Molecular and biophysical analysis of the non-catalytic PH, TH, SH3 and SH2 domains of Bruton tyrosine kinase (Btk) protein

Michael P. Okoh

Department of Biosciences, Division of Biochemistry,
University of Helsinki;
Institute of Medical Technology, University of Tampere;
and
The National Graduate School in Informational
and
Structural Biology, Åbo Akademi University, Turku, Finland.

Academic dissertation

To be presented for public criticism, with the permission of the Faculty of Science of the University of Helsinki, in the auditorium PIII in Portania, Yliopistokatu 3, Helsinki On November 22nd 2002, at 12 o'clock noon.

Helsinki, 2002

Supervisor: Professor Mauno Vihinen
Institute of Medical Technology
University of Tampere
Tampere
Finland

Reviewers: Professor Veli-Pekka Lehto
Department of Pathology
Haartman Institute
University of Helsinki
Helsinki
Finland

Professor Dennis Bamford
Department of Biosciences
Division of genetics
Faculty of Science
University of Helsinki
Helsinki
Finland

Opponent: Professor John E. Eriksson
Department of Biology
Laboratory of Animal Physiology
University of Turku
Turku
Finland

ISBN 952-10- 0321-9
ISBN (e-thesis) 952-10-0322-7
Helsinki 2002
Yliopistopaino

“My Dear....See Meeeeee”

Dedicated to my Father

CONTENTS

Original Publications

Abbreviations

Abstract

1 Introduction 10
1.1 Primary immunodeficiencies (PIDs) 10
1.1.1 X-linked agammaglobulinemia (XLA) 12

2 Signal transduction 14
2.1 Non-receptor protein kinases 14
2.2 Tec family of cytoplasmic tyrosine kinases 15
2.2.1 The regulation of Btk 18

3 Btk non-catalytic domains 23
3.1 PH domain 23
3.2 TH domain 25
3.3 SH3 domain 25
3.4 SH2 domain 28

4 Prelude to experimental approaches: Spectroscopic methods in brief 30

5 Aims of the study 31

6 Experimental approaches 32
6.1 Mutation scanning using base excision sequence scanning (BESS) method 32
6.2 Molecular cloning and expression 32
6.3 Protein purification using chromatography 33
6.4 Molecular modeling/sequence analysis 33
6.5 Biophysical studies of molecular interactions 33
6.5.1 CD spectroscopy 33
6.5.2 Determination of dissociation constant (K_d) 34
6.5.3 NMR spectroscopy 34
6.5.4 Temperature gradient gel electrophoresis 34

7 Results 36
7.1 XLA causing mutations in the non-catalytic domains of Btk 36
7.2 Structural interpretations of XLA causing mutations in the gene encoding Btk PH domain region and comparison with the Tec family PH domains 37
7.3 TH/SH3 domains partners in the downstream regulation of Btk interactions 37
7.3.1 interactions of Btk TH and SH3 domains 38
7.3.2 Intermolecular interactions involving the TH domain 38
7.4 Stability and unfolding of the Btk non-catalytic domains 39

8 Discussion 40
8.1 XLA causing mutations 40
8.2 Intra/intermolecular interaction(s): a mode for Tec family kinase regulation 40
8.3 The biological implications of Btk SH3 domain interactions 44
8.4 The dynamics of the Btk N-terminal domains 46

9 Future perspectives and conclusions 47

10 Acknowledgements 48

11 References 50

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numbers I-V:

I. Okoh, M.P. and Vihinen, M. (1999) Pleckstrin Homology Domains of Tec Family Protein Kinases. *Biochem Biophys Res Comm.* **265**, 151-157

II. Okoh, M.P. and Vihinen, M. (2002) Interaction Between Btk TH and SH3 Domain. *Biopolymers* **63**, 325-334

III. Okoh, M.P., Kainulainen, L., Heiskanen, K., Nizam Isa, M., Varming, K., Ruuskainen, O. and Vihinen, M. (2002) Novel insertions of Bruton tyrosine kinase in patients with X-linked agammaglobulinemia. *Human mutat*. in press

IV. Hansson, H., Okoh, M.P., Smith, C.I.E., Vihinen, M., and Härd, T. (2001) Intermolecular interactions between the SH3 domain and the proline-rich TH region of Bruton's tyrosine kinase *FEBS Lett*. **489**, 67-70

V. Okoh, M.P., Lappalainen, I., Stenberg, K., Pasanen, T. and Vihinen, M. (2002) Ligand Binding and Stability of Four N-Terminal Domains of Btk. Manuscript

Abbreviations

ADA	Adenosine deaminase
BCR	B-cell receptor
BESS	Base excision sequence scanning
Bmx	Bone marrow kinase gene on the X chromosome
Btk	Bruton tyrosine kinase
CD	Circular dichroism
CGD	Chronic granulomatous disease
EBV	Epstein-Barr virus
FcåRI	High affinity IgE receptor
GSH	Glutathione
GST	Glutathione S-transferase
HOMO	Highest occupied molecular orbital
HSQC	Heteronuclear single quantum coherence
IL-5	Interleukin-5
IL-12	Interleukin-12
Ins(1,2,3,4)P_4	Inositol (1,2,3,4)-tetrakisphosphate
ITAM	Immunoreceptor tyrosine activation motif
Itk	IL2-inducible T-cell kinase
kDa	kiloDalton
K_d	Dissociation constant
KM	Michaelis-Menten constant
LUMO	Lowest occupied molecular orbital
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
PDB	Protein Data Bank
PH	Pleckstrin homology
PID	Primary immunodeficiency disorder
PI3K	Phosphatidylinositol 3-kinase
PRR	Proline-rich region
PtdIns(3,4,5)P_3	Phosphatidyl (3,4,5)-triphosphate
PTK	Protein tyrosine kinase
SH2D1A	SH2-domain encoding gene
SH	Src homology
TGGE	Temperature gradient gel electrophoresis
TH	Tec homology
Txk	T and X cell expressed kinase
WAS	Wiskott-Aldrich syndrome
XCGD	X-linked form of chronic granulomatous disease
Xid	X-linked immunodeficiency in mice

XLA	X-linked agammaglobulinemia in human
XLP	X-linked lymphoproliferative syndrome

Abstract

Bruton tyrosine kinase (Btk) belongs to the Tec family of non-receptor protein tyrosine kinases. Mutations in the gene coding for Btk lead to a hereditary immunodeficiency, X-linked agammaglobulinemia (XLA), in man. This family of tyrosine kinases shares a common domain composition, with an N-terminal Pleckstrin homology domain, the Tec homology (TH) domain, Src homology 3 (SH) and SH2 domain, and a C-terminal Src kinase domain. The TH domain adjacent to the SH3 domain in Btk consists of two motifs, the Btk motif and proline-rich regions (PRR) that conform to the canonical SH3 binding motifs.

This study aimed at elucidating the role of the N-terminal non-catalytic domains (PH, TH, SH3 and SH2) in the regulation of the kinase activity of the Btk protein, and exploring the nature of mutations in Btk encoding gene in Finnish patients with XLA. Molecular modelling of Btk and its family members with mutational analysis of Btk gave insights on the structural and molecular consequences of amino acids alteration in the PH domain of the Btk. Using base excision sequence scanning methodology followed by direct sequencing, several families with XLA were studied. The role of the SH3 domain was studied using circular dichroism (CD) and fluorescence spectroscopy by analysing binding of peptides corresponding to segments of the PRRs, to the SH3 domain. The N-terminal half of the PRR was shown to bind specifically, whereas the C-terminal peptide had no detectable affinity. The TH domain had about four times lower affinity for the SH3 domain (17.0 μM) than the N-terminal peptide (3.9 μM). For the PRR-SH3 domain, neither of the two peptides could compete for the binding to the construct. However, the TH domain binding to the PRR-SH3 domain had two times lower affinity than to the SH3 domain alone. By using intermolecular NMR cross-relaxation techniques combined with gel filtration chromatography profiles, a dimer formation involving the PRR and the SH3 domain was identified. Further, using CD, fluorescence spectroscopy and temperature gradient gel electrophoresis (TGGE), the unfolding process and stability of the N-terminal (PH, TH, SH3 and SH2) domains of Btk was studied. These results have helped us to gain insights into the function and regulation of the Btk protein. Moreover, the involvement of the non-catalytic domains in signalling and the concomitant disease phenotype upon amino acid alterations is further elucidated.

1 Introduction

1.1 Primary immunodeficiency disorders (PIDs)

Primary immunodeficiency disorders (PIDs) affect the functioning of the immune system. They arise mainly due to mutations in genes, which are involved in the production of the components of the immune system. Due to these intrinsic defects, affected patients are highly susceptible to persistent and recurrent infections (Buckley et al., 1997; Smith and Notarangelo, 1997; Vihinen et al., 2001). PIDs were first identified some 50 years ago (Smith and Notarangelo, 1997). In molecular terms, the gene whose defects cause the X-linked form of chronic granulomatous disease (XCGD), was the first to be identified using positional cloning technique (Royer-Pokora et al., 1986). X-linked agammaglobulinemia (XLA) was the first reported human disorder in which a mutation in the gene encoding a cytoplasmic protein tyrosine kinase was found (Tsukada et al., 1993; Vetrie et al., 1993). Adenosine deaminase (ADA) deficiency was the first primary immuno-deficiency in which gene therapy was attempted (Blaese et al., 1995; Bordignon et al., 1995). Bovine enzyme replacement therapy has usually been accompanied with polyethelene glycol (PEG), in the treatment of patients with ADA deficiency, hence, the clinical impact of gene therapy has been difficult to assess. Cavazzana-Calvo et al. (2000) reported the first successful gene therapy trial for a patient with X-linked severe combined immunodeficiency. A significant advancement over the pioneering studies of Bordignon et al. (1995), using gene therapy in the treatment of ADA deficiency has also been recently reported (Aiuti et al., 2002a; Aiuti et al., 2002b).

Most PIDs are characterized by an increased susceptibility of the individual to opportunistic infections (Lederman and Winkelstein, 1985; Arkwright et al., 2002). Patients with B-cell deficiency are liable to bacterial infections, whilst patients with T-cell defects tend to be susceptible to various parasitic, viral and fungal infections (Buckley et al., 1997; Vihinen et al., 2001). Diseases that afflict phagocytes cause mainly skin and oral bacterial infections and the formation of granulomas due to the spread of the causative bacteria to such organs as the liver (Vihinen et al., 2001). In these patients fungal infections tends to cause severe complications. Patients with certain PIDs are known to be susceptible to some specific pathogens. Patients with X-linked lymphoproliferative syndrome (XLP), a disease caused mainly due to mutation in the gene (SH2D1A) (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998), are prone to Epstein-Barr virus (EBV) infections. Whereas those with mutations in interleukin 12 (IL-12), with the disrupted interferon-γ signaling, are susceptible to some forms of idiopathic mycobacterial and *Salmonella* infections (de Jong et al., 1998).

Some primary immune disorders are now known to result in autoimmunity. The basis of such autoimmunity has been attributed to the inability of the host to eradicate microbial pathogens and their antigens completely through the usual immune pathways (Arkwright et al., 2002). Some specific examples of PIDs that are associated with autoimmunity include those leading to phagocyte disorders such as chronic granulomatous disease (CGD) or leukocyte adhesion molecule deficiency (LAD), (for review see Arkwright et al., 2002). Mutations in the apoptosis mediator genes TNFSF6, and TNFRS6 have also been shown to lead to autoimmunity (Ochs et al., 1999). Some primary immune disorders predispose to certain forms of cancer. Due to EBV infection in patients with the X-linked Wiskott-Aldrich syndrome (WAS) the prevalence of EBV-driven lymphomas is high (Cotelingham et al., 1985).

Genes associated with PIDs are found on a number of chromosomes (Table 1.1).

TABLE 1.1 Some primary genetic disorders of the immune system and their affected genes (adapted from Vihinen et al., 2000 with minor changes)

Disease	Gene	Chromosomal loci
Severe combine immonodeficiencies (scid) of both T and B cells	Adenosine deaminase (ADA)	20q13.11
	JAK-3	19p13.1
	RAG1/2	11p13
	IL2RG	Xq26
X-linked immunodeficiency with increased IgM (XHIM)	CD40LG	Xq13
X-linked antibody deficiencies		
X-linked agammaglobulinemia (XLA)	BTK	Xq21.3-q22
IgA deficiency	IGAD1	6p21.3
IgG immunoglobulin subclass deficiencies	IGHG1/-4	14q32.33
	IGKC	2p12
Other combined immunodeficiencies of T cell origin		
Purine nucleoside phosphorylase deficiency	Nucleoside phosphorylase	14q13.1
ZAP-70 deficiency	ZAP-70	2q12
CD3γ deficiency	CD3G	11q23
CD3ε deficiency	CD3E	11q23
Other well defined immunodeficiencies		
Wiskott-Aldrich syndrome (WAS)	WAS	Xp11.23-p11.22
DiGeorge syndrome	DGCR	22q11
Ataxia-telangiectasia	ATM	11q22.3
X-linked lymphoproliferative syndrome (XLP)	SH2DIA	Xq25
Familial hemophagocytic lymphohistiocytosis (FHL)	PRF1	10q21-22
Leukocyte adhesion deficiency 1 (LAD1)	ITGB2	21q22.3
X-linked chronic granunomatous disease (XCGD)	CYBB	Xp21.1

The majority of the genes code for multidomain proteins, although there are some e.g. ADA and SH2DIA with a single domain (for details see Vihinen et al., 2001 and references therein). Most of the protein defects implicated in PIDs have been found to be involved in diverse cellular processes ranging from signal transduction, transcription factors, phagocytosis, to cell surface receptors etc. However, for many of these proteins, with their diverse locations, their exact mechanisms of action have yet to be established.

1.1.1 X-linked agammaglobulinemia (XLA)

XLA is a disease resulting from defects in Btk protein. The disease is characterized by profound hypogammaglobulinemia, an early onset of bacterial infections, a decrease in the number of B-lymphocytes, and an almost complete lack of plasma cells (Sideras and Smith, 1995; Smith and Notarangelo, 1997). The immunoglobulin levels in affected individuals are very low. The disease afflicts about 1/200,000 males (Sideras and Smith, 1995; Ochs and Smith, 1996). The associated infections, particularly bacterial meningitis and pneumonia, are often life threatening and therefore, early diagnosis is critical for the management of XLA (McKinney et al., 1987; Ochs and Smith, 1996).

Btk was identified as the molecular defect in XLA by two different approaches, using positional cloning (Vetrie et al., 1993) and in a search for novel protein kinases expressed in B lymphocytes (Tsukada et al., 1993). The gene was mapped to the Xq21.3-22 region in the mid-portion of the long arm of the X-chromosome (Kwan et al., 1986; Ott et al., 1986). The human gene encompasses 37.5 kb and is organized into 19 exons (Hagemann et al., 1994; Rohrer et al., 1994), including a 5' untranslated region (exon 1). Btk is a 659 amino acid long protein in both man and mouse (Vetrie et al., 1993; Tsukada et al., 1993). It encodes a cytoplasmic protein tyrosine kinase (PTK).

The study of XLA has profoundly contributed in the understanding of the immunological basics of the humoral immunity. Btk is expressed throughout the development in B cells except the plasma cells (Sideras and Smith, 1995). In XLA, the production of Btk is blocked at the transitional stage from pro-B to pre-B cells resulting in B cell apoptosis (Anderson et al., 1996) (see Fig 1.1). Moreover, as was recently shown in xid mice, the first manifestation of Btk deficiency occurs in the transition from pro-B to pre-B stage (Cancro et al., 2001). Thus, the initial effects of xid in mice and XLA in man are at the same stage of B cell development (Cancro et al., 2001). Various types of XLA causing mutations have been identified in all Btk domains (Vihinen et al., 1999).

The analysis of Btk expression in primary cells of XLA patients has shown that majority of the individuals do not express the protein, regardless of the mutation type (Gasper et al., 2000). So far, it has not been possible to predict how specific mutations at critical sites in Btk affect the function and the concomitant XLA phenotype (Gasper et al., 2000; Gasper et al., 1998). Further, correlation between the protein expression level and differences in the clinical and immunological phenotypes have not been possible. Many individuals previously diagnosed as having common variable immunodeficiency or immunoglobulin subclass deficiency, have been shown to have Btk deficiency (Futatani et al., 1998; Vórechvsky, 1994). The majority of the described mutations lead to a truncated protein. Whilst the large majority of mutations are found in the coding region, over 12% of mutations have been found to affect splice site recognition sequences (Vihinen et al., 1995a; Conley et al., 1998). Several XLA causing mutations in Btk have been described and are collected in the BTKbase that is available at http://www.uta.fi/imt/bioinfo/Btkbase (Vihinen et al., 1995a; Vihinen et al., 1999). In addition to describing the mutations, the database also contains clinical information regarding to patients.

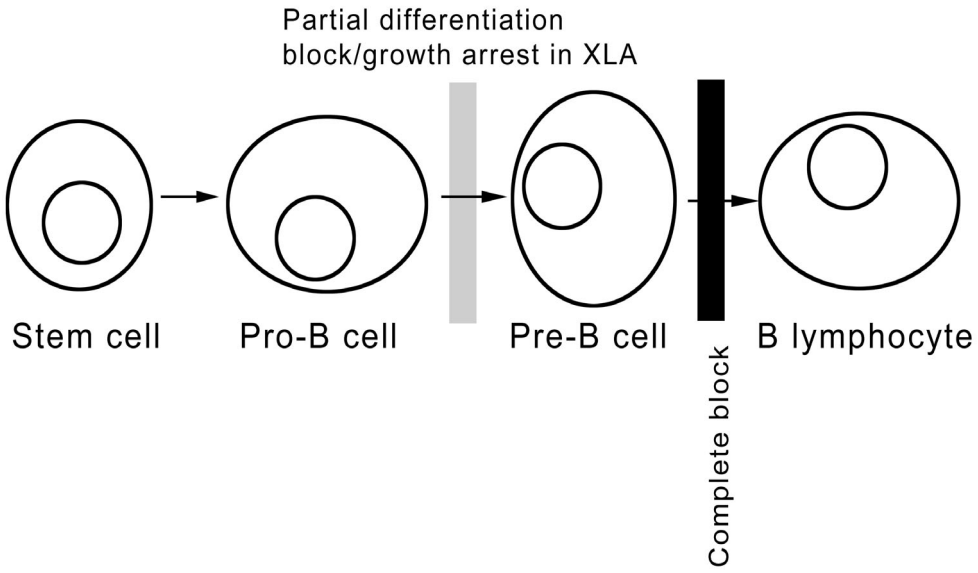


Figure 1.1 The schematic illustration of B-cell development. Btk is expressed throughout B cell development, but down regulated in plasma cells. Upon Btk mutation, there is a block in the development at the transitional stage from pro- to pre- B cell leading to XLA in man and xid in mice.

