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MOLECULAR BIOLOGY OF BRUTON'S TYROSINE KINASE

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ABSTRACT

X-linked agammaglobulinemia (XLA) is caused by an arrest in the B lymphocyte differentiation. XLA patients lack mature B-cells and subsequently antibody production. The defective gene causing XLA is the *BTK* gene, which codes for the protein Bruton's tyrosine kinase (Btk), a cytoplasmic enzyme related to the Src family of kinases. Btk is only expressed in the hematopoietic cells, except for T lymphocytes and plasma cells.

In an attempt to investigate the function of Btk, we have studied how mutations and other proteins affect the activity, phosphorylation and localization of Btk. XLA causing mutations in the Src homology 2 (SH2) domain of Btk expressed in *E. coli* were found to disrupt the binding to phosphotyrosine, indicating a necessary function for the Btk protein. Only the mutant directly involved in the binding did not significantly deviate structurally from the native SH2 domain.

Btk can phosphorylate its own tyrosine 223 in the SH3 domain. We found that the site Y223 could also be phosphorylated by c-Abl. Btk and c-Abl did also co-localize and interact in cells. Phosphorylation of Y223 has been suggested to downregulate the activity of Btk. Moreover, we found that the substrate specificity for Btk and the protein family members Itk, Bmx and Tec are different. Btk preferentially phosphorylates the SH3 domain of Btk and Bmx, while Itk phosphorylated Itk and Btk SH3 domains, whereas Tec could phosphorylate the SH3 domain of Btk and Tec, but also with reduced efficiency Bmx and Itk. The phosphorylation sites in the SH3 domains were also mapped. Tec was phosphorylated on a conserved tyrosine, which differs from the conserved Y223, and was the site of phosphorylation in the other family members.

A region in the PH domain binds phospholipids in the cell membrane. Changing negative charged amino acids to positive induce microspikes structures on the cell membrane of the cells. D43R and the previously reported gain-of-function, E41K, showed an increased cell proliferation. These mutations also made IL-5 dependent cells proliferate in the absence of IL-5, indicating a transforming potential.

In summary, these studies show some important functions of the Btk protein that are necessary for B-cell development and illustrate the complexity of these mechanisms.

Keywords: X-linked agammaglobulinemia, XLA, Bruton's tyrosine kinase, Btk, signal transduction, phosphorylation, pleckstrin homology domain, PH, SH2, SH3.

LIST OF PUBLICATIONS

This thesis is based upon the following publications. They are referred to in the text by their roman numerals.

I. Pekka T. Mattsson, Ilkka Lappalainen, **Carl-Magnus Bäckesjö**, Eeva Brockmann, Susanna Laurén, Mauno Vihinen and C. I. Edvard Smith. 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.* 2000, 164 (3): 4170-4177.

II. **Carl-Magnus Bäckesjö**, Leonardo Vargas, Giulio Superti-Furga and C. I. Edvard Smith. Phosphorylation of Bruton's tyrosine kinase by c-Abl. *Biochem. Biophys. Res. Commun.* 2002, 299 (3): 510-515.

III. Beston F. Nore, Pekka T. Mattsson, Per Antonsson, **Carl-Magnus Bäckesjö**, Anna Westlund, Johan Lennartsson, Henrik Hansson, Peter Löw, Lars Rönnstrand and C. I. Edvard Smith. Identification of phosphorylation sites within the SH3 domain of Tec family tyrosine kinases. *Biochim. Biophys. Acta.* 2003, 1645 (2): 123-132.

IV. **Carl-Magnus Bäckesjö**, Leonardo Vargas, Alar Aints, Birger Christensson, Anna Berglöf, Mauno Vihinen and C. I. Edvard Smith. Transforming effect of pleckstrin homology (PH) domain mutations in Bruton's tyrosine kinase (Btk). *Manuscript.*

