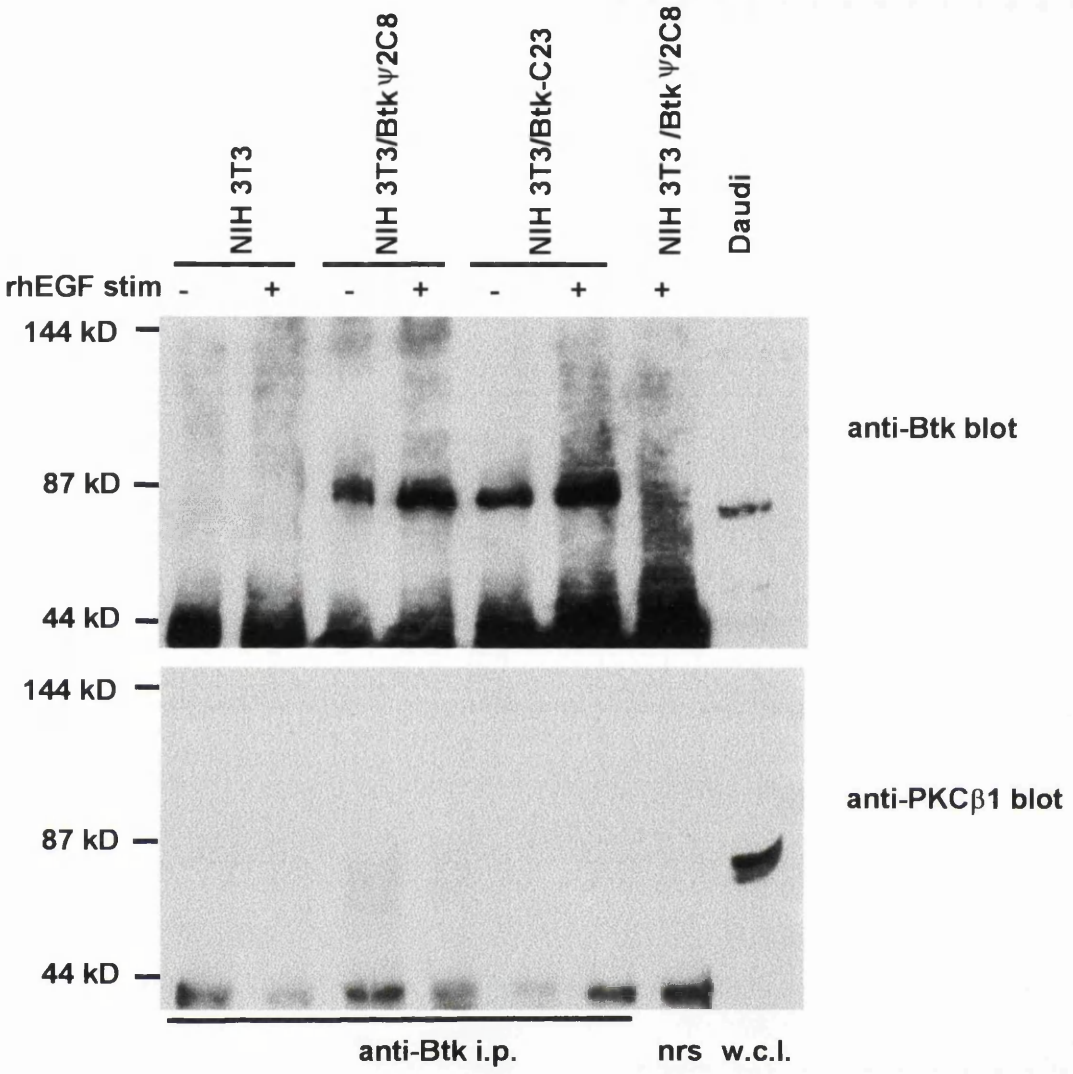


Fig. 6.5 PKCβ1 is not immunoprecipitated with Btk in transfected NIH3T3 cells *Transfected cells were stimulated with rhEGF and lysed. H360B anti-Btk antisera was used to immunoprecipitate Btk from whole cell lysates. Immunoblotting the immunoprecipitates with H360B confirms Btk immunoprecipitation (upper panel) but immunoblotting the same immunoprecipitates with PKCβ1 does not show any evidence of PKCβ1 coimmunoprecipitation (lower panel).*

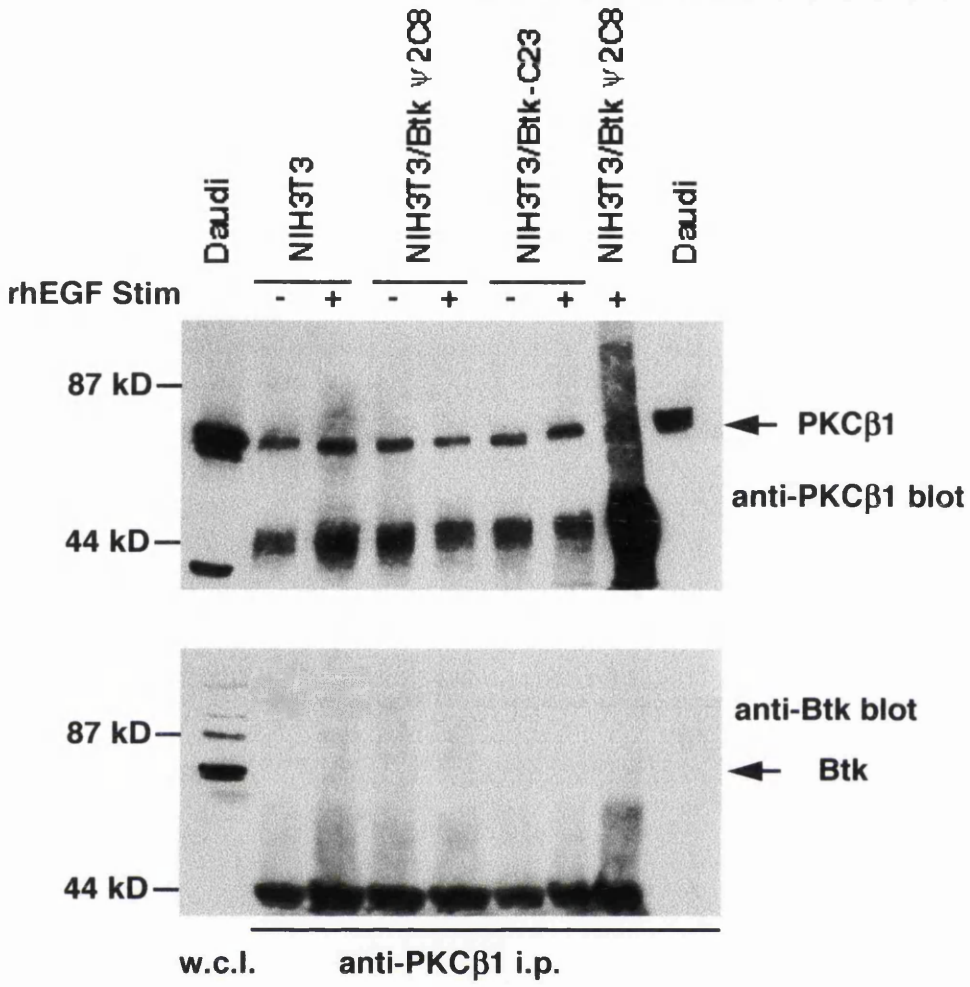


Fig. 6.6 Btk is not immunoprecipitated with PKCβ1 in transfected NIH3T3 cells *Transfected cells were stimulated with rhEGF and lysed. PKCβ1 antisera was used to immunoprecipitate Btk from whole cell lysates. Immunoblotting the immunoprecipitates with H360B does not show any evidence of Btk coimmunoprecipitation (lower panel).*

6.2.2 Btk/PI-3 kinase co-immunoprecipitation studies

Similar experiments were used to study the binding of the p85 subunit of PI-3 kinase to Btk. Btk immunoprecipitates from both stimulated and unstimulated Daudi cells and transfected 3T3 fibroblasts, having previously been shown to have successfully immunoprecipitated Btk (Figs 6.2 and 6.5) were then immunoblotted with an anti-p85 antibody. However in both sets of experiments no co-immunoprecipitation of p85 with Btk was seen (data not shown). The same experiments were attempted in the reverse direction i.e. immunoprecipitating with anti-p85 and immunoblotting with anti-Btk. However, western blot analysis of these immunoprecipitates with anti-p85 did not show successful p85 immunoprecipitation (data not shown) and thus it was not possible to study Btk co-immunoprecipitation.

6.2.3 Btk/PLC-γ2 co-immunoprecipitation studies

Btk immunoprecipitates from stimulated and non stimulated Daudi cells were also immunoblotted with PLC-γ2 antisera to look for association with Btk. However as with the previous experiments no physical association between these molecules was seen (data not shown). The PLC-γ2 antibody did not successfully immunoprecipitate PLC-γ2 and so it was not possible to study binding using PLC-γ2 immunoprecipitates.

6.3 Discussion

Our studies reported above have not been able to show any *in vivo* associations of Btk with the PKCβ1, p85 or PLC-γ2 proteins. A number of lines of evidence have suggested that these proteins may be associated with Btk in B lymphocyte signalling. Indeed in the case of PKCβ1, studies in mice have shown a physical association between these two proteins. Two general conclusions can be drawn from these experiments: either that these associations do not exist or secondly that the physical associations do exist but the methods used to demonstrate them were not capable of doing so. The latter possibility is discussed first.

6.3.1 Possible reasons for lack of Btk binding

The antibody used for both immunoprecipitation and immunoblotting, designated H360B, was a fusion protein construct raised against residues 163-218 in the PH

domain of Btk. It has already been shown that the PH domain is involved interactions with other proteins, especially PKCβ1 and the βγ subunits of heterotrimeric G proteins. Binding of a high affinity antibody to the Btk PH domain could well have interfered with the binding of other proteins to this domain. Although Tsukada et al. (1994) have shown that only subdomains 5 and 6 of the Btk PH domain (residues 94-137) are necessary for βγ subunit binding, the presence of the H360B antibody at residues 163-218 may interfere with this interaction. Alternatively, PH domain binding ligands may inhibit the binding of the H360B antibody to its epitope thus resulting in the immunoprecipitation of only uncomplexed Btk. The affinity of binding ligands to Btk may be less than that of a highly specific antisera such as H360B, such that in competition only binding of the immunoprecipitating antibody was seen.

These problems could have been tested using an antibody directed against an alternative Btk epitope. A commercially available polyclonal goat Btk antibody (Santa Cruz Biotech.) directed at a C terminal epitope of Btk was purchased and used in Btk co-immunoprecipitation studies. However, Btk was not successfully immunoprecipitated using this antibody (data not shown). Alternatively the use of an epitope tagged Btk protein, with antisera directed against the tag used for immunoprecipitation studies, may allow unhindered binding of ligands to Btk domains.

The interactions studied above may occur only transiently *in vivo*. Btk-ligand complexes and unbound Btk may exist in a dynamic equilibrium. Depending upon the state of activation of the cell there may be a high rate of dissociation of ligands from Btk such that only a small proportion of total cellular Btk remains in a complexed form. Under these circumstances it may be difficult to detect the very small amounts of ligand that are indeed bound to Btk.

Cell lysis was performed using a detergent based lysis buffer (1% NP-40 lysis buffer). It is possible that the use of such a strong lytic agent may have disrupted any protein associations prior to immunoprecipitation. Detergent based buffers solubilse cell membranes and thus may disrupt Btk interactions that are dependent upon

membrane anchoring or localisation. This may be especially important in those interactions involving the PH domain which has been implicated in membrane localisation. Alternative hypotonic lysis buffers were used for other Btk co-immunoprecipitation studies in this laboratory but no *in vivo* Btk interactions were detected using these conditions.

Finally, it has been shown that Btk is autophosphorylated on Y223 (Park et al., 1996). This tyrosine residue is predicted to be part of the ligand binding surface of the SH3 domain of Btk. Phosphorylation at this site may lead to the disruption of SH3 domain binding and dissociation of putative SH3 domain ligands. Our experiments show that even in the absence of cell stimulation, there is still significant Btk *in vitro* kinase activity (Fig. 6.2b). The process of immunoprecipitation may lead to clustering of Btk and transphosphorylation and activation *in vitro* thus preventing the co-immunoprecipitation of SH3 domain interaction proteins such as PI-3 kinase.

Such problems might have been overcome in the experiments performed with the Btk- construct transfected into fibroblast cells, although it can be seen that there was still *in vitro* kinase activity present upon immunoprecipitation. This may have arisen a result of non-specific phosphorylation by other kinases.

6.3.2 Molecular interactions of Btk

Since our studies have not been able to demonstrate the binding of Btk to certain candidate proteins, the question must be asked as to whether Btk does indeed bind to these proteins. Review of more recent evidence suggests that at least in the case of PI-3-kinase and PLC-γ2, Btk is associated in pathways involving these two proteins albeit indirectly. The case of Btk involvement with PKC appears less convincing.

6.3.2.1 Btk and PKCβ1

In initial experiments involving stimulation of murine bone marrow mast cells by crosslinking of FcεRI, Btk was shown to be tyrosine phosphorylated and activated (Kawakami et al., 1994). In this same report it was suggested that its activation occurred prior to the increase in intracellular Ca^{++} and PKC activation. The same authors, again using a murine bone marrow mast cell line, then showed a direct

interaction between the Btk-PH domain and PKCβ1 and also an *in vivo* physical association of full length Btk with PKCβ1 (Yao et al., 1994). It has been suggested that in the latter experiment, the PKC preparations used were commercially obtained and reported to be '>95% pure'. It is possible that the PKC preparations contained small amounts of protein that may have linked Btk to PKC (Shaw, 1996).

More recently, an *in vivo* functional link has been suggested by the similarity in the immunological phenotypes of the PKCβ1 deficient and *xid* mice (Leitges et al., 1996), but no further studies have demonstrated a direct interaction between these two molecules. No studies in human cells have as yet been reported.

6.3.2.2 Btk and Phosphoinositide binding

In our studies we have not been able to demonstrate a direct interaction between Btk and PI-3 kinase. However, recent reports have suggested that Btk may play a significant role in PI-3 kinase associated pathways via PH domain mediated interactions. It has previously been suggested that PH domains act as general membrane localisation signals. Harlan et al. (1994) showed that the N-terminal domains of pleckstrin and a variety of other PH domains bound to the phospholipid membrane molecule PI-4,5-P_2. Using a biosensor-based assay, Salim et al. (1996) investigated the binding of the Btk-PH domain to a variety of inositol phospholipids and were able to demonstrate a specific interaction between the Btk-PH domain and PI-3,4,5-P_3. Moreover mutation of R28 to tyrosine (the mutation in *xid* mice is R28C and in certain XLA patients R28H) abolished the binding to PI-3,4,5-P_3, emphasising the importance of this residue in ligand recognition.

The above information suggests an interacting pathway for Btk and PI-3 kinase. PI-3 kinases are activated following sIgM stimulation but how they exact a cellular effect is not known. However, from the data above one might speculate that PI-3 kinase mediated conversion of PI-4,5-P_2 to PI-3,4,5-P_3 may lead to a PH-domain mediated recruitment of Btk to the cell membrane. Furthermore this may anchor Btk and thus facilitate Btk interaction with other membrane bound substrates. This is further discussed in Chapter 8.3.