2 Signal transduction

The term signal transduction implies the conversion of an input signal received at the extracellular face of the plasma membrane into an intracellular signal that, ultimately, leads to alteration of gene expression. The correct transmission of the signal is vital for both unicellular and multicellular organisms; the stimulation of cells from outside, due to signaling molecules, can trigger a cascade of events leading to diverse cellular responses such as, differentiation, growth and movement (Hunter, 1995). Various types of proteins, including transmembrane receptors, G-protein subunits, adaptor proteins, kinases and phosphatases, are involved in signaling.

The plasma membrane plays an important role as a meeting point for signaling proteins. The association of signaling proteins with the membrane can be direct or indirect (Mumby, 1997). The binding of a cytoplasmic protein to the membrane could be achieved, for example, by modification of the proteins by lipid (Mumby, 1997). Further, with specific protein-protein interactions, signals can also be transduced through the membrane via an indirect mechanism. These processes are regulated through covalent protein modification such as phosphorylation, one of the hallmarks of intracellular signaling (Carpenter and Cantley, 1996).

Btk protein is a non-receptor PTK known to be involved in B-cell survival, cell cycle progression and proliferation in response to B cell receptor (BCR) stimulation (Petro et al., 2000). The phosphorylation of a tyrosine residue in the activation loop of Btk is essential for its participation in BCR signaling (Kurosaki, 1997).

2.1 Non-receptor protein tyrosine kinases

Approximately 20% of the ~32,000 human genes are known to encode proteins that are involved in signaling (Blume-Jensen and Hunter, 2001). Among them are, for instance the transmembrane receptors, adaptor proteins, kinases and phosphatases. Included in the latter groups are the PTKs, a group of enzymes that have evolved to play key roles in transmitting signals in multicellular organisms. PTKs are found only in metazoans (Blume-Jensen and Hunter, 2001). In the human genome, over 90 genes encode for PTKs and these can be divided into two subgroups, receptor and non-receptor PTKs. Currently, there are 30 identified mammalian non-receptor tyrosine kinases which can be divided into 11 families based on amino acid sequence identities (Burkhardt and Bolen, 1996). In addition to the kinase domain, PTKs have other conserved domains, which

are also found in several distinct signaling pathways. These conserved domains are thought to regulate signaling through their ability to mediate protein-protein interactions. These domains have consequently become the building blocks for the formation of complex networks of interacting proteins (Saouaf, 1995).

Signaling domains are generally dynamic and plastic, and can be modified. They mostly occur as part of domain assemblies in modular proteins. Although the catalytic domain of most PTKs has low substrate specificity, the non-kinase domains attached also interact with regulators and substrates in the context of specific signaling processes and the cellular environment. Moreover, intra and intermolecular interactions between domains can critically affect the kinase activity of the enzyme.

The role of modular proteins in signaling has been a focal point in signal transduction research over the last decade. Discoveries of proteins with variable domains such as PH, SH3, SH2, SH1 or the kinase domain, PDZ, C1 and C2 (Hurley et al., 2002) and elucidation of their properties and functions, have contributed greatly to the current understanding of signaling pathways. The importance of the domain concept is reflected in the fact that each domain mentioned above occurs in a large number of different signaling proteins. Hence, understanding the function of a particular domain in one protein can be used to infer its function in many other proteins that contain the same domain. Thus, domain information provides a simple and most powerful conceptual bridge in attempts to understand the vast and complex sequence data from the human genome (Hurley et al., 2002).

2.2 Tec family of cytoplasmic tyrosine kinases

The Tec family of PTKs is characterized by similar amino acid sequences (Fig. 2.1) and cells of the immune system express them predominantly. They have a characteristc domain architecture comprising the N-terminal PH domain followed by a Tec homology (TH) domain, which contains a Zn^{2+} binding Btk motif and a proline-rich region, a SH3, SH2, and the C-terminal kinase domain. Atypical member of the Tec family PTKs is Txk, which lacks the PH and part of the TH domain. Moreover, the *Drosophila* type 1 splice variant of Btk29A, also lack the PH domain. The structural similarity (Fig. 2.2) shared among the Tec family members suggest that these proteins may have overlapping functions.

Tec family kinase homolog are also expressed in other species, such as *Drosophila melanogaster*, skate and zebrafish (Roulier et al., 1998; Baba et al., 1999; Haire et al., 1997; Haire et al., 1998). Btk29A, the *Drosophila* member

of Tec kinases, exists in two different splice variants i.e. types 1 and 2 (Baba et al., 1999). Type 2 of Btk29A is composed of 786 amino acids, hence it has the highest molecular weight among Tec kinases (Baba et al., 1999). Detailed information relating to the Tec family protein tyrosine kinases is presented in the Table 2.2

Table 2.2 Tec family PTKs

PTK	Species	Chromosomal location	Gene size (kb)	Number of exons	Protein size (amino acid residues)	Gene accession number
Btk	Human Mouse	Xq22 Synt.Xq22	37.5 43.5	19 19	659 659	X58957 L29788
Tec	Human Mouse	4p12 5	 86	 17	631 602-630	D29767
Itk/Tsk/Emt	Human Mouse Zebrafish	5q34 15			620 625 616	D13720/S65186 L00619 AF016326
Bmx	Human Mouse	Xp22.2 X	~ 48	18	675 651	X83107 AF012104/U88091
Txk/Rlk	Human Mouse	4p12 5	~ 70 ~ 70	15 15	527 and 502 527 and 473	L27071 U16145
Btk29A	Drosophila melanogaster	2-[24]	~ 40	9 for type 1 12 for type 2	603 786	AB009840 AB009841
Skate Ptk	Raja eglanteria				628	U85659

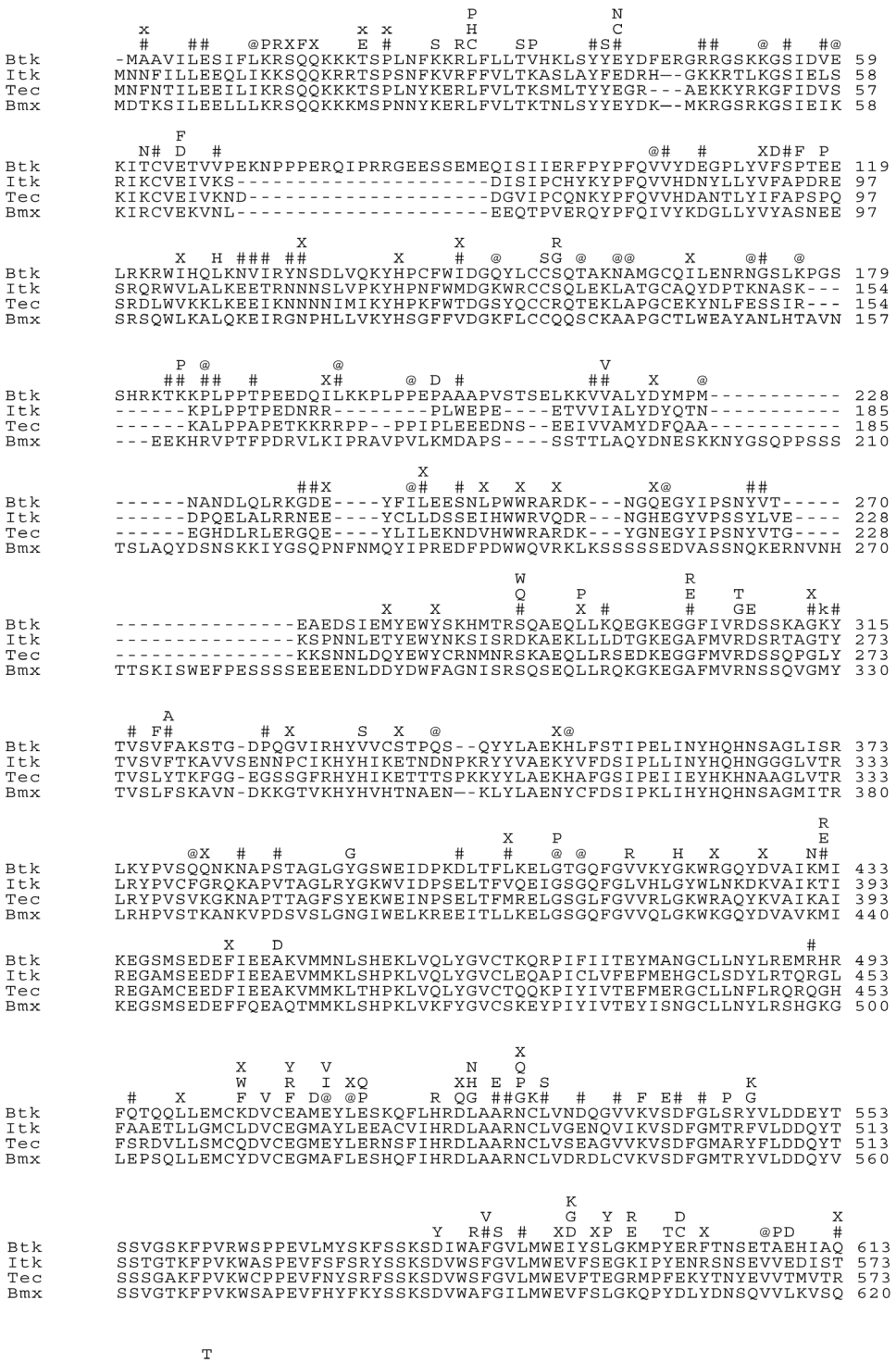

Btk -MAAVILESIFLKRSQQKKKTSPLNFKKRLFLLTVHKLSYYEYDFERGRRGSKKGSIDVE 59
Itk MNNFILLEEQLIKKSQQKRRTSPSNFKVRFFVLTKASLAYFEDRH--GKKRTLKGSIELS 58
Tec MNFNTILEEILIKRSQQKKKTSPLNYKERLFVLTKSMLTYYEGR---AEKKYRKGFIDVS 57
Bmx MDTKSILEELLLKRSQQKKKMSPNNYKERLFVLTKTNLSYYEYDK--MKRGSRKGSIEIK 58
Btk KITCVETVVPEKNPPPERQIPRRGEESSEMEQISIIERFPYPFQVVYDEGPLYVFSPTEE 119
Itk RIKCVEIVKS----------DISIPCHYKYPFQVVHDNYLLYVFAPDRE 97
Tec KIKCVEIVKND----------DGVIPCQNKYPFQVVHDANTLYIFAPSPQ 97
Bmx KIRCVEKVNL----------EEQTPVERQYPFQIVYKDGLLYVYASNEE 97
Btk LRKRWIHQLKNVIRYNSDLVQKYHPCFWIDGQYLCCSQTAKNAMGCQILENRNGSLKPGS 179
Itk SRQRWVLALKEETRNNNSLVPKYHPNFWMDGKWRCCSQLEKLATGCAQYDPTKNASK--- 154
Tec SRDLWVKKLKEEIKNNNNIMIKYHPKFWTDGSYQCCRQTEKLAPGCEKYNLFESSIR--- 154
Bmx SRSQWLKALQKEIRGNPHLLVKYHSGFFVDGKFLCCQQSCKAAPGCTLWEAYANLHTAVN 157
Btk SHRKTKKPLPPTPEEDQILKKPLPPEPAAAPVSTSELKKVVALYDYMPM---------- 228
Itk ------KPLPPTPEDNRR--------PLWEPE----ETVVIALYDYQTN---------- 185
Tec ------KALPPAPETKKRRPP--PPIPLEEEDNS--EEIVVAMYDFQAA---------- 185
Bmx ---EEKHRVPTFPDRVLKIPRAVPVLKMDAPS----SSTTLAQYDNESKKNYGSQPPSSS 210
Btk ------NANDLQLRKGDE----YFILEESNLPWWRARDK---NGQEGYIPSNYVT----- 270
Itk ------DPQELALRRNEE----YCLLDSSEIHWWRVQDR---NGHEGYVPSSYLVE---- 228
Tec ------EGHDLRLERGQE----YLILEKNDVHWWRARDK---YGNEGYIPSNYVTG---- 228
Bmx TSLAQYDSNSKKIYGSQPNFNMQYIPREDFPDWWQVRKLKSSSSSEDVASSNQKERNVNH 270
Btk ---------------EAEDSIEMYEWYSKHMTRSQAEQLLKQEGKEGGFIVRDSSKAGKY 315
Itk ---------------KSPNNLETYEWYNKSISRDKAEKLLLDTGKEGAFMVRDSRTAGTY 273
Tec ---------------KKSNNLDQYEWYCRNMNRSKAEQLLRSEDKEGGFMVRDSSQPGLY 273
Bmx TTSKISWEFPESSSSEEEENLDDYDWFAGNISRSQSEQLLRQKGKEGAFMVRNSSQVGMY 330
Btk TVSVFAKSTG-DPQGVIRHYVVCSTPQS--QYYLAEKHLFSTIPELINYHQHNSAGLISR 373
Itk TVSVFTKAVVSENNPCIKHYHIKETNDNPKRYYVAEKYVFDSIPLLINYHQHNGGGLVTR 333
Tec TVSLYTKFGG-EGSSGFRHYHIKETTTSPKKYYLAEKHAFGSIPEIIEYHKHNAAGLVTR 333
Bmx TVSLFSKAVN-DKKGTVKHYHVHTNAEN--KLYLAENYCFDSIPKLIHYHQHNSAGMITR 380
Btk LKYPVSQQNKNAPSTAGLGYGSWEIDPKDLTFLKELGTGQFGVVKYGKWRGQYDVAIKMI 433
Itk LRYPVCFGRQKAPVTAGLRYGKWVIDPSELTFVQEIGSGQFGLVHLGYWLNKDKVAIKTI 393
Tec LRYPVSVKGKNAPTTAGFSYEKWEINPSELTFMRELGSGLFGVVRLGKWRAQYKVAIKAI 393
Bmx LRHPVSTKANKVPDSVSLGNGIWELKREEITLLKELGSGQFGVVQLGKWKGQYDVAVKMI 440
Btk KEGSMSEDEFIEEAKVMMNLSHEKLVQLYGVCTKQRPIFIITEYMANGCLLNYLREMRHR 493
Itk REGAMSEEDFIEEAEVMMKLSHPKLVQLYGVCLEQAPICLVFEFMEHGCLSDYLRTQRGL 453
Tec REGAMCEEDFIEEAKVMMKLTHPKLVQLYGVCTQQKPIYIVTEFMERGCLLNFLRQRQGH 453
Bmx KEGSMSEDEFFQEAQTMMKLSHPKLVKFYGVCSKEYPIYIVTEYISNGCLLNYLRSHGKG 500
Btk FQTQQLLEMCKDVCEAMEYLESKQFLHRDLAARNCLVNDQGVVKVSDFGLSRYVLDDEYT 553
Itk FAAETLLGMCLDVCEGMAYLEEACVIHRDLAARNCLVGENQVIKVSDFGMTRFVLDDQYT 513
Tec FSRDVLLSMCQDVCEGMEYLERNSFIHRDLAARNCLVSEAGVVKVSDFGMARYFLDDQYT 513
Bmx LEPSQLLEMCYDVCEGMAFLESHQFIHRDLAARNCLVDRDLCVKVSDFGMTRYVLDDQYV 560
Btk SSVGSKFPVRWSPPEVLMYSKFSSKSDIWAFGVLMWEIYSLGKMPYERFTNSETAEHIAQ 613
Itk SSTGTKFPVKWASPEVFSFSRYSSKSDVWSFGVLMWEVFSEGKIPYENRSNSEVVEDIST 573
Tec SSSGAKFPVKWCPPEVFNYSRFSSKSDVWSFGVLMWEVFTEGRMPFEKYTNYEVVTMVTR 573
Bmx SSVGTKFPVKWSAPEVFHYFKYSSKSDVWAFGILMWEVFSLGKQPYDLYDNSQVVLKVSQ 620
Btk GLRLYRPHLASEKVYTIMYSCWHEKADERPTFKILLSNILDVMDEES---- 660
Itk GFRLYKPRLASTHVYQIMNHCWKERPEDRPAFSRLLRQLAEIAESGL---- 620
Tec GHRLYQPKLASNYVYEVMLRCWQEKPEGRPSFEDLLRTIDELVECEETFGR 624
Bmx GHRLYRPHLASDTIYQIMYSCWHELPEKRPTFQQLLSSIEPLREKDKH--- 668

Figure 2.1 Sequence alignments of Tec family members. Mutations causing XLA are indicated above the sequence, X indicating nonsense mutations, # delections, and @ insertions.

2.2.1 The regulation of Btk

The Tec family proteins are involved in a vast array of signal transduction pathways many of which form communication networks. The role of these family members in signaling has been best characterised for Btk in the context of BCR signaling (for review see Smith et al., 2001). The family members are composed of similar domains, which are the most commonly used domains involved in signaling (see Fig. 2.2).