LIST OF ABBREVIATIONS

Abl	Abelson murine leukemia oncogene
Akt	acutely transforming retrovirus
Arf6	ADP-ribosylation factor 6
Bam11	Btk associated molecule-11
BAP-135	Btk associated protein of 135kDa
Bcl-X_L	B-cell cll/lymphoma long isoform
BCR	B-cell receptor
BLNK	B-cell linker
Bmx	bone marrow tyrosine kinase gene in chromosome X
Btk	Bruton's tyrosine kinase
Cbl	CysB-like adaptor protein
CD	circular dichroism
CD95	cluster of differentiation 95
Cdc42	cell division cycle 42
CML	chronic myeloid leukemia
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
E. coli	*Escherichia coli*
EGF	epidermal growth factor
EWS	Ewing's sarcoma
FACS	fluorescence activated cell sorting
FcεR	high affinity IgE receptor
FBS	foetal bovine serum
GAP	GTPase activating protein
GDP	guanine diphosphate
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
GTP	guanine triphosphate
Grb	growth factor receptor-bound protein
GST	glutathione S-transferase
Hck	hematopoietic cell kinase
IBtk	inhibitor of Btk
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IVK	*in vitro* kinase assay
JNK	Janus kinase
kb	kilobasepairs
kDa	kilodalton
IL	interleukin
IP3	inositol 1,4,5-trisphosphate
IP4	inositol 1,3,4,5-tetrakisphosphate
Itk	interleukin-2-inducible T cell kinase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene

MAPK	mitogen activated protein kinase
mRNA	messenger RNA
NES	nuclear export signal
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa B
NLS	nuclear localization signal
PEI	polyethylene imine
PH	Pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PRR	proline rich region
PTEN	Phosphatase and Tensin homolog deleted on chromosome 10
PTK	protein tyrosine kinase
pY	phosphotyrosine
Rac1	ras-related C3 botulin toxin substrate 1
Rho	Ras homology gene family
Rif	Rho in filopodia
Rlk	resting lymphocyte kinase
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
Sab	SH3 domain-binding protein associates with Btk
Sam68	Src-associated in mitosis, 68kDa
SDF-1α	stromal cell-derived factor-1a
SFK	Src family tyrosine kinases
SH	Src homology
SHIP	SH2-containing inositol 5-phosphatase
siRNA	small interfering RNA
SLP-65	SH2-domain-containing leukocyte protein of 65kDa
Sp1	specificity factor 1
Src	Rous sarcoma oncogene
STAT	signal transducer and activator of transcription
Syk	spleen tyrosine kinase
Tec	Tyrosine kinase expressed in hepatocellular carcinoma
TFK	Tec family kinases
TH	Tec homology
Txk	T and X cell kinase
UTR	untranslated region
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
WASP	Wiskott-Aldrich syndrome protein
WT	wild type

INTRODUCTION

In multicellular organisms, phosphorylation, especially on tyrosine residues plays a major role in transducing signals from the cell membrane to the nucleus of the cell. In this thesis work, one intracellular signal mediator, the Btk enzyme, a member of the Tec tyrosine kinase family, has been studied at the molecular level.

X-linked agammaglobulinemia (XLA)

More than 50 years ago, in 1952, Dr Ogden C. Bruton reported a case of what he called agammaglobulinemia in an eight year old boy. The patient did not produce any gamma globulins as a response to vaccination (Bruton, 1952). This disease was the first immunodeficiency to be described. It was also found to be inherited in males and therefore linked to the chromosome X. The disease is now commonly referred to as X-linked agammaglobulinemia (XLA). The symptom that characterizes XLA is an extreme susceptibility to bacterial infections and patients suffers from recurrent infections that appear within the first years of life. The cause of the disease was further characterized with the discovery that patients with XLA lack mature B lymphocytes (Geha et al., 1973; Naor et al., 1969). Further research indicated that XLA is caused by arrested development of B-cells, between the pro B-cells and pre B-cells stage in the bone marrow (Campana et al., 1990) (Figure 1) and low or undetectable levels of all antibody isotypes, reviewed in (Sideras and Smith, 1995). XLA has a frequency of about 1 in 200,000 males (Vihinen et al., 2001). The therapy today is still the same as when Bruton started to treat his patients: a combination of antibiotics to treat infections and gamma globulin substitution as a prophylaxis against infections. However, this is not a cure and XLA patients are still highly prone to infections. A major step towards understanding the cause of XLA was taken in 1993 with the isolation of a gene which, when mutated, caused XLA (Tsukada et al., 1993; Vetrie et al., 1993). As expected, the gene was located on chromosome X. The gene was found to code for a novel protein tyrosine kinase (Vetrie et al., 1993). This protein kinase was named Bruton's tyrosine kinase (Btk) because of its role in XLA. One way to verify XLA is to analyze this gene for mutations (Vorechovsky et al., 1995). There is no apparent genotype-phenotype correlation. Btk expression levels do not predict the clinical and immunological outcome, and majority of the mutations result in a complete lack of Btk protein expression (Vihinen et al., 2000). Mutations have been found in all parts of the *BTK*

gene, and the information about these mutations are collected in a constantly updated database, known as BTKbase, where over 500 mutations are listed (Vihinen et al., 2001).