6.3.2.3 Btk and PLCγ

While previous studies have suggested that PLC-γ might be activated by protein-tyrosine kinases, it has only been shown recently that Btk is involved in this pathway. Studies were conducted using chicken DT40 B cells (Takata and Kurosaki, 1996). BCR stimulation of wild type DT40 cells and DT40 cells deficient in Btk showed that the elevation in intracellular calcium levels was completely abrogated in Btk deficient cells. Furthermore in the mutant cell line the BCR-induced inositol 1,4,5-trisphosphate generation was completely abolished. These events are known to be dependent upon the tyrosine phosphorylation of PLC-γ2 (Takata et al., 1995a), thus the tyrosine phosphorylation of PLC-γ2 was examined. It was shown that although Btk-deficient DT40 cells were still capable of PLC-γ2 tyrosine phosphorylation, its extent was lower than that seen in wild type cells. Transfection of wild type Btk into Btk deficient cells was able to restore PLC-γ2 tyrosine phosphorylation to wild type levels but this was not seen using Btk constructs with mutations in the PH, SH2 or kinase domains of Btk. These results suggest that Btk mediates tyrosine phosphorylation of residues of PLC-γ2 critical for PLC-γ2 activation. The latter experiments established the importance of the PH, SH2 and kinase domains in Btk mediated PLC-γ2 activation.

6.3.3 Summary

We have attempted to study *in vivo* binding of Btk to candidate ligands in two separate systems. No physical association of Btk with these proteins was seen. Recent evidence has suggested that Btk may well interact with these proteins but this is most likely to be an indirect association mediated by as yet unidentified molecules. Since its identification in 1993, and despite the efforts of numerous laboratories, Btk has only been shown to physically associate with PKCβ1 and more recently with BAP-135 (Yang and Desiderio, 1997) (further discussed in Chapter 8.4.1). Its elusive role in B cell signalling continues to be the subject of much research. Furthermore, even if ligands for Btk in the signalling pathways discussed are elucidated, the question of how this relates to its critical function in B lymphocyte maturation remains unanswered.

7

Development of an *in vitro* model of gene therapy for XLA

7.1 Introduction

The development of gene therapy for the primary immunodeficiencies has evolved through a number of different stages. In many cases, the first step has been to create an *in vitro* model in which to test gene transfer, expression and if possible correction of the disease phenotype. For the primary immunodeficiencies the most common target for initial gene transfer has been EBV immortalised B lymphoblastoid cell lines (EBV-LCLs) from affected individuals.

7.1.1 Models of gene therapy using B cell lines

EBV LCLs are good targets for gene transfer since they are grown easily in culture and have been shown to be readily transfected by retroviral vectors. Furthermore, such lines can be readily generated from affected individuals and can be expanded, stored and used when required.

7.1.1.1 Gene transfer in chronic granulomatous disease

Initial diseases to be studied by gene transfer into EBV-LCLs were X-linked and autosomal recessive chronic granulomatous disease (X-CGD and AR-CGD, respectively). In this condition neutrophils and macrophages are primarily affected since NADPH oxidase, a multicomponent system responsible for the reduction of molecular oxygen to superoxide (O_2) is defective. In X-CGD the gp91-phox gene product, an integral component of the NADPH oxidase system is defective while in

AR-CGD one of three proteins essential for NADPH oxidase function may be defective (Roos et al., 1996b). EBV-LCLs from CGD patients are similarly lacking in normal NADPH oxidase function and therefore function as a good surrogate model in which to test the usefulness of gene transfer. A number of studies have used retroviral mediated transfer of the deficient gene into such cell lines (Thrasher et al., 1992; Porter et al., 1993). In both the X-linked and autosomal recessive forms of the condition, reconstitution of NADPH oxidase function was demonstrated by gene transfer, thus illustrating the feasibility of genetic therapy albeit in an *in vitro* setting.

7.1.1.2 Gene transfer in X-SCID

Similar studies have been carried out more recently for X-linked and autosomal recessive forms of severe combined immunodeficiency arising from γ_c chain deficiency and JAK3 deficiency, respectively (see section 1.6.8). Mutations in the γ_c cause abnormalities in signalling through the IL-2, -4, -7, -9, and 15 receptors. In EBV-LCLs from X-SCID patients, there is abnormal expression of the γ_c on the cell surface. Furthermore, stimulation of these cells by IL-2 or IL-4 fails to tyrosine phosphorylate the non-receptor tyrosine kinases, JAK1 and JAK3, both of which are direct downstream signals of a fully functional IL-2 or IL-4 receptor. An MLV based retroviral construct encoding the γ_c cDNA and the neomycin resistance gene separated by the encephalomyelitis internal ribosomal entry sequence (IRES) was used to express the γ_c protein in EBV-LCLs cells from X-SCID patients (Candotti et al., 1996a). Reconstituted cell lines showed normal expression of the γ_c on the cell surface and furthermore demonstrated tyrosine phosphorylation of the JAK1 and JAK3 proteins after cytokine receptor stimulation. Very similar reconstitution of JAK1 and JAK3 phosphorylation was reported by similar studies, again using retroviral mediated gene transfer (Taylor et al., 1996; Hacein et al., 1996).

The importance of these studies was not only to illustrate the feasibility of gene transfer and the reconstitution of a functional cytokine receptor but to also allow new insights into IL-2R signalling pathways. It had previously been shown that JAK3 associates with the γ_c and that JAK1 constitutively associates with the IL-2Rβ. However, it was not known whether the γ_c was necessary for IL-2 mediated JAK1 phosphorylation. The results from these experiments clearly show a lack of JAK1

tyrosine phosphorylation in mutant cells, and correction upon γ_c gene transfer and expression.

7.1.1.3 Gene transfer in JAK 3 deficiency

Mutations in the JAK3 tyrosine kinase have been shown to result in an autosomal recessive form of SCID with a phenotype similar to γ_c deficiency, unsurprisingly since both share a common signalling pathway. In very similar experiments to those described above, an MLV based retroviral construct was used to transfer and express the full length JAK3 cDNA into EBV-LCLs from a JAK3 defcient patient (Candotti et al., 1996b). Analysis of transfected cells showed normal levels of JAK3 expression in comparison with control samples and also tyrosine phosphorylation of JAK3 in response to cytokine stimulation. Furthermore, reconstituted cells were able to proliferate normally following IL-2 stimulation. These data indicate that the biological defects of JAK-3 deficient cells can be efficiently corrected *in vitro* by retroviral mediated gene transfer.

In a further study, it was shown by gene transfer that although IL-2 stimulation of JAK3 deficient EBV-LCLs requires JAK3 expression for tyrosine phosphorylation of IL-2Rβ, JAK1 and STAT5, IL-4 stimulation of JAK3 deficient cells can still result in phosphorylation of JAK1, STAT6 and IRS-1. However the response to IL-4 stimulation is considerably enhanced following JAK3 expression by gene transfer (Oakes et al., 1996) suggesting that the IL-4 stimulation pathway is not dependent but is enhanced by JAK3 activity.

7.1.1.4 Gene transfer into EBV-LCLs from XLA patients

At the start of this study, there had not been any reports of Btk gene transfer into B cell lines from XLA patients. In the case of XLA, EBV-LCLs from patients appear to be even more appropriate *in vitro* models for gene transfer, since the haematopoietic lineage actually affected by the genetic abnormality is targeted. As suggested in chapter 6, gene transfer into fibroblasts was a surrogate model to test Btk binding but in EBV immortalised B cell lines, more appropriate haematopoietic restricted proteins with which Btk may interact are expressed. Thus gene transfer into XLA EBV-LCLs may provide valuable insights into Btk binding properties and signalling

pathways in a physiologically relevant system. One caveat to the use of this system is that cell lines in XLA are difficult to generate due to the lack of peripheral B cells in this patient population.

7.2 EBV-LCL retroviral gene transfer

The UD108 cell line derived from an XLA has previously been shown to have a T33P amino acid substitution in the PH domain of the protein. Analysis of protein expression and activity in this cell line by immunoblotting and autophosphorylation activity demonstrates the complete lack of protein as a result of this mutation (Genevier et al., 1994). Thus, this cell line is ideal for the reconstitution of Btk protein expression.

7.2.1 UD108 cell line transduction

UD108 cells were expanded in culture and then cocultivated with the packaging cell lines BtkC2 (wild type Btk virus producer), Btk-C23 (Btk kinase mutant virus producer) and MB.SP (mock virus producer) respectively. Cocultivation was carried out in 24 well plates in the presence of polybrene for 48 hours. This protocol had resulted in successful transduction of EBV-LCLs in previous studies (Porter et al., 1993). After cocultivation UD108 were washed, resuspended in medium and grown in increasing concentrations of puromycin. Transduced cells expressing the puromycin resistance gene were expanded and then analysed for vector integration and Btk expression.

7.2.2 Integration of Btk retroviral vectors into the UD108 cell line

Puromycin resistant cells were initially analysed by PCR analysis of genomic DNA (Fig. 7.1). A PCR reaction using primers designed to amplify a 550bp fragment from Btk cDNA was performed. Untransfected and mock transfected genomic DNA do not show the amplified product while BtkC2 and Btk-C23 transfected lines which should have integrated Btk cDNA as a result of successful vector transduction are positive for a 550bp band. Positive controls included were pMBbtk.SP plasmid DNA and UD108 cDNA, both of which were appropriately positive. This result demonstrates the presence of the provirus in puromycin resistant cells but does not in itself demonstrate successful integration.

Puromycin resistant cells were further analysed by Southern blotting for successful vector integration (Fig 7.2). Genomic DNA extracted from untransfected and transfected puromycin resistant cell lines was digested with *BamHI* which digests the provirus at the cloning sites flanking the Btk cDNA, but not within the cDNA itself, thereby leaving a 2.3kb fragment. Digested DNA was probed with a radiolabelled probe consisting of exon 8 of Btk. Southern blot analysis shows that a 2.3kb band is clearly visible in UD108 cells transduced with BtkC2 and Btk-C23. Untransduced cells and those transduced with the mock vector MB.SP, as well as control DNA derived from Daudi B cells, do not show any evidence of a 2.3kb band. Bands corresponding to hybridisation of the probe to digested genomic Btk are visible in all lanes. The Btk cDNA (obtained from purification of the 2.3kb band after *BamHI* digestion of the pMBbtk.SP vector plasmid) was also run alongside the cell line samples at decreasing dilutions (100pg to 1pg) in order to quantitate the copy number of provirus per cell. As can be seen, the intensity of the signal corresponds to a dilution of 1pg of plamid DNA which is equivalent to an integration frequency of 1 copy per cell.

7.2.3 RNA expression from integrated provirus

In order to examine mRNA expression from the provirus, RT-PCR was performed on transduced and untransduced cell lines. PCR reactions using an upstream gag sequence primer and a downstream internal Btk primer to amplify a proviral specific mRNA failed to amplify any specific bands. Therefore, primers corresponding to the ψ packaging sequence of MLV retrovirus were designed (as described by Gerard et al., 1996) and used to to amplify a 130bp product from mRNA derived from transfected and untransfected cells. Fig. 7.3 demonstrates the presence of a 130bp band in BtkC2 and Btk-C23 transfected NIH3T3 cells and BtkC2 and Btk-C23 transfected UD108 cells but not in untransfected 3T3 and UD108 cell lines. These data suggest that there is production of mRNA from proviral sequence. Northern blot analysis to quantitate and provide further evidence for mRNA expression from integrated provirus was attempted on several occassions but this technique was not successful.

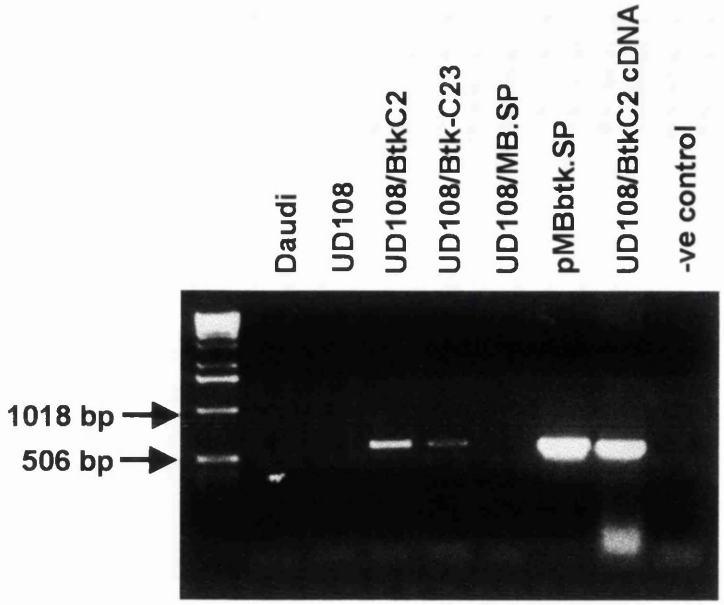


Fig 7.1 Presence of Btk encoding provirus in UD108 genomic DNA. *PCR analysis of genomic DNA from transfected and untransfected UD108 cells using Btk cDNA specific primers shows amplification of a Btk cDNA specific product in Btk.C2 and Btk-.C23 transfected lines. Positive controls include the pMBBtk.SP construct and cDNA from UD108/Btk.C2 transfected cells.*

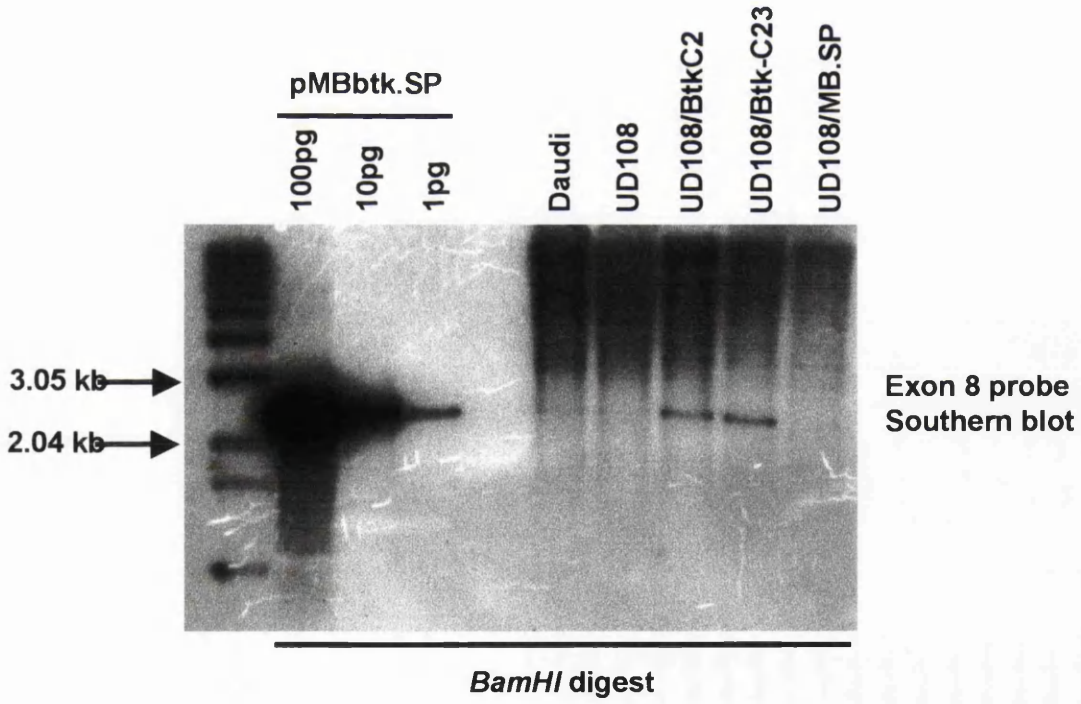


Fig 7.2 Integration of Btk vectors into UD108 genomic DNA. *Southern blot analysis of Bam HI digested genomic DNA from UD108 and Daudi cells with a radiolabelled Btk/exon 8 probe shows hydridisation of the probe to a 2.3kb band in Btk.C2 and Btk-.C23 transfected cells but not in untransfected or mock transfected lines. The pMBBtk.SP vector was digested with Bam HI and the 2.3kb BtkcDNA insert purified and run at different concentrations to allow estimation of proviral copy number*