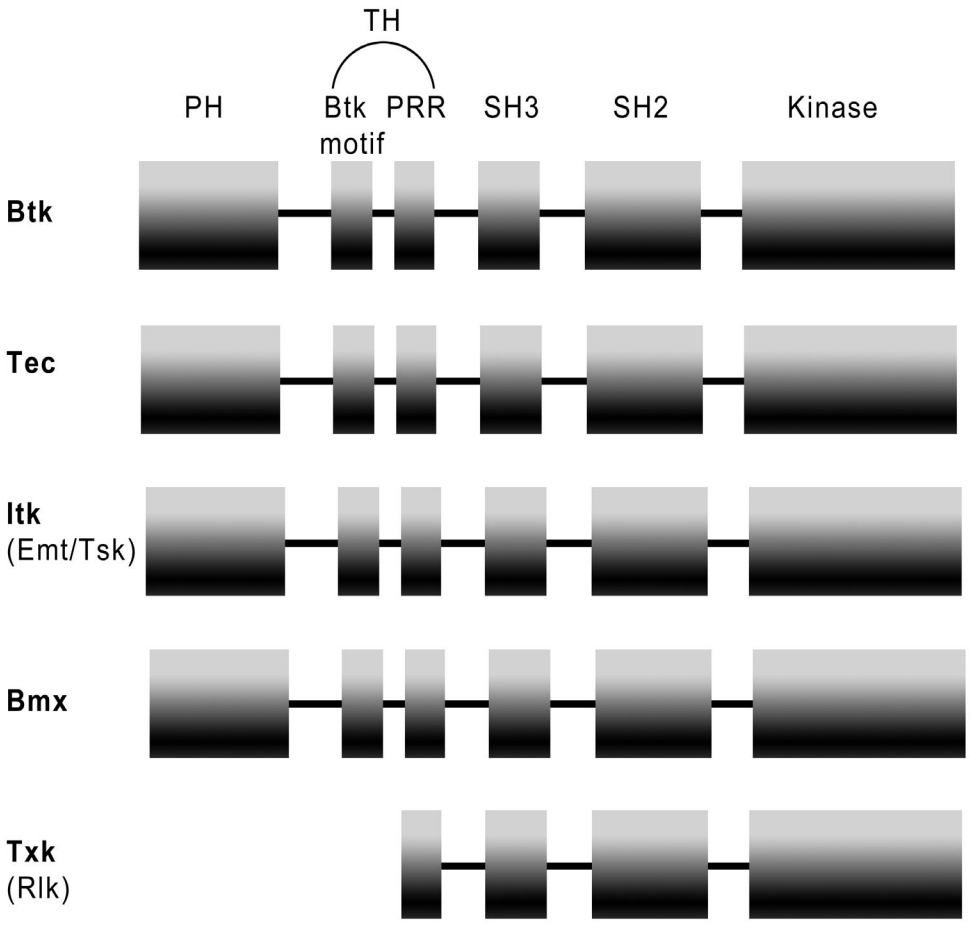


Figure 2.2 Domain organisations of Tec family protein tyrosine kinases. Txk contains in the N-terminus unique cysteine rich region.

The sequence similarity (Fig. 2.1) and expression patterns (in similar types of cells) of the Tec family members suggest that this family is involved in potentially overlapping pathways. The main pathways in which Btk has been implicated includes those initiated by the B-cell antigen receptor (BCR), the high affinity IgE receptor ($Fc_{\varepsilon}RI$), IL-5, G-protein coupled receptors (via association with $G\alpha 12$, $Gq\alpha$ or $\beta\gamma$ subunits and the CD32), collagen or thrombin receptors in platelets IL-3 (Deng et al., 1998; Sato et al., 1994; Kikuchi et al., 1995; Bence et al., 1997; Ma and Huang, 1998; Kawakami et al., 1994; Queck et al., 1998).

Binding of an antigen ligand brings about the activation of BCR which, in turn, activates Btk through a phosphorylation cascade (Park et al., 1996; Oda et al., 2000). The stimulation of BCR is intimately linked to the activation of three cytoplasmic tyrosine kinase families, i.e. the Tec family, Src family, and the Syk family (Vihinen and Smith, 1996) (Fig. 2.3). Upon BCR cross-linking, PtdIns(3,4,5)P3 is generated by phosphatidylinositol 3-kinase (PI3K) which then leads to the PH domain-mediated translocation of Btk to the membrane (Li et al., 1997). This binding to the lipid intermediate is followed by the phosphorylation of Y551, a highly conserved activation loop tyrosine, by a transient association with Lyn, a Src kinase (Fig. 2.3), causing a marked increase in Btk activity. Subsequent autophosphylation of Y223 in the SH3 domain by the kinase domain induces the full activation of Btk (Rawlings et al., 1996; Park et al., 1996).

Recently it has been shown that antigen binding and oligomerization of the BCR results in rapid translocation of BCR into lipid rafts (detergent insoluble membrane microdomains enriched in cholesterol and sphingolipids) (Cheng et al., 1999). Lyn kinase a member of the Src kinase family is concentrated within the raft region. Thus lipid rafts can serve as platforms for signaling (Cheng et al., 1999). The translocation of the BCR into lipid rafts has been shown to result in the recruitment to BCR of several associated signaling components, such as signal cascades, BLNK (B cell linker protein), PI3 kinase, VAv (a guanine nucleotide exchange factor) and Btk (Cheng et al., 2001) (Fig. 2.3).

Prior to the ligand stimulation Tec and Btk have both been observed to be constitutively associated with gp130, the signal-transducing subunit for the IL-6 cytokine (Matsuda et al., 1995). More recently, in the downstream of antigen receptors in both B and T cells, Tec was shown to be activated (Yang et al., 2001). As to the downstream effect of Btk, it is interesting that Btk has been shown to be present in the nucleus of hematopoietic cells, although in a much lower amount compared to the cytoplasm (Mohamed et al., 2000). Under certain experimental conditions e.g. due to the SH3 domain deletion, Btk has been

localized to the nucleus and perhaps being involved in regulating gene expression (Mohamed et al., 2000). In fact, both Btk and Tec are known to modulate transcription of cytokine genes in mast and T cells (Yamashita et al., 1998). Evidence linking B cell receptor stimulation with Btk nuclear response was recently found when Btk was shown to be involved in the activation of the transcription factor NF-$_K$B (Petro et al., 2000). Apart from these implied roles in transcriptional activation and reprogramming gene expression in the nucleus, Btk family kinases are also thought to have an effect on cytoskeletal reorganization and cell motility (Qui and Kung, 2000). In DT-40 cells lacking Btk, the pattern of actin expression and its ability to aggregate was shown to differ from the wild-type cells (Nore et al., 2000), suggesting that Btk is involved in cytoskeletal reorganization.

Tec and Btk share common functions during B cell development (Ellmeier et al., 2000). Itk and Txk co-exist in T cells (Schaeffer et al., 1999). The activity of Tec/Btk is required for the proper generation of immature B cells. However, Btk function seems to be more important than that of Tec as in Btk deficient mice a reduction of pre-B cells was observed, but not in those lacking Tec (Ellmeier et al., 2000). Btk has also been found to fully compensate for the loss of Tec, but Tec is not able to fully compensate for the loss of Btk in mice cells (Ellmeier et al., 2000). Itk, on the other hand, has been shown to associate directly with CD28, a T-cell costimulatory molecule (August et al., 1997). Further, Tec like Itk is activated downstream of CD28, upon the phosphorylation of Dok (a molecule that interacts with the GTPase activating protein of Ras and Ras-GAP) (Yang et al., 1999). Moreover, upon reconstitution of Btk deficient DT40 cells Itk and Tec have been shown to have an analogous function to Btk in at least PLC-γ2 signaling (Tomlinson et al., 1999).

Btk has earlier been implicated in a regulatory role in tyrosine phosphorylation of PLC-γ2 and subsequent calcium mobilization in BCR mediated signaling (Fluckiger et al., 1998) (see Fig. 2.3). In this cascade, the SH2 domain-containing inositol-polyphosphate 5'-phosphate (SHIP) (Fig. 2.3) has been implicated as an on/off switcher of Btk catalytic activity (Nore et al., 2000). In chicken DT40 cells the patterns of tyrosine phosphorylation in wild type and Btk deficient cells were found to be similar (Takata and Kurosaki, 1996). Syk (spleen tyrosine kinase) family kinase as shown in Fig. 2.3, is involved in Akt activation, and it has been shown to act as an upstream regulator of BCR induced PI3-kinase, with Btk regulating the BCR-mediated Akt activation downstream of PI3-kinase. Akt is known to be a target of PI-3K (Chan et al., 1999). Further, the activation of other Tec family members e.g. Itk upon Src/CD28-induced activation in response to interleukin-6 stimulation has been found to also re-

quire PI3-kinase activity (Lu et al., 1998). The evidence that null Btk and xid mice are similar to p85a null mice further supports the concept that Btk and PI3-kinase are within the same pathway(s). Both mice were found with reduced numbers of mature peripheral B cells and diminished proliferative response to anti-IgM, anti-CD40, and liposaccharide (Fruman et al., 1999).

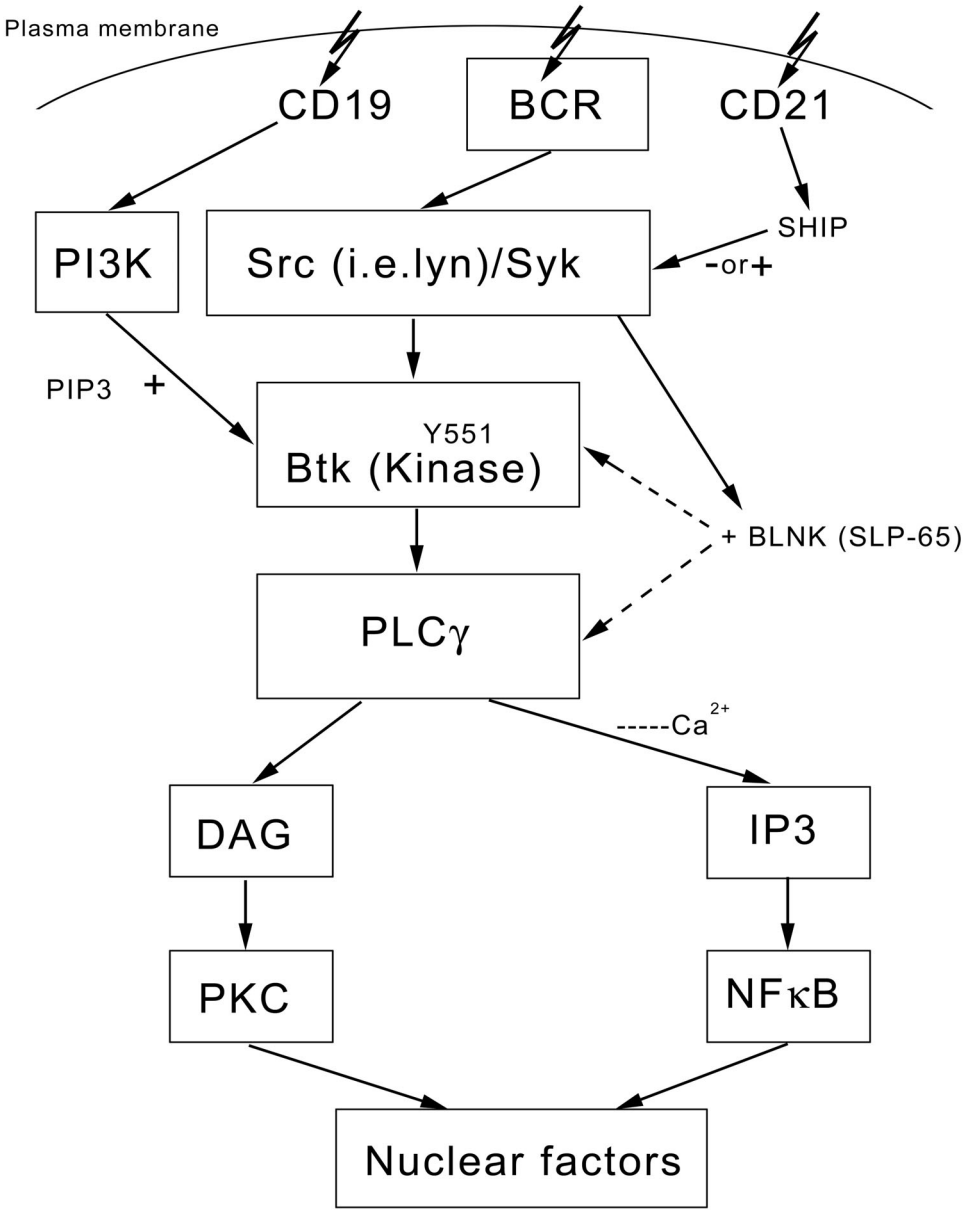


Figure 2.3 Schematic representations of the signaling pathways regulating Btk.

Tec family signaling generally involves the control of complex survival and differentiation events (Smith, et al, 2001). The phenotype of a Btk deletion mutant could exhibit a similar pattern with its pronounced differentiation block. However, under differential experimental conditions this kinase has the ability to protect against (Solvason et al., 1998), or induce apoptosis (Islam, et al, 2000; Uckun et al., 1996). Further, a constitutively active mutant form of Btk promotes tumorigenesis in cell lines (Li et al., 1995), whilst, in transgenic mice, the constitutive expression of the same mutant results in abnormalities of the lymphoid organs with arrest in B lymphocyte development (Dingjan et al., 1998). Bmx induces apoptosis in myeloid progenitor cells in the presence of G-CSF (Ekman et al., 2000) and the combined deficiency of Itk and Txk leads to a decrease in the sensitivity of CD-3 mediated apoptosis (Schaeffer et al., 1999). When put together, these observations seem to imply that the cell phenotype plays a key role on whether Tec kinases will induce cell survival or promote apoptosis (Islam and Smith, 2000).

The Src family members Blk, Fyn, Lck, and Lyn are present in B cells, where they are involved in signal transduction induced by binding of an antigen ligand to the BCR (Reth and Wienands, 1997). They interact via their SH2 domain with Syk, and also bind to other signaling molecules. Activation of the Src family kinases and Syk are the key events in the early steps of BCR signaling (Fig. 2.3). It is known that Syk phosphorylates tyrosine residues in the ITAM (immunoreceptor tyrosine based activation motif) motifs in BCR (Flaswinkel and Reth, 1994), and hence sets into motion the activation of PTK signaling cascades (Flaswinkel and Reth, 1994). The two SH2 domains of Syk are likely to be involved in the binding to the double tyrosine-phosphorylated ITAMs of the BCR (Fig. 2.3.). The deletion of the gene coding for Syk in a chicken cell line clearly implicates Syk in the signaling pathways between BCR and phospholipase Cγ (Craxton et al., 1999). Src family kinases are also involved in the phosphorylation of Btk (Mahajan et al., 1995).

3 Btk non-catalytic domains

As discussed above, the Tec family kinases are predominantly expressed in cells of the immune system. The expression patterns demonstrate their ability to play diverse regulatory roles in various physiological processes. Table 3.1 summarizes some of the molecules known to interact with the non-catalytic domains. Apart from the subtle sequence differences, it is most likely that the versatile nature of the constituent domains and their various binding partners are what permits the Tec family kinases to participate in such diverse actions. Below, the Btk non-catalytic domains are discussed in more detail.

Table 3 A summary of some of the molecules known to interact with the N-terminal domains of Tec family cytoplasmic protein tyrosine kinases. Some binding partners have only been shown to interact with specific Tec kinases, and this is indicated in parentheses.

PH domain	TH domain	SH3 domain	SH2 domain
PIP_3 PLC-γ PI3K G$\beta\gamma$ (Btk) Fas (Btk) PKC (Btk) TFII-I (Btk) F-actin (Btk) FAK (Bmx) STAT3 (Bmx) PTPD1 (Bmx)	Gα12 (Btk, Tec, Bmx) Gαq (Btk) G$\beta\gamma$ (Btk) Vav (Tec) c-Kit (Tec) LAT (Itk) Grb-2 (Itk) Fyn (Btk) Lyn (Btk)	Vav Sab (Btk) Sam68 (Btk) EWS (Btk) Syk (Btk, Itk) WASP (Btk, Itk) c-Cbl (Btk, Itk) CD28 (Itk, Tec) hnRNP-K (Itk) p^{120cbl} (Btk) PLCγ (Itk)	SLP-76 BLNK/SLP-65 Vav Dok-1 (Tec)

3.1 PH domain

The PH domains consisting of about 120 residues are highly divergent, and have been found in a number of signaling and cytoskeletal proteins including protein tyrosine and serine/threonine kinases and their substrates, phospholipase C, GTPase activating proteins, guanine nucleotide releasing factors, and adaptor proteins (Shaw, 1996). Although the precise function of the PH domain is not fully understood, several lines of evidence suggest a critical role in Btk function. In many cases, it has been found to act as a membrane localization domain (Fukuda et al., 1996; Hyvönen and Saraste, 1997).

The structure for several PH domains including Btk has been determined (Hyvönen and Saraste, 1997). Despite very low sequence identity, the structures have the same fold consisting of a ß-barrel formed of two ß-sheets and a C-terminal α-helix that caps one end of the ß-barrel. PH domains are highly

polar (Blomberg and Nilges, 1997). The structure of the Btk PH domain reveals R28 to be one of the most highly conserved residues in this motif. Several charged residues including R28 and E41 form a cleft near the N-terminus of the domain. These residues are directly involved in the ligand binding (Baraldi et al., 1999). The inositol compound binds between loops ß1-ß2 and ß3-ß4 where both the R28 and E41 are located (Baraldi et al., 1999). The binding of E41K mutant was studied in a cocrystal with Ins(1,3,4,5)P_4 (Baraldi et al., 1999). In another study, R28C substitution was found to have reduced binding to Ins(1,2,3,4)P_4, presumably because the substitution removes the basic arginine from the ligand-binding cleft and this affects the strength of the electrostatic polarization (Fukuda et al., 1996).

Residues implicated in the binding of the PH domain to phosphoinositide compounds have been localized in Btk, pleckstrin, and PLC δ1 (Baraldi et al., 1999; Harlan et al., 1995; Ferguson et al., 1995). The positively charged surface of the PH domain binds to the negatively charged phosphate groups of the phosphoinositol molecules (Baraldi et al., 1999). Several XLA-causing mutations including the Xid mutant R28C are located within the PH domain (Vihinen et al., 1998; Vihinen et al., 1999, de Weers et al., 1994). The knockout variant of R28C in mice exhibited a much milder phenotype (Thomas et al., 1993; Kerner et al., 1995). In humans, patients with missense mutation of the corresponding residue (R28) as in xid have a severe B cell deficit and classical XLA (de Weers et al., 1994).

By using a retroviral passage mutagenesis scheme, a gain of function mutant (E41K) of Btk, located in the PH domain, was isolated (Li et al., 1995). The mutant allows the constitutive expression of Btk due to its increased affinity for phosphoinositol compounds. The expression of this mutant was shown to drive the growth of NIH3T3 cells (Li et al., 1995). It was found to enhance transphosphorylation of Y551 by endogenous Src family tyrosine kinases, and autophosphorylation at position Y223 within the SH3 domain. The combined phosphorylation(s) and autophosphorylation(s) lead to constitutive membrane association via binding by phosphoinositol products of PI3K, e.g PI(3,4,5)P_3 and 1,3,4,5-tetrakisphosphate resulting in the activation of Btk (Rawlings et al., 1996; Baraldi et al., 1999). The interaction between the PH domain and PIP_3 is presently the best-characterized requirement for membrane targeting of the protein. Btk PH domain has also been found to interact with proteins, including F-actin and focal adhesion kinase (FAK), suggesting multiple mechanisms for regulating Tec kinases (Qiu and Kung, 2000; Lewis et al., 2001). Further, the carboxy terminus of the PH domain has been shown to modulate binding to the βγ subunit of heterotrimeric G proteins and, in conjunction with the Btk motif, to

facilitates the interaction of Btk with the Gα12 subunit of heterotrimeric G proteins (Tsukada et al., 1994; Jiang et al., 1998).