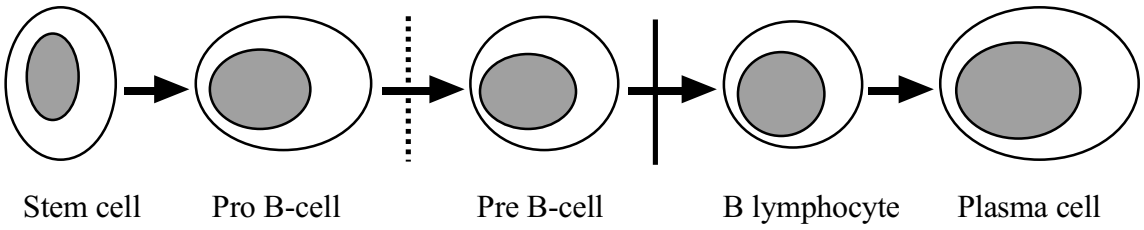

Figure 1. A schematic overview of the B-cell development. XLA patients have a partial block after the pro B-cell stage, and a complete block after the pre B-cell level, resulting in lack of mature B-cells.

X-linked immunodeficiency (Xid)

Xid is the XLA disease in mice, characterized by abnormal responses to polysaccharide antigen in mice (Amsbaugh et al., 1972). Xid mice have a naturally occurring mutation in the amino-terminal region of the Btk protein, called the Pleckstrin homology (PH) domain. This mutation alters the conserved amino acid, arginine at position 28 to a cysteine (R28C) (Rawlings et al., 1993; Thomas et al., 1993). Compared to XLA, the Xid phenotype is a less severe form of B-cell deficiency. Xid mice have reduced numbers of mature B-cells, and only the subset of B lymphocytes designated B1 cells, the isotypes IgM and IgG3 are significantly decreased. Lymphocytes from these mice fail to produce antibodies upon immunization of T-cell type II independent antigens and do not synthesize DNA in response to mitogenic stimulation of the surface IgM receptor (Rigley et al., 1989; Wicker and Scher, 1986). Targeted disruptions (knockout) of the *btk* gene in mice have been generated by different groups. Mice lacking the Btk protein turned out to have the same phenotype as mice with the R28C mutation (Hendriks et al., 1996; Kerner et al., 1995; Khan et al., 1995), while humans with the R28C mutation show the classical XLA phenotype (Vihinen et al., 1998). This indicates that there are species-specific differences concerning the function of the Btk protein in humans and mice. An interesting observation is that mice deficient in PLCγ2 (Wang et al., 2000), p85α (Fruman et al., 1999; Suzuki et al., 1999), BLNK/SLP-65 (Jumaa et al., 1999; Pappu et al., 1999) and PKCβ (Leitges et al., 1996), which are molecules just like Btk, downstream of the B-cell receptor show an Xid-like phenotype.

The *BTK* gene

The *BTK* gene was mapped to the long arm of the X-chromosome in the region Xq21.3-22 (Kwan et al., 1986) (Figure 2), and later cloned independently by two groups in 1993 (Tsukada et al., 1993; Vetrie et al., 1993). The gene is divided into 19 exons ranging from 55 to 560 bases, and spanning a genomic region of about 38 kb. The first exon comprises of a 5' untranslated region (UTR) whereas the following eighteen exons code for the protein. Exon 19 encodes part of the catalytic region of the protein as well as the 3' UTR (Rohrer et al., 1994; Sideras et al., 1994).

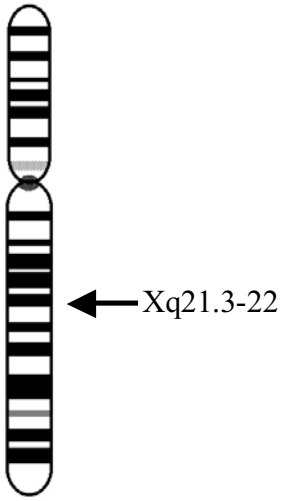

Figure 2. Localization of the *BTK* gene on chromosome X.