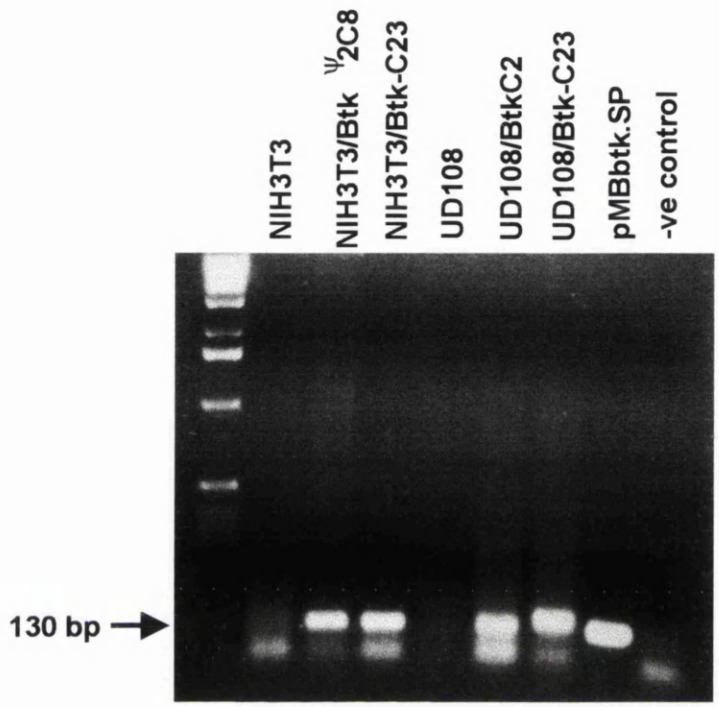


Fig 7.3 mRNA expression from integrated provirus. *RT-PCR analysis, using primers specific to the gag sequence of MLV retroviruses, was used to amplify RNA from transfected and untransfected cell lines. A 130bp band is seen in transfected UD108 and NIH3T3 cells but not in untransfected controls. The pMBbtk.SP plasmid DNA was used as a positive control.*

7.2.4 Lack of Btk protein expression in transfected lines

Successful integration of provirus containing Btk and kinase mutant Btk has been demonstrated by Southern blot analysis and we have also been able to show mRNA production from both integrated proviruses by RT-PCR. We then analysed cells for Btk and Btk- protein expression by western blot analysis. Whole cell lysates (equivalent to the lysate of 10^5 cells) from transfected and untransfected lines were immunoblotted with the H360B anti-Btk polyclonal antisera used successfully in previous experiments (see Chapters 4 and 6). A 77kD band was clearly seen in lysates from Daudi cells but BtkC2 transfected UD108 cells were not positive for this signal. This experiment was repeated using 10^6 cell lysate equivalent in case the amount of Btk expressed in transfected lines was below the limits of detection. Daudi control samples and lysate from the BtkC2 packaging cell line showed significant levels of Btk expression but Btk.C2 and Btk-.C23 transfected and untransfected UD108 cells did not show any evidence of the Btk protein (Fig.7.4). Comassie blue staining of the gel showed equivalent levels of protein loading in the UD108 samples in comparison to the positive controls (data not shown).

The possibility remained that again the level of expression of the Btk protein was below the limits of detection of the techniques used. More sensitive detection of expressed protein is afforded by Btk immunoprecipitation and analysis by western blotting and kinase assay.

7.2.5 Immunoprecipitation of Btk from transfected lines

A number of immunoprecipitation experiments were performed. Btk was immunoprecipitated using the H360B antisera which has been shown to successfully immunoprecipitate Btk from Daudi cells and transfected NIH3T3 cells (see Chapter 6). H360B antisera was used to immunoprecipitate Btk from the lysate of 20 -30 x 10^6 cells. The immunoprecipitate was then immunoblotted with the same Btk antisera for the presence of the protein. Despite varying cell numbers and using a variety of reagents and conditions, Btk was never conclusively shown to be immunoprecipitated from the BtkC2 transfected UD108 cell line. A sample immunoblot is shown in Fig. 7.5.

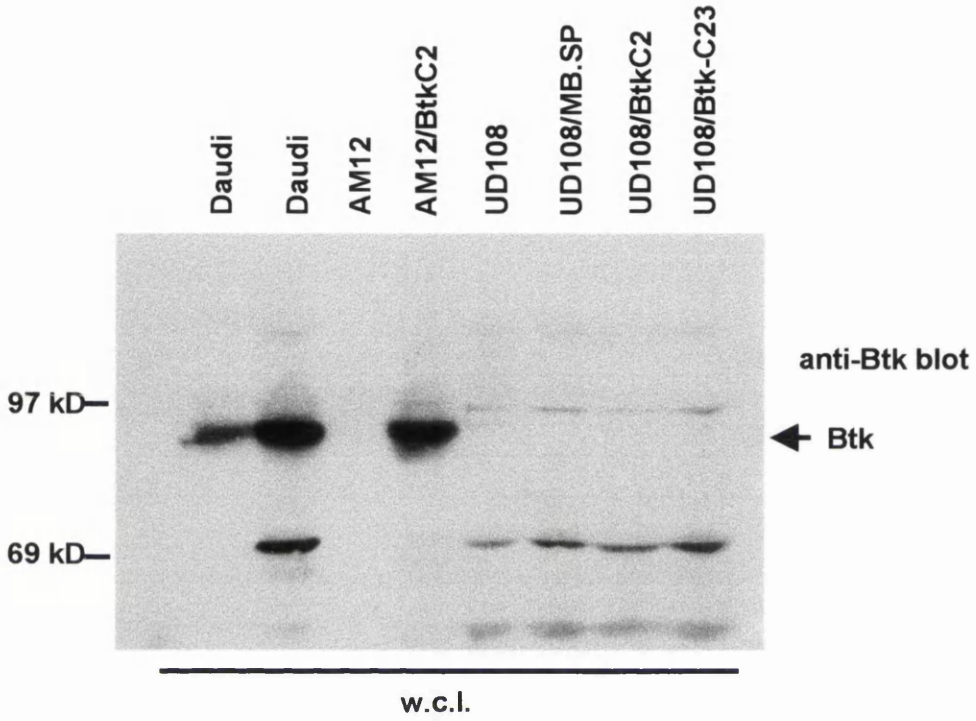


Fig. 7.4 Lack of Btk expression from transfected cell lines. a). *Immunoblotting of cell lysates with the anti-Btk antibody H360B shows the presence of a ~ 77kd band in control Daudi cells and in the packaging cell line Btk.C2. However untransfected and Btk.C2 transfected UD108 cell lines do not show evidence of Btk expression.* **b)** *The same experiment as described above was carried out using a greater amount of cell lysate (equivalent to the lysate of 106 cells). Immunoblotting with H360B still shows the absence of Btk expression in Btk.C2 and Btk-.C23 transfected UD108 cell lines.*

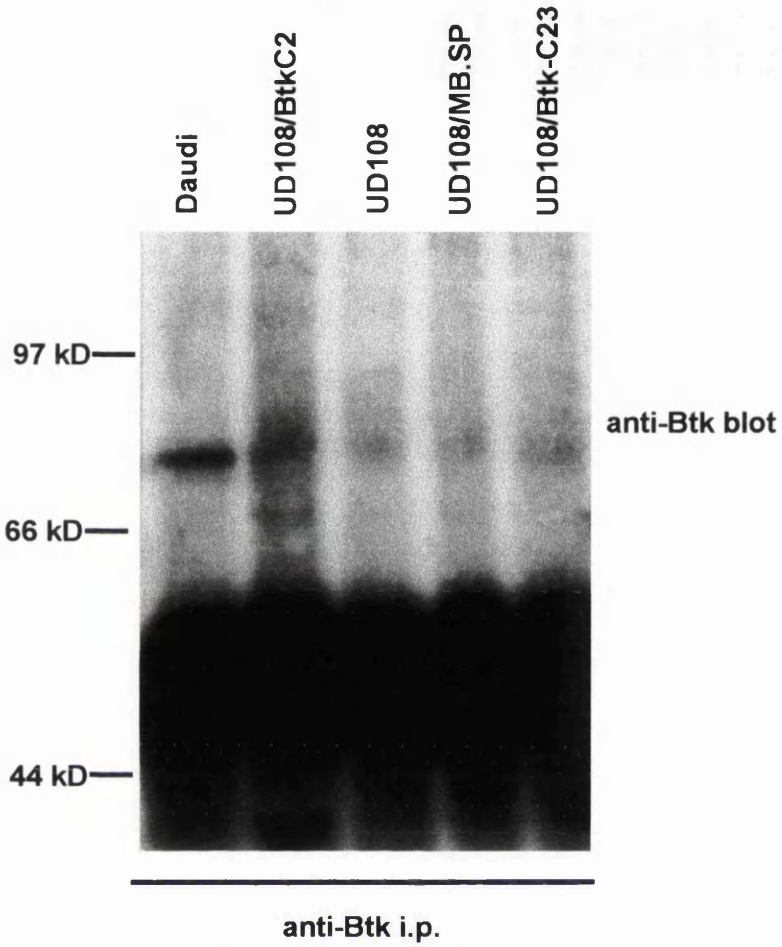


Fig. 7.5 Immunoprecipitation does not show evidence of Btk expression. *Transfected and untransfected lines were lysed and the lysate incubated with the H360B antisera. Immunoprecipitated protein was run on an 8% polyacrylamide gel and immunoblotted with H360B. Btk is shown to be successfully precipitated from control Daudi cells but no Btk is seen tranfected and untransfected cell UD108 cells.*

7.2.6 Autokinase assay of Btk from transfected lines.

To increase the sensitivity of Btk detection even further, transfected and untransfected UD108 cells were stimulated via a number of cell surface stimulants and Btk immunoprecipitated and analysed by autophosphorylation assay. Initially a non specific cell surface stimulant, PMA, was used followed by more specific B cell stimulants, IL-7 and CD38. However in all 3 experiments, no consistent activation of Btk was seen even in the control Daudi cell line. There was no evidence of kinase activity in stimulated or unstimulated cells from transfected or untransfected UD108 cells (data from the PMA stimulation experiment are shown in Figs. 7.6).

7.2.7 Btk is not expressed from transfected lines

The above series of experiments clearly demonstrate that despite the integration of the provirus into the UD108 cells the levels of Btk expression are below the level of detection of the methods used. A number of experiments have been performed to maximise the detection of even minimally expressed protein but these too have not shown any Btk expression. Having been convinced that this is not a problem of detection, the conclusion to be drawn therefore is that there is a significant abnormality in protein expression from the provirus in UD108 cells.

The capacity of the recombinant virus to express virus following integration into the target cell genome has previously been shown from transduction of NIH3T3 cells. Furthermore, previous experience in our laboratory using the X-CGD gene, has also shown that the pMB.SP viral vector is capable of gene expression in EBV-LCLs (Porter et al., 1993). Indeed, this was one of the reasons that this vector was chosen for our experiments. However, a major difference exists between the experiments in X-CGD lines and those involving XLA patient derived cell lines. The very nature of the XLA defect means that the B cells immortalised will be at a very early stage in B cell maturation. In the other conditions previously mentioned, mature B cells are immortalised. Previous phenotypic studies on UD108 cells have demonstrated that this cell line lacks surface expression of sIgM and cytoplasmic μ chain expression and also has lack of Ig light chain gene rearrangement suggesting that it has the characteristics of an immature pro-B cell line (Genevier et al., 1994). It was therefore hypothesised that the maturity of the B cell line may influence the activity of the viral LTR and hence gene expression.

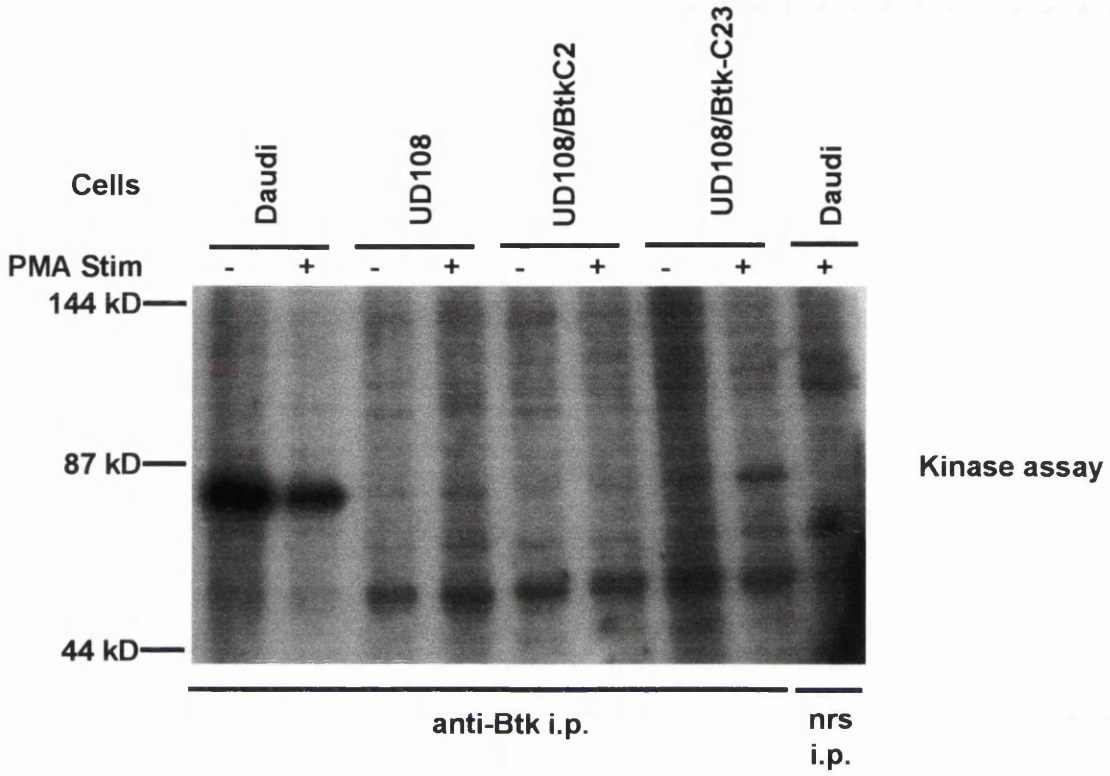


Fig. 7.6 Autophosphorylation assay does not show Btk activity after PMA stimulation. *10^6 cells were stimulated or unstimulated with PMA for 20 minutes prior to lysis. H360B antisera was used to immunoprecipitate Btk from cleared antisera and the immunoprecipitate subjected to autophoshorylation assay. Btk kinase activity is seen in control Daudi cells but not transfected or untransfected UD108 cell lines. A normal rabbit serum (NRS) immunoprecipitation was performed to exclude non-specific immunoprecipitaion.*

In order to investigate this, we used a reporter gene under the control of a similar promoter and assessed its expression in B cell lines of differing maturity.