3.2 TH domain

The TH domain in Btk consists of two motifs, the Btk motif and the proline rich region (PRR) (Vihinen et al., 1994a; Vihinen et al., 1997). The complete TH domain can be found only in the Tec protein family. The Btk motif follows the PH domain in several forms of Ras GTPase-activating protein 1 and also in a putative interferon γ-binding protein (Vihinen et al., 1995b). The function of the TH domain is not fully understood, although SH3 domains bind to PRR *in vitro* and the Btk motif seems to be essential for interactions with G proteins (Clapham and Neer, 1997).

The Btk motif consists of a highly conserved stretch of amino acids found in Tec kinases, Ras GAP, and IGBP (Vihinen et al., 1997). It structure has been determined in a cocrystal with the PH domain (Hyvönen and Saraste, 1997; Baraldi et al., 1999). The motif has a novel fold with a coordinated Zn^{2+} ion binding which packs against the ß-sheet of the PH domain. This motif seems to be crucial for the function of Tec family PH domains (Hyvönen and Saraste, 1997). A finding that in Txk, which is devoid of the PH domain, the Btk motif is also missing also supports functional linkage.

Btk contains a duplicated sequence of proline rich stretches at the C-terminal portion of the TH domain. In other Tec family kinases (except for Bmx), a homologous stretch is also found. Bmx contains a canonical SH3 domain-binding PXXP motif, however, the surrounding residues do not conform to the SH3-binding fingerprint. The proline rich region in the TH domain of Itk was shown to make an intramolecular contact with the SH3 domain (Andreotti et al., 1997). The two PRRs in the TH domain of Btk interact with the SH3 domains of Fyn, Lyn and Hck (Yang et al., 1995). Site-directed mutagenesis of the proline residues in the PRRs of Btk abolishes the interaction with SH3 domains (Yang et al., 1995).

3.3 SH3 domain

The SH3 domain consists of approximately 60 amino acids. They are present in a wide variety of proteins ranging from cytoskeletal proteins, such as myosin 1 and the α-chain of spectrin, to signal transduction proteins, e.g non-receptor tyrosine kinases and phospholipase C. Recent data from the genome sequencing projects further suggest that 63 proteins in *Drosophila*, 55 in *Caenorhabditis*

elegans, and 25 in *Saccharomyces cerevisiae* contain at least one form of this domain (Rubin et al., 2000). SH3 domains bind to proline rich peptides and proteins. Both biochemical and genetic evidence have demonstrated that SH3 domain-mediated interactions are necessary for proper signaling by e.g. receptor tyrosine kinases (Pawson, 1995). The solution structure of the Btk SH3 domain has been determined using nuclear magnetic resonance (NMR) spectroscopy (Hansson et al., 1998). The structure of the Btk SH3 domain consists of two tightly packed anti-parallel β-sheets that form a sandwich-like fold.

The structural basis for the interactions between the proline-rich peptide ligands and SH3 domains are well understood (Lim et al., 1994). Based on the mode of binding, two classes of ligands (class 1 and 2) have been defined, distinguished by the orientation of the bound ligand (Mayer and Eck, 1995). Ligands of both classes contain a PXXP core sequence that anchors the ligand to the receptor, in the polyproline type II (PPII) helical conformation. The core sequence of the ligand, provides the promiscuity of binding to different SH3 domains, the flanking residues are unique to individual SH3 domains and bind to specific pockets (Lim et al., 1994). These findings combined with successive phage display experiments have provided a framework for an algorithm to predict specific binding sites for any SH3 domain from its primary sequence (Brannetti et al., 2000). In the Btk SH3 domain the potential ligand-binding site is formed of residues Y223-Y225, D232, W251, P265, and Y268 (Hansson et al., 1998).

Recent studies show that SH3 domains, under certain circumstances bind ligands, which lack the PxxP core binding motifs. For example, the peroxisome Pix-SH3 domain in Pak, the sequence that recognizes the SH3 was found to be PPPVIAPRPETKS (Manser et al., 1998). On the other hand, PxxDY provides the site for the binding of the SH3 domain of the epidermal growth factor receptor substrate Eps8 (Mongiovi et al., 1999). The SH3-domain of the immune receptor adaptor molecule SKAP55, recognizes a novel tyrosine based RKxxYxxY motif (Kang et al., 2000). Further, in YAP VPMRLR is the minimal sequence requirement for YAP recognition by the SH3 domain of p53 protein (Gorina and Pavletich, 1996). Conversely, as many SH3 ligands containing the PxxP core motifs have been detected by direct filter binding and yeast two hybrid systems, it is safe to assume that for most SH3 domains the PxxP core motif is the target recognition sequence. The recent comparison of the yeast SH3 domains with several other SH3 domains showed the SH3 domains, which recognize canonical PxxP motifs, to be the most numerous (Cesareni et al., 2002).

In Itk, a intramolecular regulatory association has been identified between the

PRR and the adjacent SH3 domain (Andreotti et al., 1997). More recently, by using 2D-NOESY in combination with HSQC spectra measurements, an intermolecular interaction between the SH3 and SH2 domains of Itk was found (Brazin et al., 2000). The interaction provides an insight on the possible mechanism for displacement of the intramolecular interaction between the PRR and SH3 domain (Brazin et al., 2000; Ogawa et al, 2002). Possibly, the intermolecular interaction releases the SH3 domain-binding site that could be blocked by the intramolecular interaction, a step that may be necessary for the full activation of the Tec family members. A point mutation in the Itk SH3 domain, W208K, completely abrogates the intermolecular interaction and, also eliminates self-association of Itk (Brazin et al., 2000). Similarly, by introducing two additional lysines i.e. N383K, A384K (present in the Btk SH2 domain), using C-terminal rational modification of Btk SH3-SH2 protein, combined with C337 substitution (C337S and C377M) in the surface of the SH2 domain, the solubility and homogeneity of the Btk SH3-SH2 construct was improved (Nera et al., 2000). The addition of lysines at the N- or C-terminus has previously been shown to increase protein solubility (Ingley and Hemmings, 1999). The engineering of the Btk SH3-SH2 domains benefited from the observations gathered from the Btk SH2 model structure (Vihinen et al., 1994b). The dimer formation observed between Btk SH3-SH2 proteins could be intramolecular due to disulfide bridge formation, rather than intermolecular, since mutations C377S and C377M within the SH2 domain abrogate dimerization (Nera et al., 2000). The SH3-SH2 dimerization requires in addition to proper sequence, complementarity in structure and also complementarity outside the actual recognition site (Vihinen and Smith, 1998). Obviously, it will be essential to carry out further analysis on Btk SH3-SH2 to study the nature of this interaction.

A critical event in the catalytic activation of Btk is transphosphorylation of Y551 by Src in the catalytic domain. This is followed by autophosphorylation of Y223 in the SH3 domain. The observed self-association described above that involves the proline rich region open up the possibility that the autophosphorylation of Y223 could occur in either *trans* or in *cis*. The substitution Y223F in the Btk SH3 domain results in enhanced fibroblast transformation (Park et al., 1996), suggesting that the SH3 domain has a negative regulatory role (Park et al., 1996). The scenario of *trans* or *cis* activation does have implications for the understanding of the activation of Btk and other Tec family proteins. Thus, targeted mutants changing some of the residues within this domain and the adjacent TH domain would be vital to reveal the important residue(s) that are critical for either *trans* or *cis* activation. However, as recently shown (Hansson et al., 2001), changing the critical prolines in both the N-terminal PRR (Btk residue P189 and P192) and the C-terminal PRR (P203 and P206),

that are thought to be critical in making van der Waals contact in the binding pockets, do not completely abrogate intermolecular interactions. This implies that other residues distal from these might contribute to the SH3 domain interactions.

3.4 SH2 DOMAIN

SH2 domains contain about 100 amino acids. They play a central role in a number of cellular processes, including growth, immune response, metabolism, mitogenesis, motility and gene transcription (Kuriyan and Cowburn, 1997). SH2 domain-containing proteins take part in the coordination of signal transduction pathways by binding to phosphotyrosine molecules. Binding specificities of several SH2 domains have been determined by using phoshotyrosine (pY) peptide libraries and phage display (Songyang et al., 1994; Cochrane et al., 2000). Most of the XLA-causing mutations in Btk SH2 domain disrupt the pY peptide binding sites (Vihinen et al., 1994; Vihinen and Smith, 1996; Vihinen et al., 1995).

The three-dimensional structures of several SH2 domains have been determined (e.g. Waksman et al., 1992; Waksman et al., 1993; Xu et al., 1995; Eck et al., 1993). The conserved fold contains a large central antiparallel α-sheet and an associated smaller α-sheet, flanked by α-helices on each side. The pY-containing ligand binds to the SH2 domain in an extended conformation. The crystal structures of SH2 domains in complex with high-affinity peptides have shown that several residues are involved in the phosphotyrosyl peptide binding. The number of residues binding, in addition to pY, varies from three to six or seven. Regions outside the actual pY binding region can be essential for affinity and specificity (Vihinen and Smith, 1998). The most important conserved interaction between the SH2 domain and its ligand is the direct interaction between the pY phosphate group and the guanidinium group of an arginine (R307 in Btk). Other important residues for pY binding in Btk are R288 and S309. Residues forming a second ligand-binding pocket for the residue pY+3 are found in αA, βB, βD, and the loop connecting strands B and C. These residues provide the specificity for pY+1 and pY+3 affinity (Kuriyan and Cowburn, 1997).

The Btk SH2 domain contains a single cysteine residue, C337. According to the model of SH2 domain, C337 is situated at the surface of the domain (Vihinen et al., 1994). In Grb2, oxidation and subsequent dimer formation impair protein crystallization (Cochrane et al., 2000). Using mutagenesis to replace the Btk C337 with Ser or Met, dimer formation was prevented (Mattsson et al., 2000). The C337S or C337M substitutions do not impair phosphotyrosine binding nor do they affect the secondary structure of the Btk SH2 domain (Mattsson et al.,

2000).

A recently identified B-cell linker protein (BLNK or SLP-65) as one of the major Btk SH2 binding proteins explains the role of Btk SH2 domain in calcium signaling (Hashimoto et al., 1999; Su et al., 1999). Upon BLNK phosphorylation by Syk (Spleen tyrosine kinase), BLNK is thought to provide docking sites for Btk SH2 domain as well as for PLC-γ2. Thus, this provides a scenario where, Btk and PLC-γ2 are brought into close proximity (Hashimoto et al., 1999; Kurosaki and Tsukuda, 2000). When Btk is activated via the mechanisms earlier discussed (see also Fig. 2.3) it phosphorylates PLC-γ2 leading to its full activation (Kurosaki and Tsukuda, 2000). In this process, phosphorylated BLNK was found to be vital further implying that BLNK is the direct connector between Btk and PLC-γ2. However, the effect of Btk on PLC-γ2 was abrogated when a mutant variant of Btk SH2 domain was used in a heterologous transfection system (Hashimoto et al., 1999).

4 Prelude to Experimental approaches: Spectroscopic methods in brief

CD Spectroscopy: Circular dichroism (CD) spectroscopy is a powerful tool for the studies of biochemical systems in solution. CD is sensitive for the shape of a protein molecule. The principles of CD spectroscopy are based on the chirality of a protein backbone and the side chains (For a complete details see Fasman, (1996)). This method can provide information about the secondary and tertiary structure of a protein molecule.

Fluorescence spectroscopy: Fluorescence spectroscopy can reveal information about the intrinsic environment of a biological molecule. The method is a sensitive mode to view the structural and dynamic characteristics of macromolecules (for details see Gore (2000)). Fluorescence measurements are based on the ability of a molecule to absorb electromagnetic radiation. Upon the absorption via a quantum mechanical process, the molecule is transformed from a ground state to an excited state. The absorption of light results in an electronic transition in the molecule. Through a process that is determined from the Beer-Lambert law, the electron is transformed from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO).

The fluorescence properties of proteins are highly individual; hence, it has long been viewed as a sort of fingerprint of the individual protein. The aromatic amino acids, phenylalanine, tyrosine, and tryptophan absorb light in the ultraviolet spectral range. Among these, Y with molar absorptivity of 270= 1405 m^{-1} cm^{-1} and W 280 = 5579 m^{-1} cm^{-1} has been used extensively.
Using the intrinsic fluorescence properties of tryptophan, an indole amino acid, with excitation wavelength at 280 nm and emission at 345 nm, we studied the affinity of the two-proline rich regions within the TH domain for the Btk SH3 domain. With this approach, the dissociation constant of the reactions was calculated from the Michaelis-Menten equation.

5 Aims of the study

Bruton tyrosine kinase plays a crucial role in B cell development. Both genetic and biochemical methods have been used to illustrate the physiological importance of Btk in B cell signaling. This has been clearly demonstrated with the finding that mutations in the genes encoding Btk lead to immunodeficiency in both man (XLA) and mice (xid).

The present study focuses on the role of the non-catalytic PH, TH, SH3 and SH2 domains in the cooperative regulation of the activity and interactions of the full-length protein.

The detailed aims of the study were:

1) to extend the knowledge relating to the structural dynamics of Btk.
2) to study the role of the SH3 domain in intra/intermolecular inter actions of Btk.
3) to elucidate the nature of the Btk mutation(s) in Finnish patients with XLA.
4) to analyze the thermal/chemical stability and the unfolding process of the non-catalytic PH, TH, SH3 and SH2 domains.

6 Experimental approaches

6.1 Mutation scanning using base excision sequence scanning (BESS) method (I and III)

The upstream primer for each exon (1-19) was radioactively labelled as described [I and III]. Samples were incubated at 37°C for 30 minutes, and then inactivated by heating at 70°C for 5 minutes. Each of the nineteen *BTK* exons was amplified using the improved primers as described by Vorechovský et al. (1995). The PCR reactions were carried out as described in ref. I & III.

The amplified products were excised using BESS excision enzyme reaction (Epicentre Technologies, USA), and subjected to 8% polyacrylamide sequencing gel electrophoresis. Exons revealing differences after scanning with G- and T-tracker were reamplified without radiolabel. The PCR products were resolved in a 1% agarose gel stained with ethidium bromide. The band of interest was excised and purified using QIAquick PCR purification kit (Qiagen, Germany). DNA sequencing was carried out for both strands using an automated ABI PRISM™ 310 Genetic Analyser (PE Applied Biosystems), with the same primers as used in the PCR reactions. Detected mutations were confirmed by comparison of the resulting sequence with the reported coding sequence (Vetrie et al., 1993).

6.2 Molecular cloning and expression (II, IV and V)

cDNAs encoding the desired domains were amplified using polymerase chain reaction (PCR). Cleavage sites for selected endonucleases were included in the designed primers. The amplified PCR fragments were digested and cloned into a vector with a poly-histidine (His)-tag for the TH domain and glutathione-S-transferase (GST)-fusions for the SH3, PRR-SH3 and PH-SH2 domains, respectively. The PH-SH2 constructs were cloned into a pBAT vector (Peränen et al., 1996). The authenticity of all constructs was verified by DNA sequencing. Cloning was carried out in *Escherichia coli* DH5a strain.

The different domains were expressed in *E. coli* strain BL21 (DE3) or BL21/pUBS20 strain for the PH-SH2 construct. The domains were expressed by fermenting the cells in 2xTY medium containing 2% glucose as a catabolic repressor at 37°C, or 30°C for the TH domain. Expression was induced with isopropyl-D-thiogalactopyranoside (IPTG) when absorbance at 600 nm reached 1.8 nm. Induction was carried out for 4 hours with the exception of the PH-SH2 construct, which had 20 hours induction. The harvested cells were lysed using

Bead beater cell breaker.

6.3 Protein purification using chromatography (II, IV and V)

The cDNAs encoding the recombinant proteins were cloned to GST/His-tag fusion vectors except for the PH-SH2 construct. The over-expressed proteins in the supernantant were first purified using an affinity column containing GSH-Sepharose or Ni^{2+}-NTA-agarose respectively. The bound fusion products were digested overnight with thrombin (for GST fusion) at room temperature, and the proteins eluted with PBS (phosphate buffer saline) buffer. After the affinity purification, the proteins were further purified by gel-filteration on a Superdex 75 column. Then, SDS polyacrylamide gel electrophoresis and N-terminal amino acid sequencing were used to confirm the identity of the purified proteins. For further studies, the proteins were concentrated using membrane filters with a 10 kDa cut-off. The protein concentrations were determined as described in (II).

6.4 Molecular modeling/sequence analysis (I and III)

Molecular modeling was carried out essentially as described in reference I. Briefly, the computer assisted modeling of the Tec family PH domain was done by utilizing the 3D structure of the Btk PH domain (Hyvönen and Saraste, 1997) as a template. The models were generated using InsightII. Upon searching loops from the database that contained a representative selection of the PDB files, deletions and insertions were modeled. Sequence analysis was carried out using programs such as gap as contained in the GCG sequence analysis package (Devereux et al., 1984). ClustalW (Higgins et al., 1994) was used for multiple sequence alignment analysis.

6.5 Biophysical studies of molecular interactions

6.5.1 CD spectroscopy (II and V)

The CD measurements were made on a Jasco J-720 instrument. The measurements were performed as described in ref II & V respectively. CD spectra are often reported in terms of molar ellipticity. In the work presented in this thesis, the ellipticity data were converted to molar ellipticity (θ) (in units of deg cm^2 dmol-1) using the equation:

$\{\theta\} = \{\theta\}_{obs}(MRW)/10lc$,

where {θ} is ellipticity, {θ}obs is the observed ellipticity in degrees. MRW is the mean residue weight of the protein, c is the concentration of the protein in $mgml^{-1}$, and L is the optical pathlength of the measuring cell in centimeters. The unfolding transition of the protein (PH-SH2) domains was acquired by recording the CD signal at 222 nm from 25 to 70°C, with a scanning slope of 65°C/hour, 0.5 nm bandwidth and 0.1 cm cuvette.

6.5.2 Determination of dissociation constant (K_d) (II and V)

A simple binding association scheme was assumed for the reaction. The binding of the SH3 domain to either the peptide or the TH domain was assumed to be reversible and to form a complex such that SH3 + P ⇆ SH3–P. The fluorescence changes (ΔF) were directly related to the fraction of SH3–P or protein complex formed.