The mouse gene was cloned simultaneously with the human gene, and the amino acid sequence was 98.3% identical to the human sequence (Rawlings et al., 1993). The genomic organization of the human and mouse genes are also very similar (Sideras et al., 1994). The 280 bp upstream of the major transcriptional start site is part of the Btk promoter that contains three known binding sites for transcription factors. These are a GC-box and a GT-box binding the transcription factors Sp1 and Sp3, and a PU-box binding the transcription factors PU.1 and Spi-B (Himmelmann et al., 1996; Müller et al., 1996) The importance of the PU-box has been verified by mutation analysis of an XLA patient where a point mutation was found in this region (Holinski-Feder et al., 1998). Tissue-specific expression seems to be provided by the Spi-B/PU.1 factors, which are selectively expressed among hematopoietic cells (Hromas et al., 1993).

Btk is expressed in the majority of the hematopoietic cells (de Weers et al., 1993; Smith et al., 1994). In the lymphoid compartment Btk is expressed from very early stages throughout B-cell development, but expression of Btk in the most mature form of antibody

producing lymphocytes, the plasma cell, has not been detected. In the T-lymphocytes, no expression of Btk has been found after the pro-T-cell stage (de Weers et al., 1993; Genevier et al., 1994; Müller et al., 1999; Smith et al., 1994) The expression pattern in eosinophils is still unknown (Figure 3). Despite the documented defect in B-cells, no malfunctions in the other Btk expressing cells have been shown to contribute to the XLA phenotype.