7.2.8 Lac Z expression in B cell lines

7.2.8.1 Microscopic analysis of LacZ expression

The Lac Z reporter system is used frequently to assay the efficiency of gene expression following gene transfer in differing gene therapy protocols. The Lac Z gene encodes the gene for *E. Coli* β-galactosidase which hydrolyses the substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) to generate galactose and soluble indoxyl molecules which in turn are converted to insoluble indigo thus allowing visualisation by conventional light microscopy. An MFG retroviral vector, pMFGnlsLacZ, was constructed in which the Lac Z gene was under the control of a hybrid MLV/MPSV LTR promoter. Recombinant replication defective retrovirus was generated by the use of the FLY A13 amphotropic packaging cell line (Patience et al., 1998).

A number of differences exist between the pMFGnlsLacZ vector used in these experiments and the pMB.SPBtk vectors used for Btk gene expression. The MFG vector construct does not contain a selectable marker. In addition the MFG vector encodes natural viral splice acceptor and donor sites and yields increased levels of spliced transcript which may lead to enhanced expression (Byun et al., 1996; Krall et al., 1996) (discussed further in 7.3.3.1). However the purpose of this experiment was to compare the efficiency of gene expression from a similar promoter and in this regard the two vectors were comparable.

A number of different cell lines were used to test Lac Z expression. The UD108 cell line was compared with an EBV-LCL from another XLA patient, P276, which had been characterised as expressing sIgM, CD79β, and having Ig light chain gene rearrangement (Genevier et al., 1994) and was therefore considered phenotypically to be similar to a pre-B cell line. Also used for comparison was the mature B cell line Daudi and for control purposes the myeloid cell line PBL 295. All cell lines were co-cultivated with the producer packaging cell line and then expanded and analysed for β-galactosidase expression. Light microscopy of stained cells demonstrates that in all

four cell lines there were similar number of cells transfected and expressing β-galactosidase (Fig 7.7). This result suggests that the efficiency of gene transfer into the different cell lines is similar.

7.2.8.2 Quantitative analysis of Lac Z activity

To assess this more accurately, we analysed transfected immature and mature B cell lines in a quantitative manner. In this assay transduced cells are incubated with an alternative substrate to X-gal, ONPG (*0*-nitrophenyl-β-D-galactopyranoside) which is hydrolysed by β-galactosidase to *0*-nitrophenol which is yellow in colour. The amount of *0*-nitrophenol produced and hence indirectly the activity of β-galactosidase is measured by photospectrometry by adsorbance at 420nm. The activity of Lac Z in transduced UD108 cells was compared with activity in the mature B cell line, Daudi at 48 hours after transduction. As illustrated in Fig. 7.8 no β-galactosidase activity is seen in untransduced cells whereas transduced Daudi and two independently transduced sets of UD108 cells show equivalent levels of enzyme activity. These two experiments suggest that the activity of the MLV/MPSV hybrid promoter is equally active in early B lymphoid cells through to mature B cells.

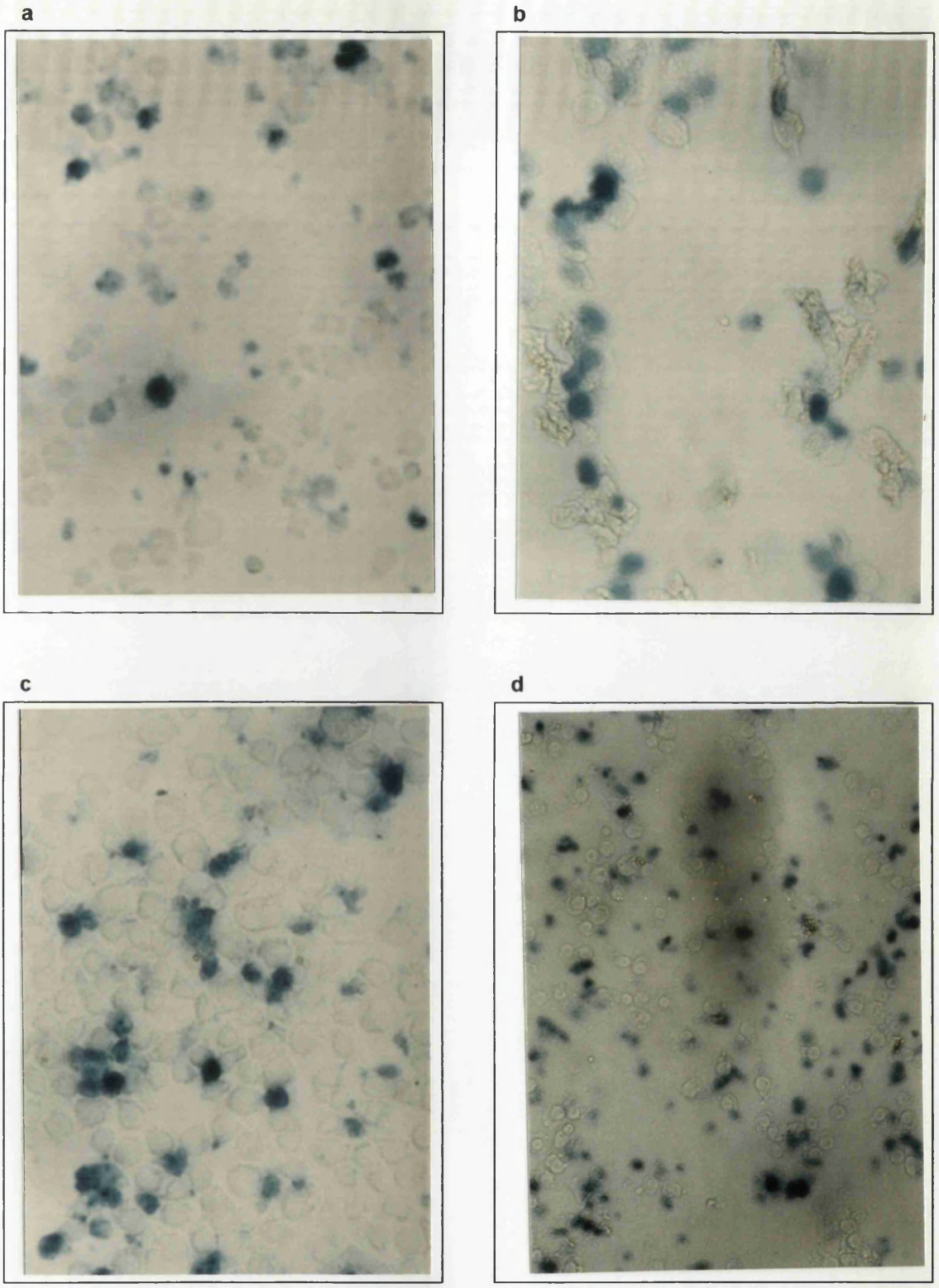


Fig. 7.7 Lac Z expression from the hybrid MLV/MPSV promoter is similar in B cell lines at different stages of maturation. *An MFG construct encoding the nlsLacZ gene under the transcriptional control of an MLV/MPSV promoter was used to infect UD108 (a), BLCL276 (b), Daudi (c) and PBL295 (d) cells. After cocultivation with the producer cell line for 48hrs cells were cytospun, fixed and stained for β-galactosidase activity. All cell lines show similar number of cells expressing β-galactosidase.*

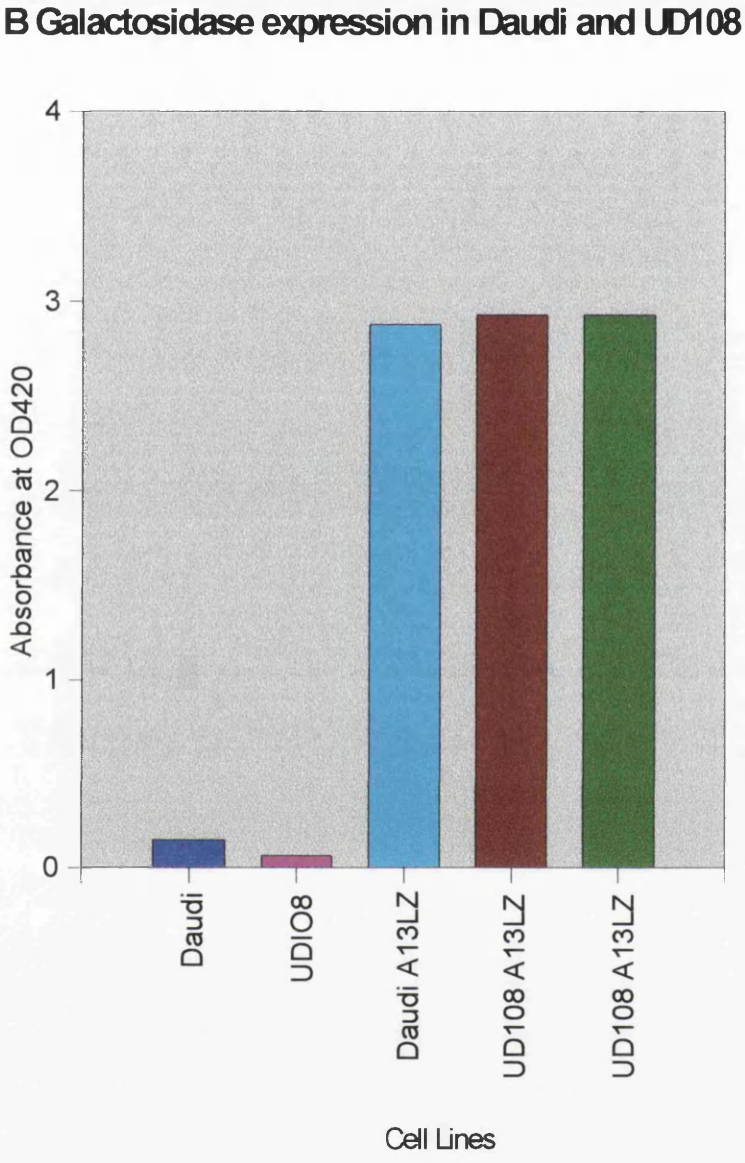


Fig. 7.8 Lac Z activity is similar in both immature and mature B cell lines. *Immature UD108 cells and mature Daudi cells transfected with MFGnlsLacZ were analysed quantatively for LacZ activity. After incubation with ONPG, the level of 0-nitrophenol produced by β-galactosidase was measured by photospectrometry. The level of β-galactosidase activity is similar in both Daudi cells and two independently transfected UD108 cell lines.*

7.2.9 Promoter interference inhibits Btk expression

The design of the retroviral construct used is such that the expression of the Btk gene is driven by the retroviral LTRs and the puromycin resistance gene is transcribed by the internal heterologous SV40 promoter. A number of reports have documented promoter interference between the heterologous promoter and the retroviral promoter(Bowtell et al., 1988; Apperley et al., 1991; Wu et al., 1996). In the experiments so far described, all cells have been selected initially by the expression of the puromycin gene and only then tested for Btk expression. We hypothesised that the activity of the SV40 promoter may interfere with Btk expression from the viral LTR. Therefore a further experiment was conducted whereby the wild type Btk vector BtkC2 was used to transduce UD108 cells as previously described by cocultivation. However, after transduction, the UD108 cells were split into two equal quantities and then grown in parallel with one group selected in puromycin and the other group unselected.

After expansion of cell numbers, immunoblotting of both whole cell lysates and of Btk immunoprecipitates from both transfected and untransfected cells was performed. Fig. 7.9 shows that there is a lack of Btk expression in whole cell lysates from all cell lines. However, immunobotting of H360B precipitates with H360B shows that there is a band visible in the unselected cell line that runs at exactly the same height as a band in the control Daudi cell line. To confirm this we immunoblotted the immunoprecipitates with a second anti-Btk antibody (denoted 7498). This also showed presence of the 77kD band in the puromycin unselected UD108 cell line (Fig. 7.10).

These experiments strongly suggest that there is expression of Btk from the cell lines which have been transduced and grown without puromycin selection. The level of expression appears to be low since only by enriching for Btk by immunoprecipitation can expression be detected. The absence of Btk expression in cells which have been selected in puromycin suggests that the process of selection and the activity of the internal SV40 promoter may have interfered with Btk expression.