The differences in fluorescence due to ligand binding were analysed using the plot of A/V against A with the equation:

$$\frac{A}{V} = \frac{Km}{V} + \frac{1}{V} \cdot A$$

where A is the concentration, V is the fluorescence change due to ligand binding and K_M is the Michaelis constant. This plot has been shown to be more accurate than the double reciprocal plot.

6.5.3 NMR Spectroscopy (IV)

NMR measurements were carried out using Varian Inova 500, Bruker DRX 500 and DRX 600 spectrometers equipped with 5 mm triple-resonance 1H/15N/13C probes respectively. The measurements were performed as described in (IV).

6.5.4 Temperature gradient gel electrophoresis (V)

The temperature gradient gel electrophoresis (TGGE) was performed on a Biometra TGGE system. 8% polyacylamide gels with 0-4 M concentration of urea were cast on a polybond film as recommended by the manufacturer (Biometra, Göttingen, Germany). The gel was placed on the horizontal gradient block and connected to the electrode tanks using pre-soaked electrode wicks.

The temperature gradient was set from 28 to 65°C. The sample was loaded onto the polyacrylamide gel attached to the polybond film, and the sample subjected to a pre-run electrophoresis for 10 minutes at room temperature enabling the sample to migrate onto the gel. The system was disconnected, the empty slot of the gel rinsed with the running buffer, and the polyacrylamide gel covered with a pre-cut film. The electrode wicks were placed on top of the cover film and the sandwich covered with an acryl-glide treated cover glass plate. After completion of the electrophoresis, the gel was stained using silver staining method.

7 Results

7.1 Mutations XLA causing in the non -catalytic domains of Btk (I and III)

Btk contains the PH, TH, SH3, SH2, and the kinase domains. Mutations in the genes encoding the Btk domains lead to XLA in man. Several XLA-causing mutations in genes encoding the above mentioned domains in Btk have been described (Vihinen et al., 1999; Vihinen et al., 2001). Using BESS method combined with direct sequencing, we studied the prevalence of mutations in patients with XLA disease. We identified XLA causing mutations in seven families out of eight. In seven unrelated families we observed distinct mutations of which six were novel. Carrier detection was performed in four families. Four of the seven mutations affected the kinase domain gene region, three were found in PH and one in SH2 domain (Table 7.1). There was a high proportion of insertions in the investigated patients. There were a frameshift causing insertions in six patients, four were in introns, one in the upstream noncoding region and one in exon 18 leading to a frameshift mutation and the consequent truncation of the protein. In addition to the disease-related mutations a previously described silent polymorphism, C/T (Bradley et al., 1994; Rodriguez et al., 2001), at the 2031 cDNA position was identified. The clinical profiles of all the patients showed classical XLA. Some of the patients showed a positive family history of the disease, but in one case the mutation was found to be new.
Table 7.1

Table 7.1 BTK mutations in XLA patients and carriers

No	PIN	Accession No	Localization	Protein Domain	Mutation	Amino acid change	Mother status
1	Intron 13 (6)	A0794	Intron 13	TK	g. 64512_64513 ins C		N.D
2	Upstream (4)	A0795	Exon 2		c.108_109 ins G	Upstream	Not a carrier
3	@A523X538(1)	A0796	Exon 16	TK	c.1700_1701 ins ACTACAG	p.A523fsX538	Carrier
4	Intron 4(6)	A0797	Intron 4	PH	g. 51375_51376 GC>TG		Carrier
5	Intron 13(7)	A0798	Exon 13	SH2	g. 63991_63992 ins GGTAGAAAAAA		N.D
6	Silent mutation		Exon 18	TK	c.2031 C>T	No change	Carrier
7	@H609X649(1)	A0799	Exon 18	TK	c.1956_1957 ins CA	p.H609fsX649	Carrier
8	V105X122(1)	A0541	Exon 5	PH	c.442 ins T	Stop	Carrier

7.2 Structural interpretations of XLA causing mutations in the gene encoding Btk PH domain region and comparison with the Tec family PH domains (I)

Pleckstrin homology domains are known to be involved in different interactions including binding to inositol compounds, protein kinase C isoforms, and heterotrimeric G proteins. Most Tec family PTKs contains a PH domain. The sequence alignment of the Tec family PH domains and the modeling of the three dimensional structure of the domains enabled us to compare their similarity and gene mutation pattern. The majority of the disease-causing changes in Btk PH domain are located in the inositol-binding region. Many of the alterations within this region either reduce or reverse the electrostatics of the domain. In the four Tec family members that we compared, similar fold and electrostatic properties were observed but the binding regions were dissimilar.

Many PH domain alterations have been shown to alter the binding of the PH domain to IP_4 because of the removal of a positive charge from the binding pocket. IP_4 interacts with Btk via residues in β1-3 and in the loop between β1 and β2 of PH (Baraldi et al., 1999). The minimal PKC binding region (Yao et al., 1997) contains residues R28 and Y39 of this region. Thus, this region contains essential amino acids that impair Btk function when altered.

The IP_4 molecule was docked to the domain models according to the binding site in Btk. Using the structural models we interpreted the effects of the mutations. It was found that the substitutions K12R and S14F most likely prevented binding to IP_4 due to the introduction of a bulky side chain into the binding pocket. The alterations T33P, V113D, S115F, T117P all were found to caused a sterical clash with the ligand. L19 is in the immediate proximity to the ligand-binding pocket and Y39 is a ligand-binding residue. Thus, the alterations L19E, Y40C or Y40R disturb the electrostatic properties of the domain, and hence reduce binding.

7.3 TH/SH3 domain partners in the downstream regulation of Btk interaction (II and IV)

Enzymatic activities of protein kinases are regulated by various intra- and intermolecular interactions. By applying biophysical techniques we investigated the role(s) of SH3 domain binding sequences present in the TH region of Btk.

7.3.1 interactions of Btk TH and SH3 domains (II)

The PRR sequence/motif of the TH domain in Tec family proteins can interact with several SH3 domains (Yang et al., 1995). Tec PTKs have the SH3 domain adjacent to the TH domain. We analysed the binding of the SH3 domain of Btk to the proline rich segments by using synthetic peptides. Peptides corresponding to the two Btk PRR repeats, peptide 1 TKKPLPPTPE (Btk residues 184-193) and peptide 2 LKKPLPPEPA (residues 198-207), were synthesized. CD and fluorescence scanning spectroscopy showed, that the Btk SH3 domain interacts specifically with the N-terminal proline rich region. Similar interaction has been previously reported for the Itk (Andreotti et al., 1997). The CD spectra of both peptides were measured and both had a negative extrema at about 202 nm. Competition assays with the peptides was used to test the interaction in the PRR-SH3 construct. The CD spectra of the Btk SH3 domain (amino acids 212-275) and the N-terminally extended version (amino acids 178-275) between 190 and 250 nm were measured. The sums of the CD spectra of Btk SH3 domain, peptide 1, as compared to the mixture of the two, indicated that the combined spectrum does not obey simple additive rules. The TH domain in competition with the Btk SH3 domain was used to further characterize the interaction.

The binding affinity of the interaction between the TH and SH3 domains of Btk was determined by using fluorescence scanning spectroscopy. The affinities measured for peptide 1 binding to the SH3 domain, TH domain binding to the SH3 domain, the TH domain binding to the extended PRR-SH3, and peptide 1 binding to the PH-SH2, were found to be 3.9 μM, 17.0 μM, 36.2 μM, and 350 μM respectively. Peptide 2 did not show binding even when used in high concentrations. These results indicate that interaction between the TH and SH3 domain contribute to the function and regulation of the Tec family PTKs.

7.3.2 Intermolecular interactions involving the TH domain (IV)

The dimer equilibrum of the PRR and the SH3 domain was studied using gel permeation chromatography, intermolecular NMR cross-relaxation measurements, and [^{15}N]-NMR relaxation measurement. We showed monomer-dimer equilibrium of the Btk PRR-SH3 with a K_d of 60 μM. The gel chromatography profiles showed the Btk PRR-SH3 domain retention to be concentration dependent. The titration of a synthetic peptide corresponding to peptide 1 onto a [^{15}N] labelled sample and monitored using an NMR spectrum supported a monomer-dimer equilibrum in the PRR-SH3 that is concentration dependent.

7.4 Stability and unfolding of the Btk non-catalytic domains (V)

CD spectroscopy and temperature gradient gel electrophoresis (TGGE) were used to analyse the stability and unfolding of the PH, TH, SH3 and SH2 domains. The CD spectrum of the protein showed intense negative minimas at 208 and 222 nm, with a positive peak close to 195 nm.

The temperature-induced denaturation using TGGE for the multidomain protein gave a single T_m of 45°C. The result indicates that a single unfolding initiation process leads to the coordinated denaturation of the four domains. The ellipticity at 222 nm as a function of temperature was followed to understand the corporate dependance of the different domains. The thermal transition curves indicate that at high temperatures, the molar ellipticity is not zero: $(\theta)222 = -10{,}10$ mdeg at 75°C. This implies the presence of some residual secondary structure even at high temperatures as previously reported (Venyaminov and Yang, 1996).

Based on the CD measurements we calculated a number of parameters. The thermodynamic parameters defined include the *Tg;* which is the upper temperature at which $\Delta G0\ (T)$ is zero; ΔHg, which is the enthalpy of denaturation at *Tg*; and ΔCp, the change of heat capacity upon protein unfolding. We calculated the transition point of the enthalpy of denaturation (Tg) of the PH-SH2 protein and the van't Hoff enthalpy upon denaturation to be 322K and Hm 323 kcal/mol, respectively. Further, with the use of the van't Hoff analysis, the ΔCp was calculated to be 16.5 ± 1.9 kJmol-1K-1. These parameters were analysed with the assumption that the denaturation process follows a two state N ⇆ D transition. Previously, it has been shown that small proteins, like the SH3 domain usually unfold in a two state transition (Knapp et al, 1996; Viguera et al 1994; Fersht, 1999). Thus far, only the stability and peptide binding specificity of the Btk SH2 and SH3 domain has been reported (Tzeng et al., 2000; Knapp et al., 1998).

8 DISCUSSION

8.1 XLA causing mutations

Single stranded conformational polymorphism (SSCP) followed by direct sequencing has this far been widely used for molecular diagnosis of XLA and several other immunodeficiencies. In our study, base excision sequence scanning (BESS) method (Hawkins and Hoffman, 1997) coupled with direct sequencing was used for the mutation analysis in *BTK* gene. By using this approach, we studied eight unrelated families. The XLA causing mutations identified in our study were located in the PH, SH2 and the kinase domain (see Table 7.1). From the families we studied, different mutations were found: two insertions, two inframe insertions, an unknown mutation in the intron 14 leading to amino acid change in the tyrosine kinase domain, and a two base substitution in the intron 4. All the patients showed XLA phenotype. All the disease-causing mutations are novel. The silent polymorphism C/T at 2031 in one patient had previously been described (Bradley *et al.*, 1994; Rodriguez *et al.*, 2001). Previous work has described, XLA-causing mutations in all the Btk domains encoding gene and are collected in the BTKbase (Vihinen et al., 1999; Vihinen et al., 2001).

Most of the mutations available in the database are located in the PH, SH2 and the kinase domain and only few has thus far been seen in the TH and the SH3 domain. Indeed, it is not clear if a missense mutation in the PRR-region of the TH domain could cause the disease. A substitution has been found in this region, E205D, but this patient also had another mutation leading to truncation in the PH domain (Vórechovský et al., 1995). Another known mutation replaces T184 (in the beginning of the first PRR) with a proline. However, in this case there is again a frameshift mutation truncating a major part of the protein (Vórechovsky et al., 1997). Although, a large number of XLA-causing gene mutations have been identified, so far no causative missense mutations in the gene encoding the SH3 domain region have been found.

8.2 Intra/intermolecular interaction(s): a mode for Tec family kinase regulation

Inter and intramolecular interactions play an important role in the regulation of many PTKs. Studies have shown that the PRR of Btk interacts with the SH3 domains of Src family kinases i.e. Fyn, Lyn, and Hck (Cheng et al., 1994). In Itk

the PRR was shown to interact in an intramolecular fashion with the SH3 domain (Andreotti et al., 1997). We were interested to study the nature of this interaction(s) in Btk. With different spectroscopic techniques we were able to bring into focus the interaction pattern(s) of the TH and the SH3 domains. Since both the tested proteins and peptides were optically active, it was possible to determine binding affinities. To accurately determine equilibrium constants for binding of ligands to SH3 domain fluorescence measurements were used (Otto-Bruc et al., 1993., Cussac et al., 1994, Vidal et al., 1999). With this method, the affinity for the interaction between the peptides, TH and the SH3 domains were determined. We also used NMR spectroscopy, which is particularly useful in the study of protein structure and dynamics in solutions. When a protein structure is known, NMR can be used to study interactions between the protein and ligands. Usually, this is achieved by observing the perturbation of the protein chemical shifts upon isotope labeling. In our case, this method was used to study the intermolecular interactions between the TH and the SH3 domains. Both [^{15}N]-HSQC and NOESY-HSQC experiments were carried out, combined with gel chromatography profiles. The results showed a monomer-dimer equilibrum involving the PRR suggesting that interaction between the TH and SH3 domain contribute to the function and regulation of the Tec family kinases.

The affinity of the PRR and the SH3 domain in Tec has been studied (Pursglove et al., 2002). The N-terminal PRR (KTLPPAP), residues 155-161 in Tec, and the C-terminal PRR (KRRPPPPIPP) residues 165-174, were independently titrated into ^{15}N-labeled Tec SH3 domain. The changes in the chemical shifts of resonances in the HSQC spectrum were measured as a function of ligand concentration. The interaction surface on the SH3 domain was defined and the binding affinity estimated. For the intramolecular interaction of Tec SH3 domain with the N-terminal PRR the K_d was found to be between 1.7-2.0 μM (Pursglove et al., 2002).

For the Tec SH3 domain, an intermolecular interaction involving the PRR has also been reported by applying surface plasmon resonance (Pursglove et al., 2002). The wild-type PRR-SH3 protein was found to bind to the SH3 domain with a K_d of 68 μM (Pursglove et al., 2002). This is consistent with our measurements for Btk (K_d 60 μM). In Tec, the disruption of the N-terminal PRR by mutagenesis had no significant effect on the affinity, whereas the alteration of the C-terminal PRR had a marked effect (K_d 2.2 mM) on the ability to bind the SH3 domain surfac indicating that this region is critical for the intermolecular interaction (Pursglove et al., 2002). Thus, in Tec the C-terminal PRR is responsible for the interaction while in Btk the N-terminal PRR is essential (Fig. 8.2).

Our results clearly indicate that the N-terminal PRR of Btk interacts with the SH3 domain. The lack of spectral shift in the PRR - SH3-peptide complex suggests that Tec family members might share a similar mode of internal regulation by binding of the TH domain to the SH3 domain. Dimer formation of these domains could prevent the binding of low affinity ligands. Recently, the C-terminal PRR in Btk was shown to play a more robust role in dimer formation as compared to the N-terminal PRR (Laederach et al., 2002) (Fig.8.2 C). Although, the N-terminal PRR competed for dimer formation, it had a remarkably weaker affinity (Laederach et al., 2002). Three conformational states for dimer formation have been proposed to coexist at equilibrium (Hansson et al., 2001), i.e.

1) the N-terminal PRR binds across the dimer interface,
2) one of the N-terminal PRRs binds the other monomer while the other is uncomplexed, or
3) both PRRs bind across the dimer interface

Thus, the formation of a dimer could prevent intramolecular interactions, which are important for regulation of the protein.

The SH3 domain in the PSD-95 (post-synaptic density protein 95 kDa) of the MAGUKS (membrane-associated guanylate kinases) family has been shown to have the ability to adopt different modes in its interaction and regulation (McGee et al., 2001). In PSD-95 the SH3 domain interaction with the GK fragment differs from the other SH3 domains so that the interaction occurs via 3D domain swapping involving the C-terminal portion of the SH3. 3D domain swapping involves the exchange of complementary structures by proteins to generate dimers or higher order oligomers (Newcomer, 2001). Previous studies of protein systems that undergo swapping have indicated that the hinge or linker region between two interacting domains plays an important role in determining the preference for intra/intermolecular interaction (Newcomer, 2001; 2002; Schlunnegger et al., 1997). It would be interesting to ascertain if this also happens in the Btk TH/SH3 interaction.

To further elucidate this interaction in Tec family members, the three-dimensional structure of the full-length protein is essential. However, dimerization behavior has been observed for the Btk PH domain (Baraldi et al., 1999). These findings indicate that self-association plays a central role in the regulation of the Btk/Tec family protein tyrosine kinases.

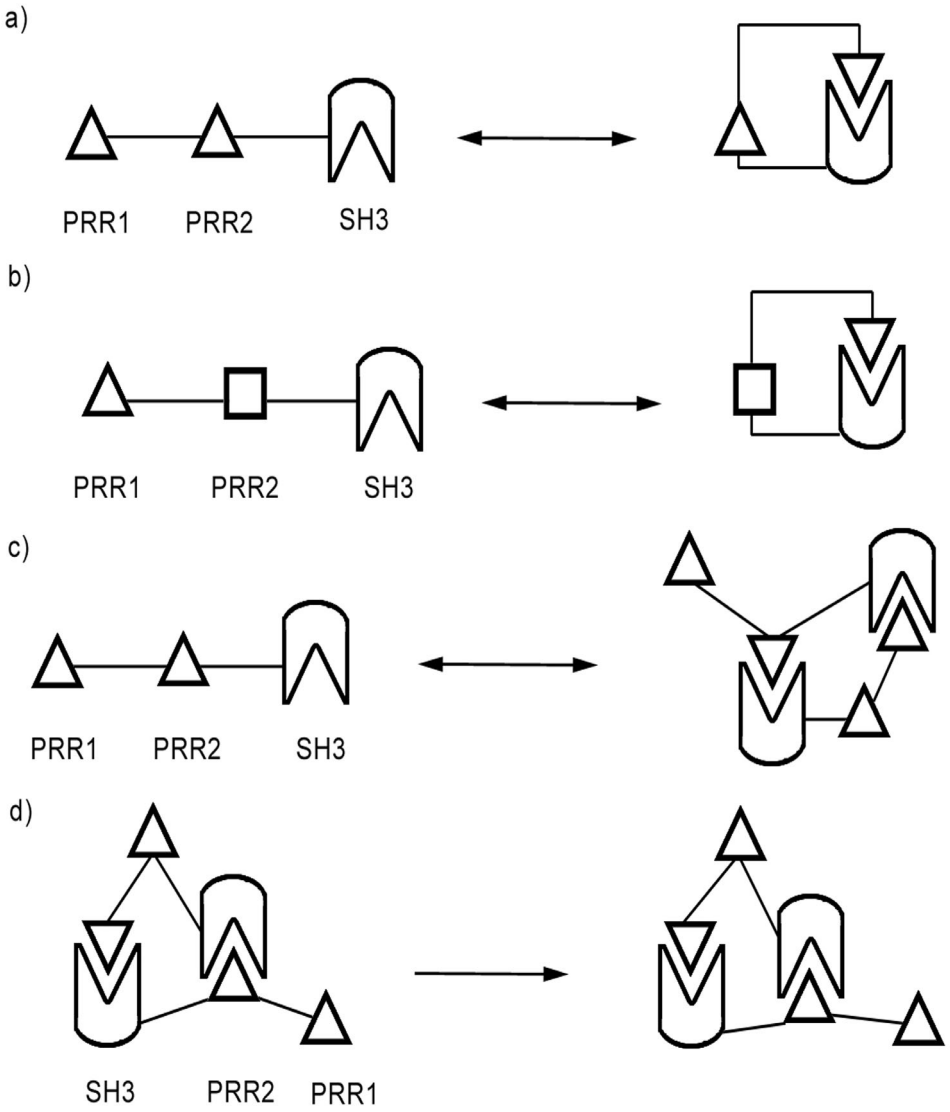


Figure 8.2 Schematic representations of the Btk PRR interactions. (A) The N-terminal PRR interacts with the SH3 domain-binding site in an intramolecular fashion. (B) Mutation of the C-terminal PRR does not show any significant effect on the interaction. (C) Proposed dimerization mode of the protein. (D) The interconvertion of the dimer in a symmetry mode.