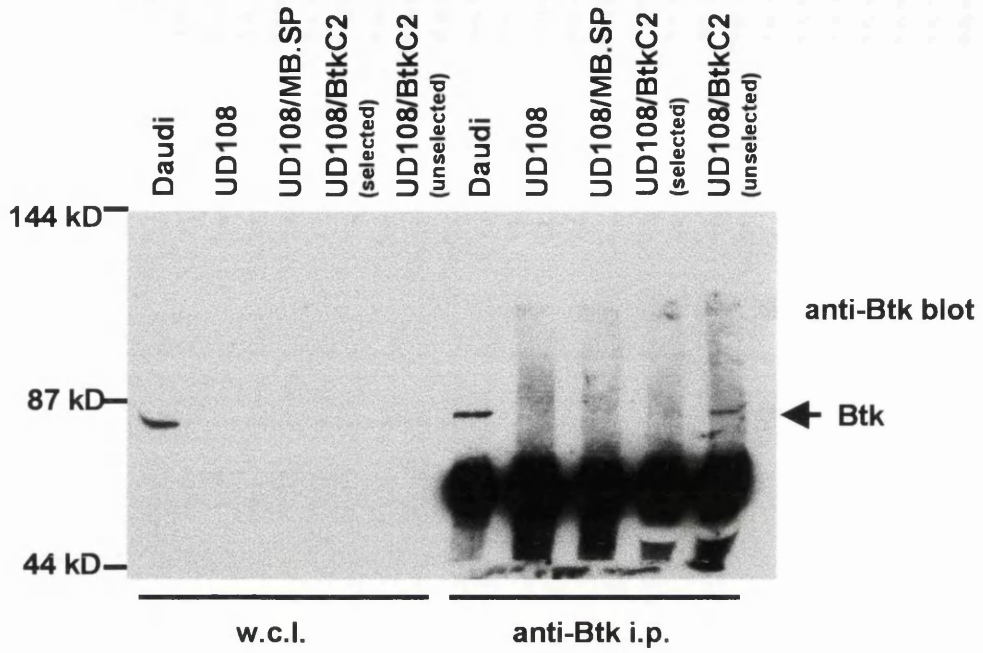


Fig. 7.9 Unselected cells express Btk. *UD108 cells were transfected with BTK.C2 as previously described and grown with or without puromycin selection. Cell lysate was either immunoblotted immediately or immunoprecipitated with H360B prior to immunoblotting. Immunoprecipitates from unselected Btk.C2 transfected cells show the presence of a 77kD band on H360B immunoblotting. No such band is seen in selected cells.*

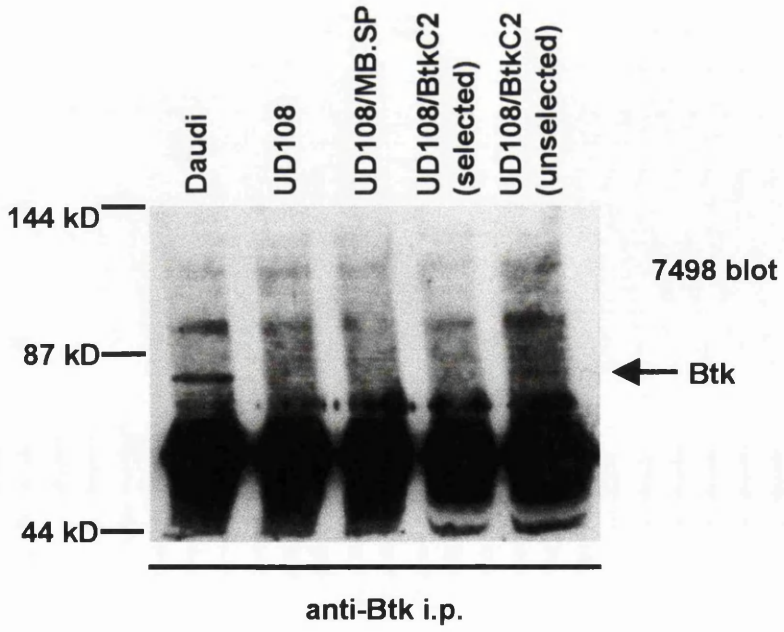


Fig. 7.10 Confirmation of Btk expression by the use of an alternative Btk antisera. *Imnunoprecipitates from transfected and untransfected cell lines were immunblotted with an alternative Btk antisera 7498. The presence of a 77kd band is seen in unselected Btk.C2 transfected UD108 cells confirming the expression of Btk in this line*

7.2.10 Surface phenotype of transfected cells

Puromycin unselected cells in which Btk was expressed were analysed for any changes in their surface phenotype. The UD108 cell line has been shown to lack surface and cytoplasmic μ chain expression. Transfected cells were therefore analysed by flow cytometry to see whether the expression of the μ heavy chain had been affected by Btk expression. However, there was no change in the lack of heavy chain expression in these cells. As a positive control CD40 expression was shown to be present in both UD108 cells and the Daudi cell line control (results not shown). The lack of phenotypic changes upon Btk expression are not altogether surprising since EBV immortalised B cell lines are transformed at a particular stage in development and are not readily differentiated.

7.3 Discussion

The results described above have attempted to express Btk in a Btk null cell line from an XLA patient. A number of difficulties have been encountered in these experiments that have prevented the primary objectives from being achieved, namely reconstitution of Btk function in a deficient cell line. This discussion will focus on the possible causes of the difficulties of gene expression in these experiments and will also discuss how these might have been overcome. Similar experiments in XLA B cell lines in which successful Btk reconstitution has been achieved are reviewed in the general discussion (Chapter 8).

7.3.1 Reasons for lack of Btk expression

The major difficulty in these experiments has been the lack of protein expression in transfected cell lines. In order to understand why this might be the case, we have attempted to analyse sequentially the steps involved in retroviral vector transduction and gene expression. Although it was initially thought that the relatively low retroviral titre of the packaging cell lines selected might hinder UD108 cell line transduction, Southern blot and PCR based experiments have shown integration of Btk and Btk- vectors into the UD108 cell line at a frequency of 1 copy per cell. In other experiments, using the same retroviral vector backbone but carrying the X-CGD cDNA this level of integration frequency has been adequate to allow gene expression (Porter et al., 1993).

The transcription of the Btk/Btk- genes from the integrated provirus was not successfully quantitatively analysed by northern blot analysis due to technical difficulties. RT-PCR of retroviral message using virus specific primers was able to show expression of the provirus but this was not performed in a quantifiable manner. Further to this, no detection of protein was possible in a succession of experiments. The possibility exists that the level of expression was below the limits of detection of the assay techniques. However, a number of experiments were performed to optimise this, including increasing the quantity of cell lysate, immunoprecipitation enrichment and autophosphorylation activity, all without success. We have therefore assumed that the problem is one of lack of expression rather than failure of detection.

7.3.2 Reasons for lack of proviral transcription

The retroviral vector used relies upon transcription of the Btk gene being directed from the retroviral promoter within the LTR. The promoter element used in our experiments is the MPSV unit which has previously been shown to have effective transcriptional activity in haemopoietic cells (Stocking et al., 1985; Laker et al., 1987; Riviere et al., 1995) and has good activity in EBV-LCLs (Porter et al., 1993). Furthermore, it must be remembered that this vector construct with the MPSV transcriptional unit was capable of Btk and Btk- gene expression in NIH3T3 fibroblasts (Fig 5.8) This suggests that host cell factors specific to this EBV cell line may have played an important role in downregulating transcriptional activity.

7.3.2.1 Transcriptional shut off in primitive cells

Studies investigating murine retroviral expression in embryonal carcinoma cell lines and embryonic stem cells found inactivity of the MLV LTR (Challita et al., 1995b) in these lines. Transcriptional repression could be overcome, however, by modifying the basic construct in three ways. Substitution of the MLV LTR for the MPSV LTR, substitution of the primer binding site and deletion of a negative control sequence at the 5' end of the LTR all led to effective expression of the reporter gene (Challita et al., 1995a).

7.3.2.2 Transcriptional shut off in early haematopoietic cells

The mechanism of repression in early haematopoietic cells was shown to be mediated in a similar fashion to that described for embryonic cells. It was demonstrated that the viral enhancer region in the U3 region of the MLV LTR, in addition to a region coincident with the PBS of MLV, limited expression in early haematopoietic cells (Baum et al., 1995). Novel retroviral vectors in which the U3 regions of the MPSV or Friend mink cell focus-forming virus were combined with the PBS of the murine embryonic stem cell virus were able to show significantly improved expression of the human multiple drug resistance gene in transduced haematopoietic cell lines (Baum et al., 1995; Baum et al., 1996).

The retroviral construct used in our experiments contained the substitution of the MPSV LTR for the MLV enhancer region, but did not exhibit any of the other additional features described above. Intact sequences in the primer binding site may have led to repression of LTR activity.

7.3.2.3 Methylation causes transcriptional inactivation

One process which has been shown to cause this effect is methylation of the viral LTRs. In a series of experiments using a retroviral construct expressing the glucocerebrosidase under the control of an MLV LTR, murine haematopoietic cells were transduced and cells serially transplanted into lethally irradiated mice (Challita and Kohn, 1994). Primary CFU-S and tissues of irradiated recipients showed high transcriptional activity but secondary and tertiary recipient CFU-S and tissues showed transcriptional inactivation despite evidence that they were derived from the same progenitor cell. A *SmaI* based restriction digest assay on proviral DNA demonstrated significant methylation of the 5' LTR in most of the transcriptionally inactive tissues from 2^0 and 3^0 recipients whereas 1^0 recipients showed no evidence of LTR methylation.

7.3.3. Assessment of MPSV LTR activity

The inherent activity of the MPSV LTR promoter in the UD108 line was compared with the B cell lines at different stages of maturation by use of the LacZ reporter system. Quantitative analysis suggested that promoter activity was indeed similar regardless of the stage of B cell maturation. This would be in contradiction to the

findings reported above. However, this is not necessarily the case since the backbone vector containing the LacZ gene is derived from the MFG construct. The intrinsic properties of the MFG vector in comparison with a pMB.SP based vector may well explain the differences seen.

7.3.3.1 Enhanced properties of the MFG retroviral construct

MFG vectors contain a small fragment of the untranslated region of the MLV env gene inserted downstream of the *gag* (ψ+) sequence and upstream of the inserted cDNA sequence thus retaining the natural *env* splice acceptor and splice donor sequences. The inserted cDNA sequence is placed as close as possible to the natural *env* translation initiation site. Together, these factors result in the production of greater levels of spliced transcript which in turns enhances the stability of the message and efficiency of translation. This is demonstrated by studies in which the level of the reporter gene chloramphenicol-acetyl-transferase (CAT) expression from an MFG construct was compared with CAT expression from a series of different MLV based LN vectors (including a construct very similar to pMBbtk.SP i.e. the CAT gene transcribed from the MLV LTR and a selectable marker transcribed from an internal SV40 promoter) (Byun et al., 1996). The MFG based vector was found to have significantly increased levels of CAT gene expression and retroviral titre over all the LN based vectors in a variety of different cell types. Northern blot analysis of mRNA produced from MFG vectors shows that the ratio of spliced to unspliced message is in the order of four fold greater than that seen in LN based constructs (Krall et al., 1996). Since spliced RNA, in which the packaging signal has been excised, is translated with greater efficiency, this may be the reason why there is enhanced protein expression in MFG vectors.

Thus, the experiments using the MFGnlsLacZ construct suggest that the activity of the MPSV LTR is similar in B cell lines at different stages of maturation, but other factors specific to the pMB.SP vector and/or the host cell, other than the promoter element, may explain why there was decreased Btk gene expression in the UD108 cell line.

7.3.4. Use of selectable markers

The use of a selectable antibiotic resistance marker has been used widely in the development of pre-clinical gene therapy protocols. The most widely employed is the neomycin resistance gene which allows transduced cells to be selected in the presence of the drug G418. Puromycin resistance has also been commonly used. The selectable marker can be transcribed from either the viral LTR or an internal heterologous promoter, such as that used in our experiments. The advantage of this approach is the ability to select only those cells that have been successfully transfected and therefore enrich for the transduced population. A number of reports have now suggested that the use of two genes in one construct may lead to promoter interference and silencing of the one or other transcriptional units. In a mouse transplant model where murine marrow was transduced with a retroviral vector expressing human ADA (hADA) driven from an internal phosphoglycerate kinase promoter, expression of hADA was seen in the majority of mice up to 6 months post transplant (Apperley et al., 1991). However, when an *in vitro* selection step was introduced using a vector encoding the gene for neomycin resistance in addition to the hADA gene (both driven by internal promoters) the level of hADA expression was significantly reduced. Furthermore it was found that expression of hADA stopped altogether after approximately 20 weeks post transplant despite the continued presence of provirus integration in DNA from peripheral blood mononuclear cells. Systematic Southern blot analysis of one recipient mouse suggested that this may have been due to the emergence of a transcriptionally silent dominant stem cell clone. Alternative studies have also observed that retroviral vectors carrying two genes are poorly transcribed *in vivo* despite the presence of provirus integration in host cells (Bowtell et al., 1988). In both the above studies the absence of selection when using the vectors encoding the selectable marker did not result in increased expression.

7.3.4.1 Puromycin selection inhibits transcription from the LTR

Our studies suggest that puromycin selection may have played a significant role in the downregulation of expression from the MPSV LTR. In contrast to the experiments above, the absence of puromycin selection in a group of cells transfected under identical conditions and grown with or without puromycin selection resulted in

expression of Btk from the MPSV LTR. Even in these circumstances, expression was only minimal and could only be detected after Btk immunoprecipitation.

We suggest that the lack of expression of Btk in our experiments may be as a result of the combination of factors, namely the characteristics of the immature B cell line targeted, the properties of the pMB.SP construct and finally the use of puromycin selection. The most important role may have been the role of the selectable marker since the absence of selection led to detectable Btk expression.

7.3.5 Alternative approaches to UD108 cell transduction

In retrospect a number of approaches could have been used to overcome the problems encountered. The choice of an alternative vector would be an important consideration. The results of LacZ expression suggest that the MFG vector construct may have given more effective transcription in this cell line. Other alternatives would be retroviral vectors which have been designed for infection of early haematopoietic cells. The Murine Stem Cell Virus (MSCV) retroviral vector which is derived from the murine embryonic stem cell virus and has a mutated primer binding site has been used in animal experiments and been shown to effectively express a therapeutic gene in both early murine and human haematopoietic progenitor cells (Hawley et al., 1994; Ding et al., 1996; Cheng et al., 1998). Moreover, transgene expression from the MSCV LTR was found to be greater than expression from the MLV LTR in direct comparison experiments (Lu et al., 1996). Another vector which has shown effective infection and gene expression in haematopoietic stem cells is the Friend murine leukaemia virus (Cohen-Haguenauer et al., 1996; Cohen-Haguenauer et al., 1998).

It would also seem more advisable to have used a vector carrying only the therapeutic gene because of the problems encountered with promoter interference. Once again the MFG construct or any of the other constructs above lacking a selectable marker would appear to be attractive choices. The other strategy designed to overcome promoter interference but still give the convenience of a selectable marker is the use of an internal ribosomal entry site (IRES) derived from a number of different viruses (e.g. polio virus, encephalomyocarditis virus) positioned downstream of the therapeutic gene and immediately upstream of the selectable marker. This bi-cistronic vector strategy results in the transcription of both genes

from the single promoter element. However the presence of the IRES sequence allows ribosomes to recognise and translate two proteins from the single RNA molecule. This system has been successfully used in phenotypic correction of X-SCID EBV-LCLs (Candotti et al., 1996a) where the therapeutic γ_c gene is coupled to a truncated nerve growth factor gene (NGF) for selection by FACS analysis. Similarly, the expression of glucocerebrosidase was coupled to the selectable marker CD24 to correct the enzyme deficiency in $CD34^+$ haematopoietic stem cells from patients with Gaucher's disease (Migita et al., 1995; Medin et al., 1996).

7.3.6 Use of alternate viral vector systems

Retroviruses have been the most widely used of all vectors in the development of gene transfer technology and in its application in clinical trials. However they are limited by their capacity to infect quiescent cells, the availability of space in the retroviral genome in which to introduce heterologous material, the titre of recombinant virus that can be generated and by the worry of replication competent virus generation. A number of different viral systems have now been developed for gene transfer each with their own advantages and disadvantages.

Adenoviral vectors have the advantage of a broad host range and very high titre of recombinant virus can be generated. Following integration, the adenoviral genome remains episomal which may make them a safer alternative to retroviral vectors but this may also only allow for short term expression. The natural affinity for the respiratory epithelium has made adenoviral based vectors a popular choice for the genetic treatment of cystic fibrosis (Crystal et al., 1994; Rosenfeld et al., 1994). However, adenovirus does not show a natural tropism for B lymphocytes and for this reason, these vectors have not been commonly used for EBV immortalised B cell studies.