8.3 The biological implications of Btk SH3 domain interactions

Our results show that the Btk SH3 domain plays a number of regulatory roles, these include inter and intramolecular regulation. Obviously the interaction of this domain with other domains within Btk is important for the overall function of Btk. Most of the PTKs exhibit similar patterns of regulation. Two different microarray chips were used to monitor gene expression patterns in a cell line with non-functional Btk. Btk itself was induced with Src kinases (Islam et al., 2002) implying similar regulation pattern for these proteins.

The interaction(s) of the Btk TH and SH3 domains, as shown by the results presented in this study, suggests a mechanism by which the accessibility of the TH and SH3 domains regulates the kinase activity of Btk. Recently, the deletion of the PRR sequence in Itk (Hao and August, 2002) was shown to result in a 50% reduction of basal activity of this protein, further implying that this region is essential for the regulation of Tec family kinases. The modulation of the SH3 domain by Y223 autophosphorylation has been predicted to block binding to the domain (Park et al., 1996; Rawlings, 1999; Yang et al., 2000). Exogenous partners that may disrupt the intra/intermolecular interactions between the TH and SH3 domains can potentially participate in the regulation of the Btk family members.

One of the roles suggested for the Btk TH and SH3 domain interactions is to regulate and suppress the kinase activity (Ma and Huang, 1998; Yang et al., 2000). The Src family kinases are known to interact with Btk TH domain via their SH3 domain (Ma and Huang, 1998). Tec family kinases have been shown to recruit other proteins, for example Src and Syk kinases, that uncouple initiated signaling via their SH3 domain (Mano, 1999). Another regulator of Btk interactions is Sab, an SH3-domain binding protein that preferentially associates with Btk by binding the Btk SH3 domain via its PxxP motif (Yamadori et al., 1999). The interaction between Sab and Btk SH3 domain results in the inhibition of Btk activity with a dramatic reduction in both early and late BCR-mediated events including calcium mobilization, IP3 production, and programmed cell death (Yamadori et al., 1999). Such interactions with proteins that have the ability to regulate the phosphorylation state of the full-length protein gives insight on the mechanism required to sustain enzyme de-/activation via the interaction between the TH and the SH3 domains.

Btk is homologous to Src in the region spanning the SH3, SH2, and the kinase domain. Although it does not possess the canonical C-terminal phosphorylation

site present in the Src family kinases, it does share a similar hydrophobic binding pocket responsible for ligand interaction (Williams et al., 1998; Vihinen and Smith, 1998). The crystal structure of the peptide encompassing the SH3-SH2 domains of Lck (a Src family member), showed that the domains dimerize (Eck et al., 1994). In Fyn, site directed mutagenesis studies demonstrated that the dimerization was phosphorylation independent. Mutations of the Fyn pY binding residue (R176K) did not prevent dimerization (Panchamoorthy et al., 1994). Furthermore, mutations of S186P and R190C, in the SH2 domain of Fyn, did not affect recognition of pY (Panchamoorthy et al., 1994). In Fyn, it seems that alterations in the phosphate-binding region do not affect SH2-SH3 domain dimerization. For most protein tyrosine kinases the binding of the SH2 domain to the pY residue (in order to repress the kinase activity) requires the SH3 domain (Williams et al, 1998; Vihinen and Smith 1998; Xu et al., 1997; Sicheri and Kuriyan, 1997; Panchamoorthy et al., 1994; Erpel et al., 1995). Previously it has been shown that the substitution K186R in Btk increases binding to Fyn (Yang et al., 1995). Using mutagenesis, P189 was found to be the most crucial residue for binding to Btk SH3 domains (Yang et al., 1995; Patel et al., 1997), notably the substitution of the neighbouring P187 alanine did not cause any significant change.

In c-Src kinase the regulation of the kinase domain by phosphorylation of the C-terminal tail was found to require both the SH3 and SH2 domains as elucidated by a coexpression experiment of Src mutants and Csk in yeast (Superti-Furga et al., 1993; Okada et al., 1994). The main intramolecular contacts that stabilize the inactive conformation of Src are those of the SH2-kinase linker with the SH3 domain, and the linker between SH2 and the N-terminal kinase lobe (Sicheri and Kuriyan, 1997). These interactions hold the enzyme in an inactive state due to limited conformational freedom (William, et al., 1998). An accessible ligand binding surface and RT (arginine and threonine) loop are essential for the function of SH3 domains (Superti-Furga et al., 1993; Okada et al., 1994; Erpel et al., 1995; Barila and Superti-Furga., 1998).

Recent data (Lowry et al., 2001) suggest that down-regulated Btk has a higher basal activity than down-regulated c-Src, possibly due to different structural conformations of the active sites and activation loops. The finding that the Btk catalytic domain cannot autophosphorylate at Y551 in the absence of the SH3/SH2 or the PH/TH modules, whilst the c-Src can autophosporylate at its equivalent site Y416 (Lowry et al., 2001), implies that the Btk catalytic domain relies on these domains to recognize the substrate or to modulate substrate accessibility. In this respect, further work is required to fully comprehend the role(s) of the TH/SH3 interaction(s) on the one hand, and the interaction between the

SH3 and SH2 domains on the other hand.

8.4 The dynamics of the Btk N-terminal domains

We studied the unfolding and the stability of the Btk N-terminal domains. Thermal and chemical induced denaturation was followed at 222 nm using CD spectroscopy and TGGE. The reverse of the unfolding experiment was not done as it has previously been reported that the refolding process of multidomain proteins *in vitro* occur at a lower rate due to the slown correct pairing of the folded units (Jaenicke, 1987). When applying CD spectroscopy to protein denaturation and unfolding studies, it is assumed that the changes observed occur due to the sensitivity of the system to the changes in the environment. And also due to the changes of chromophoric amino acids resulting from temperature perturbation and the resultant local conformational changes in the full-length protein. These observations are supported by e.g. an earlier study on myosin (Holtzer and Holtzer, 1992), where the effect of temperature on the protein was attributed to the local changes in the helical content.

Local interactions at the secondary structure level and interactions between domains and subunits are thought to contribute to the net free energy of protein stabilization (Jaenicke, 2000). Understanding the individuality of the different Btk domains in concert with stability studies would profoundly increase the understanding of the biological functions of the full-length protein. In recent times, studies of protein folding, unfolding and in particular of misfolding have been implicated in a number of so called conformational diseases i.e. transmissible spongiform encephalopathies and Alzheimer's disease (Ellis and Pinheiro, 2002) thus providing a wider relevance for such studies.

9 Future perspectives and conclusions

The three dimensional structure of Btk is vital to study the functional mechanisms and regulation in detail. The structure would help in illuminating the *in vitro* and *in vivo* observations made thus far. Moreover, the nature of the intra/intermolecular interactions between the TH/SH3 domains needs to be further addressed, as does the role of the Btk SH3 domain phosphorylation site. As revealed in this study among others, the interactions of these domains are essential for cell signalling, protein interactions etc. Using molecular and biophysical means we have successfully shown the biological significance of these domain interactions in Btk regulation. Specifically, we showed that the Btk SH3 domain can interact with the PRR in the N-terminal TH domain. To understand these interactions in the Tec family kinases further experiments are needed to elucidate the molecular determinants of the various conformational states of the Tec family kinases. Experiments, which explore the differences in the SH3 domains, the linker region, and the proline rich region would be vital in this respect.

The diversity of the molecules interacting with Btk and other family members suggest functions, which are significant and strictly controlled. Several signal mediators are now known as effectors of Tec family signalling. An understanding of the molecular interactions of the Tec family is essential for addressing issues related to the basis of signal transduction and diseases arising from mutations in the involved genes. In this regard, the combination of multiple methods will be vital in attempting to unravel the true complexity of these proteins. One of the fundamental questions this study answered is, how the N-terminal Btk (PH, TH, SH3 and SH2) domain interactions are coordinated and regulated to achieve both spatial and specific interaction.

10 Acknowledgements

This study was carried out at the Department of Biosciences, Division of Biochemistry, University of Helsinki. The academy of Finland, EU BIOTECH BIO4-CT98-0142, the Sigrid Juselius Foundation, and the Medical research fund of Tampere University hospital provided the financial support. Also the National graduate school in informational and structural biology is acknowledged for the financial assistance.

I am deeply indebted to my supervisor Professor Mauno Vihinen, who provided me with the opportunity to study in Helsinki. The driving engine behind this work from the inception has been your exemplary skills as a researcher and your eventful ideas. Your friendship during the bad and good times of this study and your confidence in my ability has been inspiring. You are to me a good friend indeed and for this I thank you without reservations. My sincere gratitude goes to Professor Carl G. Gahmberg, the head of the Division of Biochemistry for providing me with an unfettered access to the excellent facilities in the Division. I also wish to thank Professor Kari Keinänen for his supportive and friendly attitude toward my work and me.

I would like to thank Professors Veli-Pekka Lehto and Dennis Bamford for their quick but critical review of the manuscript. Their suggestions and comments were invaluable in improving this work. Professor Lehto is especially thanked to have introduced me to the world of protein signaling. Dr Sarah Coleman is also acknowledged for the language corrections and advice.

To our group members Ilkka, Juha, Kaj and Johanna, I thank you guys for I would not have been able to make it without your cooperations. To Ile and Juha I am particularly grateful for if not for you guys my work would have been more difficult knowing how naive I was in computer applications when I came from Oulu. To the other group members in Tampere especially, Jukka, Tomi, Hannu, Bairong, Jouni, Mansour and Martti I say thank you all for the wonderful times we always manage to have on the few occasions that we get together.

I cannot thank enough the people at the Division of Biochemistry, for their friendly attitude and comradeship. Especially I owe thanks to Drs Mathias, Ragna, Erkki, Pirjo and Matti. Also Drs Tian and Leena formally of our Division are thanked for their friendship. Most shout out to Aki, Asko, Esa, Heli, Mikael and Michaelis for your friendship. To L Kuoppasalmi, I say thank you for your help. My sincere gratitude is also extended to the secretarial staff of the Division.

All my coauthors are acknowledged. Thank you all for sharing your scientific prowess with me. My sincere thanks also extend to Professor Mark Johnson and Kaija Soderlund Director and coordinator respectively of the National graduate school in informational and structural biology for their support. Dr Carola of Orion pharma is thanked for taking her time to teach me the all-important HPLC method and also for allowing me to use her lab at any short time notice.

My sincere gratitude also goes to Mensu Ize-Okhai for believing in my ability and for the elderly advice. I also wish to thank all my friends that have shared their time with me both before and during this study.

My story is about to end but not without mentioning the people who are the most important in my life; Mrs Q, Zakari, Mary and Dora; my most senior, immediate senior and junior sisters respectively. Thank you all for your understanding and support...I love you all. Mohan-Klem, Okoh my senior brother and my best friend thank you for everything so numerous to narrate here. Suffice though to say I am glad I have you as my best friend, my mentor and a brother....I love you. To my Mother thank you for your unwavering support throughout the period of this study. Thaddeus, can you see me? RIP boy. I wish my Father was alive he would have upon seeing this thesis say "**My Dear....See Meeeeee**". Thank you Dad for everything, most of all thank you for your unconditional love and support to me throughout your lifetime even though I was not a very easy son. **To you I dedicate this thesis**.

This thesis is a testimonial to the biblical saying that all things worketh good for those that beliveth. To God be the glory for providing me with the inspiration, I thank him for making me to be just me…



11. References

Aiuti, A., Vai, S., Mortellaro, A., Casorati, G., Ficara, F., Andolfi, G., Ferrari, G., Tabucchi, A., Carlucci, F., Ochs, H.D., Notarangelo, L.D., Roncarolo, M.G. and Bordignon, C. (2002a) Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat Med*. **8,** 423-5

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M.G. and Bordignon, C. (2002b) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296,** 2410-3

Anderson, J.S., Teutsch, M., Dong, Z. and Wortis, H.H. (1996) An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. *Proc Natl Acad Sci USA.* **93,**10966-71

Andreotti, A.H., Bunnell, S.C., Feng, S., Berg, L.J. and Schreiber, S.L. (1997) Regulatory intramolecular association in a tyrosine kinase of the Tec family. *Nature* **385,** 93-7

Arkwright, P.D., Abinum, M. and Cant, A.J. (2002) Autoimmunity in human primary immunodeficiency diseases. *Blood* **99,**2694-2702

August, A., Sadra, A., Dupont, B. and Hanafusa, H. (1997) Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc Natl Acad Sci USA.* **94,** 11227-32

Baba, K., Takeshita, A., Majima, K., Ueda, R., Kondo, S., Juni, N. and Yamamoto, D. (1999) The Drosophila Bruton's tyrosine kinase (Btk) homolog is required for adult survival and male genital formation. *Mol Cell Biol.***19,** 4405-13.

Baraldi, E., Carugo, K.D., Hyvönen, M., Surdo, P.L., Riley, A.M., Potter, B.V., O'Brien, R., Ladbury, J.E. and Saraste, M. (1999) Structure of the PH domain from Bruton's tyrosine kinase in complex with inositol 1,3,4,5-tetrakisphosphate. *Struct Fold Des.* **7,** 449-60

Barila, D. and Superti-Furga, G. (1998) An intramolecular SH3-domain interaction regulates c-Abl activity. *Nat Genet*. **18,** 280-2

Bence, K., Ma, W., Kozasa, T. and Huang, X.Y. (1997) Direct stimulation of Bruton's tyrosine kinase by G(q)-protein alpha-subunit. *Nature* **389,** 296-299

Blaese, R.M., Culver, K.W., Miller, A.D., Carter, C.S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., et al. (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270**, 475-80

Blomberg, N. and Nilges, M. (1997) Functional diversity of PH domains: an exhaustive modelling study. *Fold Des*. **2,** 343-55

Blume-Jensen, P. and Hunter, T. (2001) Oncogenic kinase signalling. *Nature* **17,** 355-65

Bordignon, C., Notarangelo, L.D., Nobili, N., Ferrari, G., Casorati, G., Panina, P., Mazzolari, E., Maggioni, D., Rossi, C., Servida, P., et al. (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* **270,** 470-5

Brandley, L.A.D., Sweatman, A.K., Lovering, R.C., Jones, A.M., Morgan, G., Levinsky, R.L. and Kinnon, C. (1994) Mutation detection in the X-linked agammaglobulinemia gene, Btk, using single strand conformation polymorphism analysis. *Hum Mol Genet.* **3,**79-83

Brannetti, B., Via, A., Cestra, G., Cesareni, G. and Helmer-Citterich, M. (2000) SH3-SPOT: an algorithm to predict preferred ligands to different members of the SH3 gene family. *J Mol Biol*. **298,** 313-28

Brazin, K.N., Fulton, D.B. and Andreotti, A.H. (2000) A specific intermolecular association between the regulatory domains of a Tec family kinase. *J Mol Biol*. **302,** 607-23

Buckley, R.H., Schiff, R.I., Schiff, S.E., Markert, M.L., Williams, L.W., Harville, T.O., Roberts, J.L. and Puck, J.M. (1997) Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. *J Pediatri.* **130,** 345-6

Burkhardt, A.L. and Bolen, J.B. (1996) Signaling through hematopoietic antigen receptors. Signal transduction, Chapman & Hall pp. 51-78

Cancro, M.P., Sah, A.P., Levy, S.L., Allman, D.M., Schmidt,M.R. and Woodland, R.T. (2001) xid mice reveal the interplay of homeostasis and Bruton's tyrosine kinase-mediated selection at multiple stages of B cell development. *Inter Immunol.* **13,**1501-1514

Carpenter, C.L. and Cantley, L.C. (1996) Phosphoinositide kinases. *Curr Opin Cell Biol.* **8,** 216-222

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L., Bousso, P., Deist, F.L. and Fischer A. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288,** 669-72.

Cesareni, G., Panni, S., Nardelli, G. and Castagnoli, L. (2002) Can we infer peptide recognition specificity mediated by SH3 domains *FEBS Letters* **513,** 38-44.