The human single stranded DNA parvovirus adenoassociated virus (AAV) has also been developed as a potential tool for gene delivery (reviewed in Flotte and Carter, 1995; Kotin, 1994). The advantageous properties of AAV as a gene transfer vector include stable and efficient integration of viral DNA into the host genome, lack of any associated human disease and the ability to infect growth arrested cells. This latter property has generated much interest with regard to transduction of

haematopoietic $CD34^+$ stem cells. However, although early reports have suggested that AAV can infect this stem cell population (Goodman et al., 1994), subsequent studies have not been able reproduce these findings. Another feature of AAV thought to be an attractive property was the ability of wild type AAV to integrate in a site specific manner into chromosome 19q13.3-qter thereby eliminating the risk of insertional mutagenesis or oncogenesis. For recombinant AAV minimal requirements for site specific integration are the rep gene products and the AAV terminal repeats. AAV has been shown to successfully transduce EBV-LCLs with therapeutic genes for CGD. In our laboratory reporter genes such as LacZ have been successfully transferred and expressed in the UD108 cell line (M. de Alwis unpublished obvervations) suggesting that this vector might have been an alternative system to use.

Retroviral vectors, adenoviruses and AAV represent the most well developed viral vector systems for use in gene transfer. In addition to these, interest has turned to the engineering of other viruses such as HIV (Lever, 1996) and herpes viruses (Efstathiou and Minson, 1995). The natural tropism of EBV for B lymphocytes might make this a candidate for the transfection of EBV immortalised B lymphocytes and primary B lymphocytes. However, the development of EBV based vectors remains at an early stage and this must first address the safety of the use of these viruses, given their association with lymphoproliferative disease and carcinoma.

8

Discussion

XLA was the first of the primary immunodeficiencies to be described. Since that time many more conditions have been characterised and the advances in molecular biology has resulted in the identification of many of the genes responsible. The challenge still remains to understand the molecular mechanisms of disease and to develop novel therapeutic strategies based on gene transfer. The work conducted in this thesis has attempted to study a number of different aspects of XLA ranging from the role of Btk in the pathogenesis of disease, at both the molecular and protein level, to the development of *in vitro* models of gene transfer.

8.1 Genotype-phenotype correlation in XLA

The identification of the molecules responsible for inherited defects has finally allowed the ability to correlate clinical disease with the molecular defect. In the case of the Btk this has not been a particularly successful exercise. Despite the establishment of an international database collecting data from over 400 individuals from multiple centres, no correlation between clinical phenotype and the genetic defect can be made. With such numbers, reports of discordant phenotypes within single pedigrees (Kornfeld et al., 1997) and relatively asymptomatic individuals with null phenotypes (Kornfeld et al., 1996), it would be reasonable to assume that no such correlation exists.

A more pertinent exercise therefore is to examine what, if not the genetic defect in Btk, determines the clinical phenotype in XLA. It would be easy to speculate that

this is multifactorial and may involve the age of the patient, past history of infections, previous treatment and compensatory genetic factors. Certainly the variation in phenotype between individuals with the same mutation, might argue strongly for such acquired influences. However, at present no reports exist to confirm or refute such an influence.

8.1.1 Compensatory mechanisms

The effect of the mutation on protein structure and function has also been the subject of much interest in order to identify phenotypic correlations, but as the results in Chapter 4 suggest, in the majority of cases mutations in Btk lead to the complete absence of protein expression. The presence of other kinases and signalling molecules might allow speculation on the ability of such proteins to act as compensatory factors. The upregulation of Syk to compensate for the absence of ZAP-70 in T cells has already been mentioned and indeed other similar mechanisms exist. In the residual peripheral T cells that develop in $Lck^{-/-}$ mice, constitutive phosphorylation of the TCR ζ chain which is normally mediated by Lck in thymocytes is still seen. It has been postulated that the tyrosine kinase Fyn which is expressed at low levels in thymocytes but upregulated in peripheral T cells could compensate for the absence of Lck (van Oers et al., 1996b). In another example, mice were generated with targeted deletions of the Src like tyrosine kinases, Hck and Fgr in order to study the role of these kinases in myelomonocytic cells. The analysis of macrophage function in single or double homozygous knockout mice showed that most assays were unaffected leading the investigators to suggest that other kinases could compensate for the lack of Hck and Fgr. Indeed, stimulation of macrophages from wild type and $Hck^{-/-}$ mice with LPS and IFN-γ showed a significant increase in Lyn kinase activity in $Hck^{-/-}$ mice, again suggesting that a compensatory role for Lyn exists (Lowell et al., 1994).

If such mechanisms exist in the absence of Btk, it is tempting to speculate which molecules might fulfil this role. Clearly, structurally homologous haematopoietic lineage restricted Btk/Tec family kinases such as Itk and Bmx are attractive candidates. This assumes that the restriction of Itk expression to the T cell lineage is less stringent in the absence of Btk allowing Itk to play a compensatory role in B cell

development. Alternative candidates to consider would be other proteins which display similar phenotypes to Btk knockout mice, thus implying common roles in B cell development. PKCβ1 and Vav would be candidate molecules to fulfil this role (Tarakhovsky et al., 1995; Leitges et al., 1996).

8.1.2 Genetic background

One might also consider the genetic background upon which the Btk mutation exists. One influence might be the role played by innate immunity as determined by mannose-binding lectin (MBL). Mutant MBL exists at relatively high frequencies in a number of different populations worldwide (0.11-0.16 in Eurasian populations (reviewed by Turner 1996) and several studies have now shown an association between mutant MBL and increased risk of infection (Summerfield et al., 1995; Summerfield et al., 1997). It is rare for low levels of MBL alone to cause disease but it has been postulated that it might serve to affect the severity of disease if the individual also manifests another immune defect (Turner, 1996).

8.1.3 Genotype-phenotype correlation in other primary immunodeficiencies

The lack of genotype-phenotype correlation has been also seen in other primary immunodeficiencies. Worldwide mutation databases have been now established for the majority of these conditions and updated reports recently published. In the cases of hyper IgM syndrome and chronic granulomatous disease no correlation could be determined (Notarangelo and Peitsch, 1996; Roos, 1996a). A recent report documenting 40 unique mutations in the Wiskott-Aldrich syndrome protein gene, suggested that milder WAS phenotypes were seen in individuals with mutations in exons 1-3 of the WASp gene (Chen et al., 1978). However, this is very much dependent on the classification of mild disease and the analysis of larger numbers of patients would be required to substantiate this idea. Contradictory to this assumption, mutations in the WASp database show that the same mutation has been found in patients with classical WAS phenotypes as well as those with atypical WAS and also in X-linked thrombocytopaenia (XLT), an attenuated allelic variant of WAS (Schwarz, 1996a). We have also studied one family in which two severely affected boys and their paternal grandfather, who had previously been labelled XLT, were all shown to carry the same mutation (unpublished observations). Since the grandfather

is now over 60 years old, and is obviously a mild phenotype, a genotype-phenotype correlation is hard to justify.

8.2 Absence of Btk protein expression in XLA

The lack of protein expression in individuals with XLA was a surprising finding. Such had been the drive for genotype-phenotype correlations that many groups had speculated on the structure and function of the mutant protein prior to its examination in patient cells. Our results suggest that while this remains a valuable exercise it should be taken in context with the expression data.

While our studies imply nearly all patients with XLA lack protein expression, clearly further patient samples need to be analysed before an accurate prediction can be made. The study performed in Japan used kinase assays to analyse all patient samples with 31 of 35 showing lack of autokinase activity (Hashimoto et al., 1996). Western blot analysis was not carried out on all patients studied and so an assessment on protein expression *per se* cannot be made. Further studies in our laboratory have analysed another 10 patients in whom mutations were known and all have shown absence of Btk expression. To date only the R288W mutation in an EBV immortalised B cell line has shown expression.

The mechanism of mRNA and/or stability still remains in doubt. One feature that remains to be explored is the variation of mutant protein stability in different systems. Certainly when mutant Btk proteins have been expressed by means of expression vectors in B cells and in other cell lines, no protein instability has been seen (Rawlings et al., 1996; Li et al., 1995) (and the R520Q Btk mutant expression in Chapter 6). Furthermore, mutant protein appears to be more stable in EBV immortalised B cell lines than in primary cells. We have recently undertaken a study to look at WASp protein expression in WAS patients and in 12 of 13 patients studied there was lack of expression of mutant WASp protein (MacCarthy Morrogh et al., 1998). In contrast a larger study examining WASp expression in the EBV-LCLs from 40 WAS patients found significant levels of WASp expression in approximately half of the patient samples (Zhu et al., 1997). Of particular note is the fact that a mutation which resulted in no protein expression in primary cells led to significant WASp

expression in EBV-LCLs. Once again, larger numbers need to be analysed in order to arrive at a definite conclusion but one might hypothesise that the mechanisms that determine mutant protein stability may differ in primary cells in comparison to EBV-LCLs. Mutant protein expressed from different expression vectors may also display greater stability. This might suggest that the process of mutant protein degradation involves an active rather than a purely passive process whereby in the physiological setting mutant protein is selectively removed by intracellular mechanisms.

8.2.1 Protein expression as a diagnostic tool

The ability of these protein assays to make a diagnosis of XLA is demonstrated by the analysis of patients 13 and 14 in chapter 4. One criticism of the assay might be that detection of Btk was abnormal due purely to the lack of B cells. However, our control samples included a female with absent B cells and agammaglobulinaemia and in whom Btk was detectable. Since then, the assay has been repeated on other children with multiple abnormalities not associated XLA but with absent B cells and agammaglobulinaemia and Btk expression has been detected. This leads us to believe that despite the lack of B cells in the peripheral circulation Btk protein expression and activity can be detected. This is most likely to be due to the expression of the protein in monocytes.

I would suggest that the protein assays described be used as an initial screening procedure for the diagnosis of XLA. The techniques described are simple, inexpensive and quick to perform and can provide an unambiguous molecular diagnosis within 24hrs of blood sampling. At present, mutation analysis for XLA involves screening the 19 exons of the Btk gene in genomic DNA by one of many techniques, of which SSCP is the most commonly used, prior to direct sequencing of the affected exon. This can be a time consuming and expensive procedure and a definitive diagnosis may take weeks to achieve. Furthermore, the sensitivity of the SSCP procedure is only ~80%, a figure which can be increased to 90-95% if a number of conditions are varied (Ravnik et al., 1994; Ushijima et al., 1995). In cases where a band shift is not found and the clinical phenotype is atypical, doubt may exist as to the diagnosis of XLA. Other screening techniques do exist, but the sensitivities are not necessarily greater than SSCP.

A rationale for the diagnosis of XLA would be as follows. All patients with suspected Btk deficiency including those with atypical XLA should initially be screened by the protein assays described. A definitive diagnosis can thus be made rapidly. Those with abnormalities on protein analysis should then be analysed by SSCP. Furthermore, if no band shifts are initially seen, the conditions can be varied to increase SSCP sensitivity in the knowledge that the diagnosis has already been proven. This approach would offer a more rapid diagnostic service and improve the efficiency of the SSCP procedure.

The ability to make a rapid molecular diagnosis has many benefits. The spectrum of XLA is now much greater than previously suspected. In the study by Hashimoto et al. (1996), patients who had previously been labelled as CVID or IgG subclass deficiency were found to have mutations in Btk and demonstrated abnormalities of protein function and expression. Moreover, it has also been shown that the XLA phenotype can arise as a result of defects in molecules other than Btk. Defects in the μ heavy chain (Yel et al., 1996) and, more recently, in the λ5/14.1 surrogate light chain (Minegishi et al., 1998) have been identified as other molecular causes of congenital agammaglobulinaemia (as discussed in section 1.6.1). Other individuals with similar phenotypes but as yet unidentified molecular causes have also been described (Meffre et al., 1996)

One of the limitations of the protein analysis described is that it is not possible to offer carrier status to mothers and female relatives of affected boys. However, in some cases of XLA it is possible that a protein assay could offer such information. In XLA, over 65% of the mutations result in premature termination of translation either by nonsense mutations or by deletion/insertion mutations which lead to premature stop codon formation. Use of the protein truncation test (Gardner et al., 1995; Hogervorst et al., 1995) would detect the presence of a truncated protein in affected boys and the presence of both abnormal and wild type protein in carrier females. For a certain proportion of women, this would again offer a more rapid diagnostic procedure than either SSCP or X inactivation analysis.

8.2.2 Protein expression in other primary immunodeficiencies

From the evidence outlined in these studies, it would be interesting to note if the fate of mutant protein is the same in other primary immunodeficiencies, since this might offer a rapid screening test for these other conditions. In our study on 13 WAS patients quoted above (MacCarthy Morrogh et al., 1998), the mutation was known in 12 of the individuals, 11 of whom lacked WASp, and protein expression was used to make the diagnosis on the other. Since then, 4 further WAS patients have been identified using protein assays. CD40 ligand expression on the surface of activated T cells has been used to make a diagnosis of X linked hyper IgM. However, there have been no large patient studies as yet correlating CD40L expression with the mutation data. In the case of SCIDX1 due to γ_c deficiency, surface expression of γ_c was detectable in two-thirds of patients studied but again these analyses were carried out on EBV-LCLs (Puck et al., 1997). Analysis of primary mononuclear cells in our laboratory on 7 patients with known mutations in X-SCID has found abnormal expression in all cases (J. Gwyther unpublished data). Large studies performed on primary cells from patients with known mutations need to be performed in order to assess the usefulness or otherwise of these assays as diagnostic screening procedures for each of the immunodeficiencies mentioned above.

8.3 Btk and its role in B cell signalling

Evidence accumulated since the identification of Btk has supported the involvement of Btk in a number of different signalling pathways. This includes those studies in which Btk has been shown to be positively activated following the stimulation of different surface receptors (see section 1.5) and also those in which the involvement of Btk is inferred by changes detected in mutant models e.g. in *xid* and Btk knockout mice. In addition attempts have been made to define the role of Btk by studying its interaction with other signalling molecules.

The most convincing evidence still implicates Btk in signalling downstream of BCR stimulation. This has been shown by a number of different investigators using different cell systems. The overall picture to emerge is of the tyrosine phosphorylation of Btk at Y551 following the concerted action of one or more of the proximal tyrosine kinases (Mahajan et al., 1995; Rawlings et al., 1996). The subsequent autophosphorylation of Btk at Y223 has been elegantly demonstrated by a number of studies (Afar et al., 1996; Wahl et al., 1997). However these studies do not explain the effect of B cell activation on overall cellular function. More recently, a series of detailed reports (Bolland et al., 1998; Fluckiger et al., 1998; Scharenberg et al., 1998) have demonstrated a mechanism by which the molecular interactions of Btk lead to regulation of cellular calcium flux.

8.3.1 Btk and calcium mobilisation

Study of the events following BCR and pre BCR ligation in both B cells from *xid* mice (Rigley et al., 1989) and in bone marrow and EBV-LCLs from XLA patients (Genevier and Callard, 1997) demonstrate that there is impairment of normal calcium mobilisation, suggesting that Btk is crucial to this process. In a series of experiments using vaccinia driven transfection of wild type Btk, mutant Btk and other Tec family kinases (Fluckiger et al., 1998), ectopic expression of Btk (or other Tec family kinases) was shown to restore extracellular calcium influx in Btk deficient XLA B cell lines following BCR crosslinking. The use of mutant transfectants showed that this function was dependent on the presence of an intact SH2 and PH domain but independent of the Btk-SH3 domain. Further support for the role of Btk in this process was provided by induction of calcium flux by direct dimerisation of membrane attatched Btk in the absence of BCR crosslinking.