Chan, T.O., Rittenhouse, S.E. and Tsichlis, P.N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem*. **68,** 965-1014

Cheng, G., Ye, Z.-S. and Baltimore, D (1994) Binding of Bruton's tyrosine kinase to Fyn, Lyn or Hck through a Sac homology 3 domain-mediated interaction. *Proc Natl Acad Sci USA*. **91,** 8152-8155

Cheng, P.C., Dykstra, M.L., Mitchell, R.N. and Pierce, S.K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J Exp Med.* **190,** 1549-60

Cheng, P.C., Brown, B.K., Song, W. and Pierce, S.K. (2001) Translocation of the B cell antigen receptor into lipid rafts reveals a novel step in signalling. *J. Immunol.* **166,** 3693-701

Clapham, D.E. and Neer, E.J.(1997) G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol.* **37,** 167-203

Cochrane, D., Webster, C., Masih, G. and McCafferty, J. (2000) Identification of natural ligands for SH2 domains from a phage display cDNA library. *J Mol Biol.* **297,** 89-97

Coffey, A.J., Brooksbank, R.A., Brandau, O., Oohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wrary, P., Pavitt, R., Wilkinson, J., Leversha, M., Huckle, E., Shaw-Smith, C.J., Durham, A., Rhodes, S., Schuster, V., Porta, G., Yin, L., Serafini, P., Sylla, B., Zollo, M., Franco, B., Bolino, A., Seri, A., Lanyi, A., Davis, J.R., Webster, D., Harris, A., Lenoir, G., de St Basile, G., Jones, A., Behloradsky, B.H., Achhatz, H., Murgen, J., Fassler, R., Sumegi, J., Romeo, G., Vaudin, M., Ross, M.T., Meindl, A. and Bentley, D.R. (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in the SH2-domain encoding gene. *Nature Genet.* **20,**129-135

Conley, M.E., Mathias, D., Treadaway, J., Minegishi, Y. and Rohrer, J. (1998) Mutations in Btk in patients with presumed X-linked agammaglobulinemia. *Am J Hum Genet.* **62,**1034-43

Cotelingam, J.D., Witebsky, F.G., Hsu, S.M., Blaese, R.M. and Jaffe, E.S. (1985) Malignant lymphoma in patients with the Wiskott-Aldrich syndrome. *Cancer Invest.* **3,** 515-22

Craxton, A., Jiang, A., Kurosaki, T. and Clark, E.A. (1999) J Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *J Biol Chem*. **274,** 30644-50

Cussac, D., Frech, M. and Chardin, P. (1994) Binding of the Grb2 SH2 domain to phosphotyrosine motifs does not change the affinity of its SH3 domains for Sos proline-rich motifs. *EMBO J.* **13,** 4011-4021

de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., Boer, T., van Breda Vriesman, P.J., Kabel, P.J., Draaisma, J.M., van Dissel, J.T., Kroon, F.P., Casanova, J.L. and Ottenhoff, T.H. (1998) Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science* **280,**1435-8

Deng, J., Kawakami, Y., Hartman, S.E., Satoh, T., and Kawakami, T. (1998) Involvement of Ras in bruton's tyrosine kinase mediated JNK activation. *J Biol Chem.* **3,**16787-91

de Weers, M., Mensink, R.G., Kraakman, M.E., Schuurman, R.K. and Hendriks, R.W. (1994) Mutation analysis of the Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient xid mice. *Hum Mol Genet*. **3,** 161-6

Del Vecchio, P., Graziano, G., Granata, V., Barone, G., Mandrich, L., Manco, G. and Rossi, M. (2002) Temperature- and denaturant-induced unfolding of two thermophilic esterases. *Biochemistry* **41,**1364-71

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res*. **11,** 387-95

Dingjan, G.M., Maas, A., Nawijn, M.C., Smit, L., Voerman, J.S., Grosveld, F. and Hendriks, R.W. (1998) Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing E41K mutated from Bruton's tyrosine kinase. *EMBO J.* **17,** 5309-5320

Eck, M.J., Shoelson, S.E. and Harrison, S.C. (1993) Recognition of a high affinity phosphotyrosyl peptide by the Src homology 2 domain of p56lck. *Nature* **362,** 87–91

Eck, M.J., Atwell, S.K., Shoelson, S.E. and Harrison, S.C. (1994) Structure of the regulatory domains of the Src-family tyrosine kinase Lck. *Nature* **368,** 764

Ekman, N., Arighi, E., Rajantie, I., Saharinen, P., Ristimaki, A., Silvennoinen, O. and Alitalo, K. (2000) The Bmx tyrosine kinase is activated by IL-3 and G-CSF in a PI-3K dependent manner. *Oncogene* **19,** 4151-8

Ellmeier, W., Jung, S., Sunshine, M.J., Hatam, F., Xu, Y., Baltimore, D., Mano, H. and Littman, D.R. (2000) Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. *J Exp Med.* **192,** 1611-24

Ellis, R.J and Pinheiro, T.J.T. (2002) Danger - misfolding proteins. *Nature* **416,** 483-484

Erpel, T., Superti-Furga, G. and Courtneidge, S.A. (1995) Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. *EMBO J.* **14,** 963-75

Fasman, G.D. (1996) Circular dichroism and the conformational analysis of biomolecules. Plenum press, New York

Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B.(1995) Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* **83,** 1037-46

Fersht, A. (1999) Structure and mechanism in protein science: W.H. Freeman and Co. pp 508-539, 547-553.

Flaswinkel, H. and Reth, M. (1994) Dual role of the tyrosine activation motif of the Ig-alpha protein during signal transduction via the B cell antigen receptor. *EMBO J.* **13,** 83-9

Fluckiger, A.C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Kinet, J.P., Witte, O.N., Scharenberg, A.M. and Rawlings, D.J. (1998) Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation. *EMBO J.* **17,** 1973-85

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W. and Cantley, L.C. (1999) Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science* **283,** 393-7

Fukuda, M., Kojima, T., Kabayama, H. and Mikoshiba, K. (1996) Mutation of the pleckstrin homology domain of Bruton's tyrosine kinase in immunodeficiency impaired inositol 1,3,4,5-tetrakisphosphate binding capacity. *J Biol Chem*. **271,** 30303-6

Futatani, T., Miyawaki, T., Tsukada, S., Hashimoto, S., Kunikata, T., Arai, S., Kurimoto, M., Niida, Y., Matsuoka, H., Sakiyama, Y., Iwata, T., Tsuchiya, S., Tatsuzawa, O., Yoshizaki, K. and Kishimoto T. (1998) Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. *Blood* **91,** 595-602.

Gasper, H.B., Ferrando, M., Caragol, I., Hernandez, M., Bertran, J.M., De Gracial, X., Lester, T., Kinnon, C., Ashton, E. and Espanol, T. (2000) Kinase mutant Btk results in atypical X-linked agammaglobulinaemia phenotype. *Clin Exp Immunol*. **273,**1096-1100

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

Gaspar, H.B., Brandley, L.A.D., Katz, F.R., Lovering, C., Roifman, C.M., Morgan, G., R. Levinsky, J. and Kinnon, C. (1995) Mutation analysis in Bruton's tyrosine kinase, the X-linked agammaglobulinaemia gene, including identification of an insertional hotspot. *Hum Mol Genet.* **4,** 755-7

Gorina S. and Pavletich, N.P. (1996) Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science* **274,** 921-2.

Gore, M.G. (2000) Spectrophotometry & Spectrofluorimetry. Oxford university press.

Hagemann, T.L., Chen, Y., Rosen, F.S. and Kwan, S.P. (1994) Genomic organization of the Btk gene and exon scanning for mutations in patients with X-linked agammaglobulinemia.*Hum Mol Genet*.**3,** 1743-9

Hansson, H., Mattson, P.T., Allard, P., Haapaniemi, P., Vihinen, M., Smith, C.I.E. and Härd, T. (1998) Solution structure of the SH3 domain from Bruton's tyrosine kinase. *Biochemistry* **37,** 2912-24

Haire, R.N., Strong, S.J. and Litman, G.W. (1997) Identification and characterization of a homologue of Bruton's tyrosine kinase, a Tec kinase involved in B-cell development, in a modern kinase expressed in zebrafish kidney. *Immunogenet.* **47,** 336-7.

Hansson, H., Smith, C.I.E. and Härd, T. (2001) Both proline-rich sequences in the TH region of Bruton's tyrosine kinase stabilize intermolecular interactions with the SH3 domain. *FEBS Lett*. **508,** 11-15

Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Kishimoto, T., Kurosaki, T. and Tsukada, S. (1999) Identification of the SH2 domain binding protein of Bruton´s tyrosine kinase as BLNK – functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood* **94,** 2357-64

Hawkins, G.A. and Hoffman, L. M. (1997). Base excision sequence scanning. *Nat Biotechnol.* **15,** 803.

Haire, R.N., Strong, S.J. and Litman, G.W. (1998) Tec-family non-receptor tyrosine representative of a phylogenetically ancient vertebrate. *Immunogenet.* **46,** 349-51.

Hao, S. and August, A (2002) The proline rich region of the Tec homology domain of ITK regulates its activity. *FEBS Lett*. **525,** 53-58

Harlan, J.E., Yoon, H.S., Hajduk, P.J. and Fesik, S.W. (1995) Structural characterization of the interaction between a pleckstrin homology domain and phosphatidylinositol 4,5-bisphosphate. *Biochemistry* **34,** 9859-64

Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.*Nucleic Acids Res*. **22,** 4673-4680

Holtzer, M.E. and Holtzer, A. (1992) Alpha-helix to random coil transitions: interpretation of the CD in the region of linear temperature dependence. *Biopolymers* **32,** 1589-91

Hunter T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80,** 225-36.

Hurley, J.H., Anderson, D.E., Beach, B., Canagarajah, B., Ho, Y.S., Jones, E., Miller, G., Misra, S., Pearson, M., Saidi, L., Suer, S., Trievel, R. and Tsujishita Y. (2002) Structural genomics and signaling domains.*Trends Biochem Sci*. **27,** 48-53

Hyvönen, M. and Saraste, M. (1997) Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinaemia. *EMBO J*. **16,** 3396-40

Ingley, E. and Hemmings, A.B. (1999) Large-scale expression and purification of a soluble form of the pleckstrin homology domain of the human protooncogenic serine/threonine protein kinase PKB (c-akt) in Escherichia coli. *Protein Expr Purif*. **17,** 224-30

Islam, T.C. and Smith, C.I.E. (2000) The cellular phenotype conditions Btk for survival of apoptosis signaling. *Immunol Rew.* **178,** 49-63

Islam, T.C., Lindvall, J., Wennborg, A., Brandén, L.J., Rabbani, H. and Smith C.I.E. (2002) Expression profiling in the transformed human B cells: influence of Btk mutations and comparison to B cell lymphomas using filter and oligonucleotide arrays. *Eur J Immunol*. **32,** 982-993

Jaenicke, R. (1987) Folding and association of proteins. *Progr Biophys Mol Biol.* **49,** 117-237

Jaenicke, R. (2000) Stability and stabilization of globular proteins in solution. *J Biotech.* **79,** 193-203.

Jiang, Y., Ma, W., Wan, Y., Kozasa, T., Hattori, S. and Huang, X.Y. (1998) The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. *Nature* **395,** 808-13

Kang, H., Freund, C., Duke-Cohen, J.S., Musacchio, A., Wagner, G. and Rudd, C.E. (2000) SH3 domain recognition of a proline independent tyrosine based RkxxYxxY motif in immune cell adaptor SKAP55. *EMBO J.* **19,** 2889-2899

Kawakami, Y., Yao, L., Miura, T., Tsukada, S., Witte, O.N. and Kawakami, T. (1994) Tyrosine phosphorylation and activation of Bruton tyrosine kinase upon Fc epsilon RI cross-linking. *Mol Cell Biol*. **14,** 5108-13

Kerner, J.D., Appleby, M.W., Mohr, R.N., Chien, S., Rawlings, D.J., Maliszewski, C.R., Witte, O.N. and Perlmutter, R.M. (1995) Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity* **3,** 301-312

Kikuchi, Y., Yasue, T., Miyake, K., Kimoto, M. and Takatsu, K. (1995) CD38 ligation induces tyrosine phosphorylation of Bruton tyrosine kinase and enhanced expression of interleukin 5-receptor alpha chain: synergistic effects with interleukin 5. *Proc Natl Acad Sci USA*. **92,** 11814-18

Knapp S, Mattsson PT, Christova, P, Berndt KD, Karshikoff A, Vihinen M, Smith CIE, Ladenstein R (1998) Thermal unfolding of small proteins with SH3 domain folding pattern. *Proteins* 31, 309-319

Kuriyan, J. and Cowburn, D. (1997) Modular peptide recognition domains in eukaryotic signaling. *Annu Rev Biophys Biomol Struct.* **26**, 259-88

Kurosaki, T. (1997) Molecular mechanisms in B cell antigen receptor signalling. *Curr Opin Immun.* **9,**309-318

Kurosaki, T. and Tsukada, S. (2000) BLNK: Connecting Syk and Btk to calcium signals. *Immunity* **12,**1-5

Kwan, S.P., Kunkel, L., Bruns, G., Latt, S. and Rosen, F.S. (1986) Mapping of the X-linked agammaglobulinemia locus by use of restriction fragment-length polymorphism. .*J Clin Invest*. **77,** 649-52

Laederach,A., Cradic, K.W., Brazin, K.N., Zamoon, J., Fulton,D.B., Huang, X-Y. and Andreotti, A.M. (2002) Competing modes of self-association in the regulatory domains of Bruton's tyrosine kinase: Intramolecular contact versus asymmetric homodimerization. *Protein science* **11,** 36-45

Lewis, M.C., Christine, B., Czar, M.J. and Schwartzberg, P.L. (2001) Tec kinases: modulators of lymphocyte signalling and development. *Curr Opin Immunol*. **13,** 317-325

Lederman, H.M. and Winkelstein, J.A. (1985) X-linked agammaglobulinemia: an analysis of 96 patients. *Medicine Baltimore* **64,** 145-56.

Li, Z., Wahl, M.I., Eguinoa, A., Stephens, L.R., Hawkins, P.T. and Witte, O.N. (1997) Phosphatidylinositol 3-kinase-gamma activates Bruton's tyrosine kinase in concert with Src family kinases. *Proc Natl Acad Sci USA.* **94,** 13820-5

Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K. and Witte ON. (1995) Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity* **2,** 451-60

Lim, W.A., Richards, F.M. and Fox, R.O. (1994) Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. *Nature* **372,** 375-9

Lowry, W.E., Huang, J., Lei, M., Rawlings, D. and Huang, X.Y. (2001) Role of the PHTH module in protein substrate recognition by Bruton's agammaglobulinemia tyrosine kinase. *J Biol Chem*. **276**, 45276-81

Lu, Y., Cuevas, B., Gibson, S., Khan, H., LaPushin, R., Imboden, J. and Mills, G.B. (1998) J Phosphatidylinositol 3-kinase is required for CD28 but not CD3 regulation of the TEC family tyrosine kinase EMT/ITK/TSK: functional and physical interaction of EMT with phosphatidylinositol 3-kinase. *J Immunol*.**161,** 5404-12

Ma, Y.C. and Huang, X.Y. (1998) Identification of the binding site for Gqalpha on its effector Bruton's tyrosine kinase. *Proc Natl Acad Sci USA.* **95,** 12197-201

Mahajan, S., Fargnoli, J., Burkhardt, A.L., Kut, S.A., Saouaf, S.J. and Bolen, J.B. (1995) Src family protein tyrosine kinases induce autoactivation of Bruton's tyrosine kinase. *Mol Cell Biol*.**15,** 5304-11

Mano, H. (1999) Tec family of protein tyrosine kinases: An overview of their structure and function. *Cytokine Growth Factor Rev.* **10,** 267-280

Matsuda, T., Takahashi-Tezuka, M., Fukada, T., Okuyama, Y., Fujitani, Y., Tsukada, S., Mano, H., Hirai, H., Witte, O.N. and Hirano, T. (1995) Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. *Blood* **85,** 627-33

Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T. and Lim, L. (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell.* **1,** 183-92

Mattsson, P.T., Lappalainen, I., Backesjo, C.M., Brockmann, E., Lauren, S., Vihinen, M. and Smith, C.I. (2000) Six X-linked agammaglobulinemia-causing missense mutations in the Src homology 2 domain of Bruton's tyrosine kinase: phosphotyrosine-binding and circular dichroism analysis. *J Immunol.* **164,** 4170-7

Mayer, B.J. and Eck, M.J. (1995) SH3 domains. Minding your p's and q's. *Curr Biol* **5,** 364-7

McGee, A.W., Dakoji, S.R., Olsen, O., Bredt, D.S., Lim, W.A. and Prehoda, K.E. (2001) Structure of the SH3-guanylate kinase module from PSD-95 suggests a mechanism for regulated assembly of MAGUK scaffolding proteins. *Mol Cell* **8,** 1291-301

McKinney, E.E.S.Jr., Katz, L. and Wilfert, C.M. (1987) Chronic enteroviral meningoencephalitis in agammaglobulinemic patients. *Rev Infect Dis*. **9,** 334-56

Mohamed, A.J., Vargas, L., Nore, B.F., Backesjo, C.M., Christensson, B. and Smith, C.I.E. (2000) Nucleocytoplasmic shuttling of Bruton's tyrosine kinase. *J Biol Chem.* **275**, 40614-9

Mongiovi, A.M., Romano, P.R., Panni, S., Mendoza, M., Wong, W.T., Musacchio, A., Cesareni, G., and Di Fiore, P.P. (1999) A novel peptide-SH3 interaction. *EMBO J.* **18,** 5300-9

Mumby, S.M. (1997) Reversible palmitoylation of signaling proteins. *Curr Opin Cell Biol.* **9,**148-54

Newcomer, M.E. (2001) Trading places. *Nat Struct Biol.* **8,** 282-297

Newcomer, M.E. (2002) Protein folding and three-dimensional domain swapping: a strained relationship. *Curr Opin Struct Biol.* **12,** 48-53

Nichols, K.E., Harkin, D.P., Levitz, S., Krainer, M., Kolquist, K.A., Genovese, C., Bernard, A., Ferguson, M., Zuo, L., Snyder, E., Buckler, A.J., Wise, C., Ashley, J., Lovett, M., Valentine, M.B., Look, A.T., Gerald, W., Housman, D.E. and Haber, D.A. (1998) Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci USA.* **95,**13765-70

Nera, K-P., Brockmann, E., Vihinen, M., Smith, C.I.E. and Mattsson, P. (2000) Rational design and purification of human Bruton's tyrosine kinase SH3-SH2 protein for structure-function studies. *Protein Expr Purif.* **20,** 365-71

Nolting, B., Golbik, R., Soler-Gonzalez, A.S. and Fersht, A.R. (1997) Circular dichroism of denatured barstar suggests residual structure. *Biochemistry* **36,** 9899-905

Nore, B.F., Vargas, L., Mohamed, A.J., Branden, L.J., Backesjo, C.M., Islam, T.C., Mattsson, P.T., Hultenby, K., Christensson, B. and Smith, C.I.E. (2000) Redistribution of Bruton's tyrosine kinase by activation of phosphatidylinositol 3-kinase and Rho-family GTPases. *Eur J Immunol.* **30,**145-154

Nore, B.F., Mohamed, A.J Vargas, L., Branden, L.J., Backesjo, C.M., Vihinen, M., Christensson, B. and Smith, C.I.E. (2000) The role of Brutons's tyrosine kinase (Btk) in phosphoinositide-dependent signalling. *ACI International* **12,** 126-133

Ochs, H.D. and Smith, C.I.E. (1996) X-linked agammaglobulinemia. A clinical and molecular analysis. *Medicine* **75,** 287-299.