Investigation of the mechanism of calcium regulation by studying the effect of Btk activation on PLC-γ tyrosine phosphorylation and IP_3 production, showed marked enhancement of IP_3 production in Ramos and murine A20 B cells in which wild type Btk was overexpressed. Consistent with this mechanism, expression of Btk, Lyn or both together in NIH 3T3 fibroblasts resulted in increased tyrosine phosphorylation of PLC-γ2 suggesting that PLC-γ2 is a direct substrate of Btk. Similar results have been shown in similar experiments performed in DT40 avian lymphoma cells (Takata and Kurosaki, 1996). In contrast to these studies, in EBV immortalised B cell lines from XLA patients, following BCR crosslinking, PLC-γ activation was shown to proceed in the absence of Btk. This may reflect species differences between human and chicken lines in that there may be alternative sites of PLC-γ phosphorylation or the use of other Tec kinases to phosphorylate PLC-γ.

Further details of the proximal events in this process are provided by the study of Btk interaction with PIP_3 and PI-3 kinase (Scharenberg et al., 1998). Co-expression of Tec kinases, including Btk, with the p110 subunit of PI-3 kinase enhances BCR mediated IP_3 production and calcium signalling. This effect can be attenuated by pretreatment of cells with wortmannin (a PI-3 kinase inhibitor) emphasising the role of PI-3 kinase in Btk mediated calcium mobilisation. The mechanism for this interaction has previously by suggested *in vitro* studies demonstrating the association of the PH domain of Btk with phosphoinositide PIP_3 (Salim et al., 1996) and is further supported by *in vivo* data from these studies (Scharenberg et al., 1998). Coexpression of p110 and Btk enhanced the production of PIP_3 and was indirectly shown to be due to the binding of the Btk PH domain to PIP_3. This interaction also led to increased Btk autophosphorylation suggesting the interaction of Btk with PIP_3 also promotes Btk activation. In addition, the tyrosine phosphorylation of PLC-γ was enhanced by the co-expression of p110 and attenuated by treatment with wortmannin again implying that the pathway to calcium mobilisation via PLC-γ is PI-3 kinase dependent.

These studies together with that of Fluckiger et al. (1998) provide evidence for a molecular pathway by which Btk is involved in the regulation of calcium

mobilisation. PI-3 kinase activity results in the production of PIP_3 which, by association with its PH domain, initiates Btk activation in concert with Src kinases (and in particular Lyn). In addition it recruits Btk to the plasma membrane where via an SH2 domain dependent interaction, Btk activates PLC-γ by tyrosine phosphorylation. This in turn leads to the production of IP_3 which releases calcium from intracellular stores. This is summarised diagrammatically in Fig. 8.1.

Evidence for the regulation and inhibition of this process is now also available. The low affinity receptor for IgG, FcγRIIb1, plays a critical role in the negative feedback regulation of B cell activation and immunoglobulin production (Muta et al., 1994). Co-crosslinking of FcγRIIb1, together with the BCR, inhibited Btk dependent calcium flux and the production of IP_3 (Fluckiger et al., 1998). The mechanism of this regulatory process maybe due to the activity of SHIP (SH2-containing inositol polyphosphate phosphatase) which following FcγRIIb1 activation, is recruited to the plasma membrane and catalyses the hydrolysis of PIP_3 to PIP_2. Thus, this may interfere with PIP_3-Btk PH-domain mediated membrane localisation (Bolland et al., 1998) and subsequently the effect of Btk on calcium mobilisation via PLC-γ tyrosine phosphorylation (Scharenberg et al., 1998).

8.3.2 Btk and its association with G protein signalling

The putative interaction and activation of Btk following association with the βγ subunits of heterotrimeric G proteins in *in vitro* studies (Touhara et al., 1994) has already been mentioned (section 1.5.6.5). However, two more recent lines of evidence support the possibility that Btk is involved in signalling downstream of G protein receptors. The purified α-subunit of the G_q class of G proteins ($G_{\alpha q}$) directly stimulates the activity of Btk *in vitro* and also *in vivo* using DT40 avian lymphoma cells. The significance of this was tested by stimulation of the G_q receptor which showed activation of p38 (a member of the MAP kinase pathway) in wild type DT40cells but not in Btk deficient cells (Bence et al., 1997).

Fig 8.1 Ca^{++} mobilisation mediated by Btk. *BCR ligation results in the activation of Btk (as previously discussed in Chaper 1.5) and of PI-3 kinase. The action of PI-3 kinase results in the conversion of PIP_2 to PIP_3, which is then able to recruit Btk to the plasma cell membrane via an association with the Btk PH domain. Activated Btk phosphorylates PLC-γ2 which catalyses the hydrolysis of PIP_2 to IP_3 and DAG. The increase in IP_3 induces the release of calcium from intracellular calcium stores.*

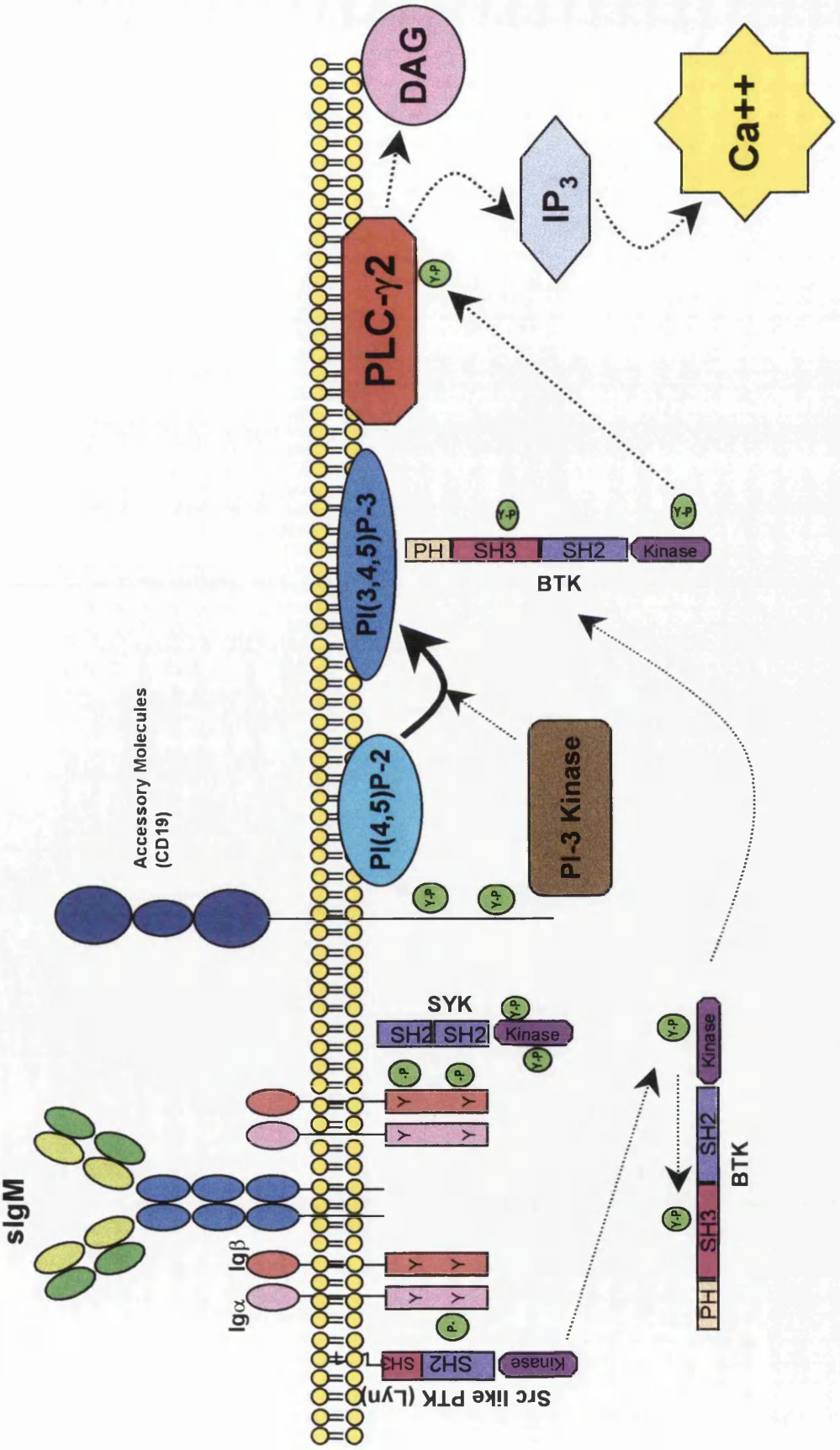


Fig 8.1 Ca++ mobilisation mediated by Btk

In a further report, stimulation of another class of G protein receptors, G_i, also showed failure of MAP kinase pathway activation (Wan et al., 1997) in Btk deficient DT40 cells. In addition these studies implied the need for other tyrosine kinases such as Lyn and Syk in MAP kinase activation following G protein receptor stimulation. Such evidence suggests that Btk may be directly coupled to heterotrimeric G proteins and that Btk along with other tyrosine kinases are required to link G protein receptor activation to the MAP kinase pathway.

8.4 Btk and apoptosis

While many interactions and pathways for Btk in lymphocyte signalling have now been defined, the mechanism by which abnormalities in Btk result in failure of B lymphocyte development remains unanswered. Evidence from a number of predominantly murine studies now implicates Btk in the regulation of pathways that control B cell survival and thus may provide some understanding of this problem.

Naturally occurring programmed cell death (apoptosis) is common during B cell differentiation with only one tenth of the daily 3-5 x 10^7 newly formed B cells being incorporated into the peripheral mature pool. Death of precursor cells by apoptosis may occur at all stages in B cell differentiation. The stochastic nature of Ig gene rearrangement results in unsuccessful assembly of heavy and light chains in the majority of precursors leading to their death in the bone marrow. At a later stage, sIgM bearing immature B cells undergo further selection based on the specificity of their IgM receptors. Those that recognise self-antigen will be eliminated by apoptosis while non self-reactive cells enter the peripheral circulation. Thereafter, encounter with antigen leads to T cell dependent affinity maturation of surface antigen receptors by somatic hypermutation resulting in the preferential selection of cells exhibiting high affinity for antigen or death by apoptosis for cells with low affinity. The exact intracellular mechanisms by which apoptosis in B cell development is regulated are not known but a number of receptor mediated pathways and molecules involved in this process have now been defined.

Engagement of sIgM, CD40L, IL4-R and CD21 surface molecules modulate programmed cell death in B cells in ways that vary according to developmental stage

and the apoptotic trigger (reviewed by Rothstein, 1996). For instance crosslinking of sIgM leads to death by apoptosis of immature B cells, whereas in mature B cells the same trigger induces B cell proliferation (Norvell et al., 1995). The pro-apoptotic sIgM signal in WEHI-231 murine cells can be counteracted by CD40 mediated signals (Choi et al., 1995; Wang et al., 1995) and also by stimulation through IL-4 (Komada et al., 1994).

The receptor mediated signals are conveyed by a number of intracellular molecules which regulate both the induction of apoptosis and the resistance of cells to this process. Prominent in this latter group are the Bcl-2 family of molecules. The role of Bcl-2 in B cell homeostasis and survival is demonstrated by the dramatic apoptosis of both mature B and T lymphocytes seen in Bcl-$2^{-/-}$ mice (Veis et al., 1993). Conversely, mice with dysregulated Bcl-2 expression as a result of an introduced transgene show significant proliferation of B lymphocytes (McDonnell et al., 1990). However, the normal maturation of B cells in Bcl-$2^{-/-}$ mice and the downregulation of Bcl-2 expression in B cell precursors (Merino et al., 1994) suggests that Bcl-2 may not necessarily be important for the anti-apoptotic mechanism in precursor B cells. Nevertheless, this function may be fulfilled by the homologous protein, Bcl-x_L, which is tightly regulated during B cell development with the highest level of expression at the pre-B cell stage (Grillot et al., 1996).

In wild type mice splenic B cells showed induction of Bcl-x_L expression on sIgM cross-linking whereas in similar experiments, *xid* mice cells failed to induce Bcl-x_L suggesting a mechanism whereby functional Btk regulates Bcl-x_L expression. In addition, sIgM stimulation resulted in the synthesis of DNA and a decrease in apoptosis in wild type mice whereas no effect on cell death was seen in *xid* mice (Anderson et al., 1996). Further evidence for an interaction between Btk and this anti-apoptotic mechanism comes from studies using transgenic mice expressing the human Bcl-2 gene under the control of the IgH enhancer. Bcl-2 transgenics were crossed with *xid* mice to produce Bcl-2/ *xid* hybrids which showed prolonged splenic B cell survival and suppressed apoptosis as well as marked expansion of the mature IgM^{lo}/IgD^{hi} B cell population normally diminished in *xid* mice (Woodland et al., 1996). It was also observed that transgene driven expression of Bcl-2 could not support the defective anti-Ig induced proliferation seen in *xid* mice. In similar

experiments, it was demonstrated that Bcl-x_L transgenics crossed with *xid* mice are able to rescue the failure of *xid* B cells to proliferate following antigen receptor cross linking, suggesting that Bcl-x_L may have a unique and non-redundant role in maintaining viability during antigen driven B cell expansion (Solvason et al., 1998). In both sets of trangenic experiments, a number of other *xid* humoral abnormalities such as the diminished CD5 B cell population and the reduction in IgM and IgG3 production remained unaffected by transgene driven expression of Bcl-2 or Bcl-x_L molecules.

While the evidence at present is inconclusive there is, nevertheless, an emerging association of Btk interaction with the Bcl family of anti-apoptotic molecules. If substantiated, this may ultimately provide a mechanism by which the evidence from BCR signalling studies can be linked to the role of Btk in B lymphocyte maturation.

8.4.1 BAP-135 is a downstream target of Btk

The downstream effects of Btk activity include the activation of PLC-γ2 and the induction of Bcl-x_L expression. It is most likely, however, that these are indirect effects of Btk function and no physical association between Btk and these two molecules exists. A molecule BAP-135 has recently been identified as an *in vivo* ligand for Btk and may function as a link between Btk and its downstream substrates (Yang and Desiderio, 1997). In experiments performed on Ramos B cells, a 135kD tyrosine phosphorylated protein was immunoprecipitated with Btk. Sequencing of the isolated protein termed Btk associated protein (BAP)- 135 does not reveal homology with any known proteins. Further studies showed that BAP-135 is a substrate for phosphorylation by Btk and that mutations in Btk that impair its activation by Src kinases abolish Btk dependent phosphorylation of BAP-135. In addition BCR crosslinking leads to BAP-135 tyrosine phosphorylation. This evidence strongly suggests that BAP-135 lies downstream of Btk in a signalling pathway originating at tbe BCR. As such, this is the first *in vivo* association of Btk in human cells to be identified.