Ochs, H.D., Smith, C.I.E. and Puck, J.M. (1999) Primary immunodeficiency disease. A molecular and genetic approach pp1-501. Oxford University Press, New York, Oxford.

Oda, A., Ikeda, Y., Ochs, H.D., Druker, B.J., Ozaki, K., Handa, M., Ariga, T., Sakiyama, Y., Witte, O.N. and Wahl, M.I. (2000) CrkL is an adapter for Wiskott-Aldrich syndrome protein and Syk. *Blood* **95**, 2663-9

Ogawa, A., Takayama, Y., Sakai, H., Chong, K.T., Takeuchi, S., Nakagawa, A., Nada, S., Okada, M. and Tsukihara, T. (2002) Structure of the carboxyl-terminal Src kinase, Csk. *J Biol Chem.* **277**, 14351-4

Okada, M., Howell, B.W. and Cooper J.A. (1993) Deletion of the SH3 domain of Src interferes with regulation by the phosphorylated carboxyl-terminal tyrosine. *J Biol Chem*. **268,** 18070-5

Otto-Bruc, A., Antonny, B., Vuong, T. M., Chardin, P. and Chabre, M. (1993) Interaction between the retinal cyclic GMP phosphodiesterase inhibitor and transducin. Kinetics and affinity studies. *Biochemistry* **32,** 8636-8645

Ott, J., Mensink, E.J., Thompson, A., Schot, J.D. and Schuurman, R.K. (1986) Heterogeneity in the map distance between X-linked agammaglobulinemia and a map of nine RFLP loci. *Hum Genet.* **74**, 280-3

Panchamoorthy, G., Fukazawa, T., Stolz, L., Payne, G., Reedquist, K., Shoelson, S., Zhou, S., Cantley, L., Walsh, C. and Band, H. (1994) Physical and functional interactions between SH2 and SH3 domains of the Src family protein tyrosine kinase p59fyn. *Mol Cell Biol*. **14,** 6372-85

Park, H., Wahl, M.I., Afar, D.E., Turck, C.W., Rawlings, D.J., Tam, C., Scharenberg, A.M., Kinet, J.P. and Witte, O.N. (1996) Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity* **4,** 515-25

Patel, H.V., Tzeng, S-R., Liao, C-Y., Chen, S-H., and Cheng, J-W. (1997) SH3 domain of Bruton's tyrosine kinase can bind to proline-rich peptides of TH domain of the kinase and p120cbl. *Proteins* **29,** 545-52

Pawson, T. (1995) Protein modules and signalling networks. *Nature* **373,** 477-80

Petro, J.B., Rahman, S.M., Ballard, D.W. and Khan, W.N. (2000) Bruton's tyrosine kinase is required for activation of IKB kinase and nuclear factor KB in response to B cell receptor engagement. *J Exp Med*. **191,**1745-54

Peranen, J., Rikkonen, M., Hyvonen, M. and Kaariainen, L. (1996) T7 vectors with modified T7lac promoter for expression of proteins in Escherichia coli. *Anal Biochem.* **236,** 371-3

Pursglove, S.E., Mulhern, T.D., Mackay, J.P., Hinds, M.G. and Booker, G.W. (2002) The solution structure and the intramolecular association of the Tec kinase Sac homology 3 domain. *J Biol Chem.* **277,** 755-762

Quek, L.S., Bolen, J. and Watson, S.P. (1998) Regulation of Bruton´s tyrosine kinase and phospholipase Cα2 in collegen-stimulated platelets. *Curr Biol*. **8,**1137-40

Qui, Y. and Kung, H.J. (2000) Signaling network of the Btk family kinases. *Oncogene* **19,** 5651-5661

Rawlings, D.J., Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M., Fluckiger, A.C., Witte, O.N. and Kinet, J.P. (1996) Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* **271,** 822-5

Rawlings, D.J. (1999) Bruton's tyrosine kinase controls a sustained calcium signal essential for B lineage development and function. *Clinical Immunol*. **91,** 243-253

Reth, M. and Wienands, J. (1997) Initiation and processing of signals from the B cell antigen receptor. *Annu Rev Immunol*. **15**, 453-79

Rodriguez, M.G., Granados, E.L., Cerdán, A.F. and Fontán, G. (2001) Molecular analysis of bruton's tyrosine kinase gene in Spain. *Hum Mut.* **18,** 84-88.

Rohrer, J., Parolini, O., Belmont, J.W., Conley, M.E. and Parolini, O. (1994) The genomic structure of human BTK, the defective gene in X-linked agammaglobulinemia. *Immunogenetics* **40,** 319-24

Roulier, E.M., Panzer, S. and Beckendorf, S.K. (1998) The Tec29 tyrosine kinase is required during Drosophila embryogenesis and interacts with Src64 in ring canal development. *Mol Cell* **1,** 819-29.

Royer-Pokora, B., Kunkel, L.M., Monaco, A.P., Goff, S.C., Newburger, P.E., Baehner, R.L., Cole, F.S., Curnutte, J.T. and Orkin, S.H. (1986) Cloning the gene for the inherited disorder chronic granulomatous disease on the basis of its chromosomal location. *Cold Spring Harb Symp Quant Biol*.**1,** 177-83.

Rubin, G.M., Yandell, M.D., Wortman, J.R., Gabor Miklos, G.L., Nelson, C.R., Hariharan, I.K., Fortini, M.E., Li, P.W., Apweiler, R., Fleischmann, W., Cherry, J.M., Henikoff, S., Skupski, M.P., Misra, S., Ashburner, M., Birney, E., Boguski, M.S., Brody, T., Brokstein, P., Celniker, S.E., Chervitz, S.A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R.F., Gelbart, W.M., George, R.A., Goldstein, L.S., Gong, F., Guan, P., Harris, N.L., Hay, B.A., Hoskins, R.A., Li, J., Li, Z., Hynes, R.O., Jones, S.J., Kuehl, P.M., Lemaitre, B., Littleton, J.T., Morrison, D.K., Mungall, C., O'Farrell, P.H., Pickeral, O.K., Shue, C., Vosshall, L.B., Zhang, J., Zhao, Q., Zheng, X.H. and Lewis, S. (2000) Comparative genomics of the eukaryotes. *Science* **287,** 2204-15

Saouaf, S.J., Burkhardt, A.L. and Bolen, J.B (1995) Nonreceptor protein tyrosine kinase involvement in signal transduction and immunodeficiency disease. *Clin Immunol Immunopathol.***76,** 151-7.

Sato, S., Katagiri, T., Takaki, S., Kikuchi, Y., Hitoshi, Y., Yonehara, S., Tsukada, S., Kitamura, D., Watanabe, T., Witte, O. et al. (1994) IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J Exp Med.* **180,** 2101-11

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., Oettgen, H., De Vries, J.E., Aversa, G. and Terhorst, C. (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* **395,** 462-9

Schaeffer, E.M., Debnath, J., Yap, G., McVicar, D., Liao, X.C., Littman, D.R., Sher, A., Varmus, H.E., Lenardo, M.J. and Schwartzberg, P.L. (1999) Requirement for Tec kinases Rlk and Itk in T cell receptor signalling and immunity. *Science* **284,** 638-641

Schlunegger, M.P., Bennett, M.J. and Eisenberg, D. (1997) Oligomer formation by 3D domain swapping: a model for protein assembly and misassembly. *Adv Protein Chem*. **50,** 61-122

Shaw, G. (1996) The pleckstrin homology domain: an intriguing multifunctional protein module. *BioEssays* **18**, 35-46

Shoelson, S.E., Sivaraja, M., Williams, K.P., Hu, P., Schlessinger, J. and Weiss, M.A. (1993) Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. *EMBO J.* **12,** 795-802.

Sicheri, F. and Kuriyan, J. (1997) Structures of Src-family tyrosine kinases.*Curr Opin Struct Biol.* **7,** 777-85

Sideras, P. and Smith, C.I.E. (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. *Adv Immunol*. **59,**135-223

Smith, C.I.E and Notangelo L.D. (1997) Molecular basis for X-linked immunodeficiencies. *Adv Genet.* **35,** 57-115

Smith, C.I., Islam, T.C., Mattsson, P.T., Mohamed, A.J., Nore, B.F. and Vihinen, M. (2001) The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. *BioEssay*s **23,** 436-46

Solvason, N., Wu, W.W., Kabra, N., Lund-Johansen, F., Roncarolo, M.G., Behrens, T.W., Grillot, D.A., Nunez, G., Lees, E. and Howard, M. (1998) Transgene expression of bcl-xL permits anti-immunoglobulin (Ig)-induced proliferation in xid B cells. *J Exp Med.* **187,**1081-1091

Songyang Z, Blechner S, Hoagland N, Hoekstra MF, Piwnica-Worms H, Cantley LC. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr Biol.* **4,** 973-82

Su, Y.W., Zhang, Y., Schweikert, J., Koretzky, G.A., Reth, M. and Wienands. J. (1999) Interaction of SLP adaptors with the SH2 domain of Tec family kinases. *Eur J Immunol.* **29,** 3702-11

Superti-Furga, G., Fumagalli S., Koegl, M., Courtneidge, S.A. and Draetta,G. (1993) Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. *EMBO J.* **12,** 2625-34

Takata, M. and Kurosaki, T. (1996) A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. *J Exp Med*. **184,** 31-40

Tomlinson, M.G., Kurosaki, T., Berson, A.E., Fujii, G.H., Johnston, J.A. and Bolen, J.B. (1999) Reconstitution of Btk signaling by the atypical tec family tyrosine kinases Bmx and Txk. *J Biol Chem.* **274,** 13577-85

Thomas, J.D., Sideras, P., Smith, C.I.E., Vorechovsky, I., Chapman, V. and Paul, W.E. (1993) Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* **261,** 355-358

Tsukada, S., Saffran, S.D., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Mohandas, T. and Quan, T., et al. (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* **72,** 279-90

Tzeng, S.R., Pai, M.T., Lung, F.D., Wu, C.W., Roller, P.P., Lei, B., Wei, C.J., Tu, S.C., Chen, S.H., Soong, W.J. and Cheng, J.W. (2000) Stability and peptide binding specificity of Btk SH2 domain: molecular basis for X-linked agammaglobulinemia. *Protein Sci.* **12,** 2377-85

Uckun, F.M., Waddick, K.G., Mahajan, S., Jun, X., Takata, M., Bolen, J. and Kurosaki, T. (1996) BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. *Science* **273,** 1096-100

Venyaminov, S.Y and Yang, J.T. (1996) Determination of protein secondary structure: in Circular Dichroism and the Conformational Analysis of Biomolecules (Fasman, G.D., Ed.) pp 69-107, Plenum Press, New York.

Vetrie, D., Vorechovský, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993) The gene involved in X-linked agammaglobulinemia is a member of the src family of protein tyrosine kinases. *Nature* **361,** 226-232

Vidal, M., Goudreau, N., Cornille, F., Cussac, D., Gincel, E. and Garbay, C. (1999) Molecular and cellular analysis of Grb2 SH3 domain mutants: interaction with Sos and dynamin. *J Mol Biol*. **290,** 717-730.

Viguera, A.R., Martinez, J.C., Filimonov, V.V., Mateo, P.L. and Serrano, L. (1994) Thermodynamic and kinetic analysis of the SH3 domain of Spectrin shows a two state folding transition. *Biochemistry* **33,** 2142-2150

Vihinen, M., Nilsson, L. and Smith, C.I.E (1994a) Tec homology (TH) adjacent to the PH domain. *FEBS Lett*. **350,** 263-5

Vihinen, M., Nilsson, L. and Smith, C.I.E. (1994b) Structural basis of SH2 domain mutations in X-linked agammaglobulinemia. *Biochem Biophys Res Commun*. **205,** 1270-7

Vihinen, M., Cooper, M.D., de Saint Basile, G., Fischer, A., Good, R.A., Hendriks, R.W., Kinnon, C., Kwan, S.P., Litman, G.W., Notarangelo, L.D., et al. (1995a) BTKbase: a database of XLA-causing mutations. International Study Group. *Immunol Today* **16,** 460-5

Vihinen, M., Zvelebil, M.J., Zhu, Q., Brooimans, R.A., Ochs, H.D., Zegers, B.J., Nilsson, L., Waterfield, M.D. and Smith, C.I.E. (1995b) Structural basis for pleckstrin homology domain mutations in X-linked agammaglobulinemia. *Biochemistry* **34,** 1475-81

Vihinen, M. and Smith, C.I. (1996) Structural aspects of signal transduction in B-cells. *Crit Rev Immunol* **16,** 251-75

Vihinen, M., Nore, B.F., Mattsson, P.T., Bäckesjö, C.M., Nars, M., Koutaniemi, S., Watanabe, C., Lester, T., Jones, A., Ochs, H.D. and Smith, C.I.E. (1997) Missense mutations affecting a conserved cysteine pair in the TH domain of Btk. *FEBS Lett.* **413,** 205-10

Vihinen, M., Brandau, O., Brandén, L.J., Kwan, S.P., Lappalainen, I., Lester, T., Noordzij, J.G., Ochs, H.D., Ollila, J., Pienaar, S.M., Riikonen, P., Saha, B.K. and Smith, C.I.E. (1998) BTKbase, mutation database for X-linked agammaglobulinemia (XLA). *Nucleic Acids Res*. **26,** 242-7

Vihinen, M. and Smith, C.I.E. (1998) Interactions between SH2 and SH3 domains. *Biochem Biophys Res Comm*. **242,** 351-6

Vihinen, M., Kwan, S.P., Lester, T., Ochs, H.D., Resnick, I., Väliaho, J., Conley, M.E. and Smith, C.I.E. (1999) Mutations of the human BTK gene coding for bruton tyrosine kinase in X-linked agammaglobulinemia. *Hum Mutat*. **13,** 280-5

Vihinen, M., Mattsson, P. and Smith, C.I.E. (2000) Bruton's tyrosine kinase (BTK) in X-linked agammaglobulinemia (XLA). *Front Biosci.* **5,** 917-928

Vihinen, M., Arredondo-Vega, F.X., Casanova, J.L., Etzioni, A., Giliani, S., Hammarstrom, L., Hershfield, M.S., Heyworth, P.G., Hsu, A.P., Lahdesmaki, A., Lappalainen, I., Notarangelo, L.D., Puck, J.M., Reith, W., Roos, D., Schumacher, R.F., Schwarz, K., Vezzoni, P., Villa, A., Valiaho, J. and Smith, C.I.E. (2001) Primary immunodeficiency mutation databases. *Adv Genet.* **43,**103-188

Vorechovsky, I. (1994) SSCP at the Btk locus. *Hum Mol Genet.* **3,** 1031

Vorechovsky, I., Vihinen, M., de Saint Basile, G., Honsová, S., Hammarström, L., Müller, S., Nilsson, L., Fischer, A. and Smith, C.I.E. (1995) DNA-based mutation analysis of Bruton's tyrosine kinase gene in patients with X-linked agammaglobulinaemia. *Hum Mol Genet*. **4,** 51-8

Vorechovsky, I., Luo, J.M., Hertz, S.S., Froland, T., Klemola, M., Fiorini, I., Quinti, R., Paganelli, H., Ozsahin, L., Hammarström, A.D., Webster, A.D. and Smith, C.I.E. (1997) Mutation pattern in the Bruton's tyrosine kinase gene in 26 unrelated patients with X-linked agammaglobulinemia. *Hum Mutat*. **9,** 418-25

Waksman, G., Kominos, D., Robertson, S.R., Pant, N., Baltimore, D., et al. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature* **358,** 646–53

Waksman, G., Shoelson, S.E., Pant, N., Cowburn, D. and Kuriyan. J. (1993) Binding of a high affinity phophotyrosyl peptide to the src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* **72,** 779–90

Williams, J.C., Wierenga, R.K. and Saraste, M. (1998) Insights into Src kinase functions: structural comparisons. *Trends Biochem Sci*. **23,**179-84

Woody, R. W. (1994) Contributions of tryptophan side chains to the far-ultra-violet circular dichroism of proteins. *Eur Biophys J.* **23**, 253-62

Xu, P., Mitchelhill, K.I., Kobe, B., Kemp, B.E. and Zot, H.G. (1997) The myo-sin-I-binding protein Acan125 binds the SH3 domain and belongs to the super-family of leucine-rich repeat proteins. *Proc Natl Acad Sci USA.* **15,**3685-90

Xu, R.X., Word, J.M., Davis, D.G., Rink, M.J., Willard, D.H. Jr. and Gampe, R.T Jr. (1995). Solution structure of the human pp60c-src SH2 domain complexed with a phosphorylated tyrosine pentapeptide. *Biochemistry* 34:2107–21

Yamashita, Y., Watanabe, S., Miyazato, A., Ohya, K., Ikeda, U., Shimada, K., Komatsu, N., Hatake, K., Miura, Y., Ozawa, K. and Mano, H. (1998) Tec and Jak2 kinases cooperate to mediate cytokine-driven activation of c-fos transcription. *Blood* **91,** 1496-507

Yamadori, T., Baba, Y., Matsushita, M., Hashimoto, S., Kurosaki, T., Kishimoto, T. and Tsukada, S. (1999) Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein. *Proc Natl Acad Sci USA* **89,** 1118-1122

Yang, W., Malek, S.N. and Desiderio, S. (1995) An SH3-binding site conserved in Bruton's tyrosine kinase and related tyrosine kinases mediates specific protein interactions in vitro and in vivo. *J Biol Chem.* **270,** 20832-40

Yang, W.C., Ghiotto, M., Barbarat, B. and Olive, D. (1999) The role of Tec protein tyrosine kinase in T cell signaling. *J Biol Chem.* **274**,607-617

Yang , W.C., Collette, Y., Nunes, J.A. and Olive, D. (2000) Tec kinases: a family with multiple roles in immunity. *Immunity* **12,** 373-382

Yang, W.C., Ching, K.A., Tsoukas, C.D. and Berg, L.J. (2001) Tec kinase signaling in T cells is regulated by phosphatidylinositol 3-kinase and the tec pleckstrin homology domain. *J Immunol.* **166,** 387-395

Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W.B, Kawakami, Y. and Kawakami, T. (1997) Interactions between protein kinase C and pleckstrin homology domains. Inhibition by phosphatidylinositol 4,5-bisphosphate and phorbol 12-myristate 13-acetate. *J Biol Chem*. **272,** 13033-9