8.5 Btk gene transfer in XLA

The latter aims of the project focused on the development of models of gene therapy for XLA by viral mediated gene transfer of Btk using the retroviral vector constructs described in Chapter 5. However, an assessment of the ability of gene transfer to correct the abnormalities observed in XLA was not possible in our studies due to inefficient gene transfer as discussed in Chapter 7.3.

Gene transfer technology has been used to study the role of Btk in B cell signalling and development by a number of other groups. Vaccinia viral constructs have been used to transfect Btk and other signalling molecules into EBV-LCLs (Rawlings et al., 1996; Fluckiger et al., 1998; Scharenberg et al., 1998) from XLA patients and into NIH3T3 fibroblasts in order to demonstrate the interactions of Btk with Lyn and PLC-γ. The studies examining heterotrimeric G protein signalling in DT40 avian lymphoma lines (Bence et al., 1997; Wan et al., 1997) used lipofectin mediated transient transfection of Btk to reconstitute function and signalling in Btk deficient cells.

8.5.1 Btk gene transfer and B cell development

While gene transfer techniques have been successfully used as a tool to examine the role of Btk in lymphocyte signalling as described above, the effect of gene transfer in overcoming the B lymphocyte maturation abnormalities in Btk deficiency has also been addressed. At present no reliably reproducible *in vitro* model of B cell development with which to effectively assess this strategy exists. Thus correction of B cell maturation defect and consequent humoral abnormalities in murine knockout models have been used. A number of targeted Btk knockout mice exist and display similar immunophenotypes to the *xid* mouse (Chapter 1.2.3) (Kerner et al., 1995; Khan et al., 1995). Surprisingly, despite the development of retroviral mediated strategies for gene therapy of other immunodeficiencies and the availability of ideal animal models in which to assess its efficacy, it is interesting to note that there are to date no reports documenting the effect of retroviral mediated gene transfer in the *xid* mouse. It is very likely that although such experiments have been attempted, they have been unsuccessful in correcting the *xid* humoral defect.

Success has however been achieved using alternative methods. While not strictly gene transfer experiments in the conventional sense, two recent studies have demonstrated the principle that ectopic Btk gene expression can overcome the B cell developmental defects seen in Btk null mice (Drabek et al., 1997; Maas et al., 1997). In one strategy, a yeast artificial chromosome (YAC) transgenic mouse strain was generated in which high level expression of human Btk was provided by endogenous regulatory cis-acting elements present on a 340kb transgene. When the YAC-Btk transgenic mice were mated with the Btk^- strain (Hendriks et al., 1996), mice which were Btk^- but with a YAC-Btk backgound were generated. Analysis of the immunophenotype in this population demonstrated restoration of normal numbers of mature B cells in the bone marrow and periphery and reconstitution of the $CD5^+$ B cell population in the peritoneum. In addition the expression of the YAC-Btk transgene restored serum Ig levels and responses to T independent antigens in Btk^- mice.

In an alternative strategy, a transgenic mouse strain was generated in which expression of the human Btk gene (cDNA) was placed under the control of the murine class II major histocompatibility complex Ea gene locus control region, which provides gene expression from the pre-B cell stage onwards (Drabek et al., 1997). Again, when these mice were mated with the Btk^- strain, the presence of the transgene on the Btk^- background resulted in the rescue of the Btk^- strain immune deficits. It would also be reasonable to suggest from these experiments that the temporal regulation of Btk expression during B cell development is critical for successful reconstitution of the maturation defect. The YAC-Btk transgene construct contains the 40kb Btk gene together with 100kb of 5' flanking DNA and 200kb of 3' flanking DNA which would be expected to contain all regulatory elements required for high level expression of Btk throughout B cell differentiation. By contrast the MHC II Ea promoter region would only allow Btk expression from the pre-B cell stage onwards. The success of this latter experiment suggests, therefore, that the Btk gene does not necessarily need to be introduced into haematopoietic stem cells or pro-B cell populations in order to restore B cell development. Gene transfer strategies aimed at transferring and expressing Btk in pre-B cells might achieve successful B lymphocyte reconstitution.

8.6 Gene therapy for primary immunodeficiencies

The initial phase of clinical trials of somatic gene therapy for primary immunodeficiency have now been evaluated and the results published. Only two diseases have been targeted in clinical trials, adenosine deaminase deficiency and chronic granulomatous disease (CGD), although pre-clinical studies and *in vivo* models of gene therapy have been developed for many of the primary immunodeficiencies. Details of the trials conducted so far are summarised in Table 8.1 below.

Table 8.1 Clinical trials of gene therapy for primary immunodeficiency

Investigators[1]	Year initiated	Disease	Vector	Target cell	No. of patients
Blaese et al. (1995)	1990	ADA deficiency	retrovirus	T cells	2
Bordignon et al. (1995)	1992	ADA deficiency	retrovirus	T depleted BM[2] and T cells	3
Hoogerbrugge et al. (1996)	1993	ADA deficiency	retrovirus	BM $CD34^+$ cells	3
Kohn et al. (1995)	1993	ADA deficiency	retrovirus	HUCB[3] $CD34^+$ cells	3
Onodera et al. (1998)	1995	ADA deficiency	retrovirus	T cells	1
Malech et al. (1997)	1995	CGD (p47-phox)	retrovirus	PBSC[4] $CD34^+$ cells	5

[1]denoted by principle author in publication of results

[2]BM= bone marrow [3]HUCB=human umbilical cord blood

[4]PBSC=peripheral blood stem cells

These clinical trials, although initiated with great expectation on the part of patients, investigators and scientific press alike, have not resulted in true therapeutic success. No one trial can claim to have effected a cure on treated patients and in most cases only limited immune reconstitution due to gene transduced cells has been seen. They have, nevertheless demonstrated the principle that long term genetic modification of human cells is possible and that gene therapy is a safe and potentially useful therapeutic strategy.

8.6.1 Clinical gene therapy trials for ADA deficiency

The first clinical trial of gene therapy was started on two girls with ADA deficiency in the United States (Blaese et al., 1995). The treatment protocol involved LASN retroviral vector supernatant transduction of cytokine stimulated peripheral T cells apheresed from each patient The transduced expanded T cells were then returned to the patients 9-12 days later by peripheral infusion and the procedure repeated 11-12 times at regular intervals for each patient over a period of 18-24 months since it was assumed there would be a natural half-life to the transduced cells. Analysis of peripheral cells by Southern blot analysis 2 years after the last infusion showed the presence of transduced vector sequences at a frequency of one copy per cell in PBMC (Mullen et al., 1996). Peripheral blood lymphocyte ADA expression was approximately one quarter of normal and vector ADA gene expression was detectable by RT-PCR. Immunological reconstitution was difficult to evaluate, partly because of the continued administration of PEG-ADA and also due to the variable results in the two patients. In both patients there was an initial rapid rise in the T cell count before stabilising in the normal range for Patient 1 and a slight increase from the pre-treatment levels for Patient 2. Cell mediated immunity was shown to improve in both patients as assessed by the development of DTH skin test reactivity to a variety of antigens and also by improvement in *in vitro* T cell immune responses. Humoral immune function improved with an increased specific antibody response to Hib and tetanus immunisation and an increase in the level of isohaemagluttinin production, although the latter decreased considerably with time.

In a Japanese trial involving one patient treated with exactly the same protocol as above (Onodera et al., 1998), less prolonged follow up showed the frequency of integrated provirus in peripheral blood to be 15% (equivalent to 0.1 –0.2 proviral copies per cell) with an associated increase in ADA activity. Again there was an increase in total T lymphocytes post gene therapy and an improvement in humoral and cellular immunity as assessed by similar parameters to those above. However, in both trials, the continued administration of exogenous enzyme replacement does not allow accurate assessment of the contribution of transduced T lymphocytes to improved immune function. One interesting observation arising from these studies is the prolonged survival of the transduced T cells suggesting a proliferative advantage to gene transduced ADA positive cells.

In the three other ADA gene trials conducted attempts were made to transduce progenitor populations. An alternative approach was taken by Bordignon and colleagues in 1992 (Bordignon et al., 1995). Two structurally identical vectors expressing the human ADA cDNA and promoter regions and distinguishable by the presence of alternative restriction sites in a non-functional region of the viral LTR, were used to transduce peripheral blood lymphocytes (PBLs) and T cell depleted bone marrow independently. Thus by restriction digest analysis, it was possible to identify the origin of transduced cells. Three patients have to date been treated by this protocol (result have been published on the initial two patients). Analysis of vector transduced cells from peripheral blood indicated that initially all vector-positive cells were derived from transduced PBLs. However, 3 years after initiation, and 1 year after discontinuation of gene therapy, peripheral lymphocytes showed the alternative restriction digest pattern indicating conversion of the circulating transduced lymphocytes from a PBL-derived to a bone marrow derived population. Further analysis of T lymphocyte clones, granulocytes and erythrocytes taken at this time point also showed a predominantly bone marrow origin. These findings suggest that there had been stable transduction of early haemopoietic progenitors capable of generating multilineage progenies. Moreover, the continued presence of transduced bone marrow cells and their differentiation into mature circulating cells appears to confirm the assumption that a small number of ADA+ cells have a survival advantage in an ADA- environment.

The immune reconstitution in these children showed an increase in absolute lymphocyte and T cell numbers in both children into the normal range and there has been an improvement in T cell proliferative responses to non-specific and specific stimuli. Isohaemagluttinin titre has also improved since the initiation of gene therapy. To further study the improvement in T lymphocyte function, the T cell repertoire was analysed by Vβ usage. RT-PCR analysis using Vβ chain specific primers showed demonstrated the development of a normal T cell repertoire. Although these results appear promising, it must be remembered that both children are still on PEG-ADA therapy, albeit with decreasing doses.

$CD34^+$ stem cell transduction was attempted in the two other trials. In the bone marrow transduction protocol (Hoogerbrugge et al., 1996), evidence of the transduced gene in the bone marrow and peripheral blood cells was lost after 6 months. The final trial to be published also targeted the $CD34^+$ population but on this occasion in cord blood derived cells (Kohn et al., 1995). Three pre-natally diagnosed infants were treated by autologous transplantation of transduced cells. Cord blood was taken from their umbilical cord at birth and $CD34^+$ cells selected prior to transduction with the LASN vector. All 3 patients were started on PEG-ADA in the first few days of life. Four years after the procedure, a recent report highlights many of the positive features and limitations of these initial clinical trials (Kohn et al., 1998). The report demonstrates the continued stable presence of gene transduced lymphocytes and granulocytes in the peripheral blood after four years, suggesting that long-lived progenitor cells were transduced and engrafted to produce cells of multiple haematopoietic and lymphoid lineages. In addition, the frequency of T lymphocytes containing the transduced ADA gene exceeded the frequency of transduced cells of other haematopoietic and lymphoid lineages again suggesting that the ADA gene corrected T lymphocytes may have a proliferative or survival advantage.

Importantly in this study, in one patient PEG-ADA was stopped temporarily. During this time the absolute number of transduced T cells increased but there was a decrease in the overall numbers of T cells as well as a deterioration in other immune parameters. Furthermore, the metabolic correction provided by PEG-ADA was not sustained by the ADA gene transduced cells alone and necessitated recommencement of PEG-ADA therapy. These data suggest that although significant levels of cells may have been transduced, the expression of the ADA gene from these cells is inadequate and may reflect poor expression of the ADA gene from the retroviral promoters.

8.6.2 Clinical gene therapy trials for CGD

Five adult patients with CGD due to p47-phox deficiency were treated by retroviral mediated transfer of the p47-phox gene into autologous PBSCs (Malech et al., 1997). Transduced gene positive cells were present in the peripheral blood with functional activity up to 6 months after treatment albeit at very low frequencies. The

disappearance of transduced cells 6 months after treatment is most likely a reflection of the low numbers of stem cells transfected by the transduction procedure.

8.6.3 Summary

The most important lessons arising from these initial trials are that stable gene transfer into somatic cells is a safe and feasible strategy. They have also shown that relatively early progenitor cell populations capable of multilineage commitment can be stably transduced. However, these trials and importantly those trials of gene therapy for other haematopoietic conditions have also highlighted the limitations of present retroviral vector systems and the relative inefficiency of current transduction protocols. Of major concern is the inability to efficiently transduce true haematopoietic stem cells. Development of improved vector systems, a greater understanding of stem cell physiology and the means by which repopulating cells can be assayed *in vivo* are required before this can be successfully achieved.

8.7 Overall summary and future prospects

The work in this thesis has examined a wide range of aspects of X-linked agammaglobulinaemia. In many ways, the work accomplished mirrors studies performed for many of the other primary immunodeficiencies. Since the identification of the respective genes, studies involving mutation analysis, genotype-phenotype correlation, protein expression and an attempt to understand the molecular pathogenesis of disease have been carried out for many of these conditions. The most striking observations concern the lack of phenotypic correlation with the genetic defect and the expression of the mutant protein. The data emerging from the study of a number of primary immunodefciencies strongly suggests that the resultant clinical phenotype is a multifactorial entity. In the discussion a number of possible influences and compensatory factors have been suggested, but as yet no experimental data has been forthcoming to substantiate or refute these ideas.

The interaction of Btk and the downstream pathways in which Btk was involved were the subject of much speculation and initially only suppported by *in vitro* data. The role of Btk in the mobilisation of intracellular calcium is now evident and this indeed ties together the previous *in vitro* data regarding the interaction of the PH domain with phophoinositides and the interaction of Btk with PLC-γ2. However, only recently, as discussed in section 8.4, has a more complete picture of the association between the B cell molecular signalling pathways and cellular events started to emerge. The suggestion that Btk may regulate the anti-apoptotic mechanism at a crucial stage in B lymphocyte differentiation appears attractive but will require further more direct evidence.

For many of the primary immunodeficiencies, the identification of the defective gene has led to the development of gene therapy for their treatment. In the cases of ADA-SCID and CGD, clinical trials have been attempted. However, in both clinical trials and in pre-clinical models, as presented in this thesis, a number of fundamental difficulties have been identified. If somatic gene therapy is to become a clinical reality, then a greater understanding of transgene expression and regulation will be necessary. This will be especially so for a condition such as XLA where precise regulation of gene expression may be required for a successful outcome.

APPENDIX

A number of terms have been used to define the different clinical and immunological manifestations of patients with XLA. For the purposes of clarification, the terms used in this thesis are defined below:

Severe/Classical XLA

These are patients with a history of recurrent bacterial infections and with an immunological profile of <1% B cells in the peripheral circulation and <1g/l of IgG detectable in the peripheral circulation at the time of diagnosis. This definition refers to the following patients in Chapter 4; P1-10, P13, P14

Leaky XLA

This term is used to define certain individuals purely on the basis of their immunological profile. These patients have at least 1% circulating B cells and the production of >1g/l of IgG. This refers to the following patients in Chapter 4; P11, P12. The respective immunological investigations at the time of diagnosis are given below:

Patient	B cell %	IgG (g/l)	IgM (g/l)	IgA (g/l)
P11	1%	8.09	0.25	0.46
P12	1%	2.75	not done	not done

Atypical/Mild XLA

This term refers to patients on the basis of their clinical phenotype. Certain patients present with less severe infective problems or present late, such as in their teens or early adulthood. This does not necessarily correlate with the numbers of B cells in their peripheral circulation or with their level of immunoglobulin production.

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