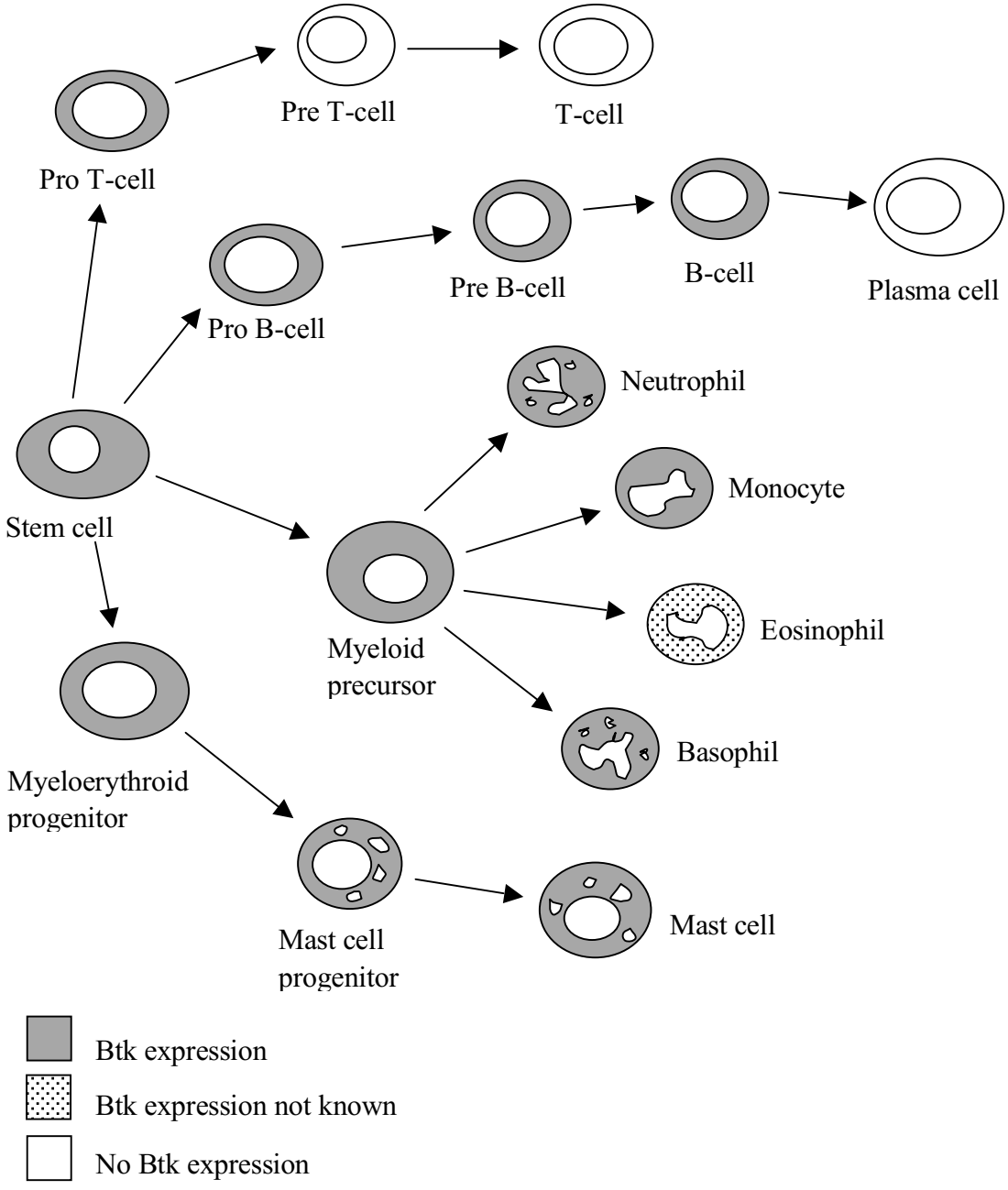

Figure 3. The Btk expression in hematopoietic cells.

The Btk protein

The Btk protein is a 659 amino acid protein with a molecular weight of 77 kDa. It is a cytoplasmic tyrosine kinase related to the Src family of tyrosine kinases (SFK) based upon the homology and composition of the C-terminal kinase domain followed by an N-terminal Src homology 2 (SH2) domain and SH3 domain (Tsukada et al., 1993; Vetrie et al., 1993). Together with Tec (Mano et al., 1990; Mano et al., 1993), Itk (Heyeck and Berg, 1993; Siliciano et al., 1992), Bmx (Tamagnone et al., 1994) and Txk (Haire et al., 1994), Btk form the Tec family of kinases (TFK). The TFK members are distinguished from the SFK by the presence of a Pleckstrin homology (PH) domain followed by a Tec homology (TH) domain N-terminal of the SH domains, reviewed in (Smith et al., 2001) (Figure 4A). Other characteristics that distinguish the TFK members from SFK are the absence of the consensus N-terminal myristoylation sequence through which SFK are coupled to the plasma membrane, and the regulatory tyrosine residue in the C-terminus, which has been implicated in negative regulation of the kinase activity (Desiderio and Siliciano, 1994). In contrast to the globular shape of Src with many intramolecular interactions (Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997), a low-resolution structure of the full-length Btk protein indicated a linear arrangement of the domains with no, or few, intramolecular interactions (Marquez et al., 2003). The domains in Btk are necessary for protein-protein or protein-lipid interaction, regulating the localization and activity of the protein. Several proteins have been shown to interact with the different domains in Btk, reviewed in (Takesono et al., 2002) (Figure 4B).

A

B

PH	TH	SH3	SH2	Kinase
PIP3, IP4	Gα12	WASP	BLNK	BAP-135/TFII-I
Gβγ	Gαq	p120 Cbl		STAT5A
BAP-135/TFII-I	Fyn	Sam68		Gβγ
Fas	Hck	Vav		Fas
PKC	Lyn	Syk		
Bam11		Sab		
IBtk				

Figure 4. Linear representation of the Btk protein and molecules interacting with Btk.

The Btk PH domain

The pleckstrin homology (PH) domain is the second largest domain in Btk, comprising 138 residues (Vihinen et al., 1994). PH domains in general have been shown to bind lipid phosphoinositides in the plasma membrane (Harlan et al., 1994). This function is believed to substitute for the myristoylation found in other molecules such as Src for binding to the membrane (Resh, 1994). The key protein for recruitment of Btk to the cell membrane is the phosphatidylinositol 3-kinase (PI3K) (Nore et al., 2000; Wymann and Pirola, 1998). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) in the 3'-position generating phosphatidylinositol 3,4,5-trisphosphate (PIP3). The PH domain specifically recognizes and interacts with PIP3 (Rameh et al., 1997; Salim et al., 1996). Btk membrane targeting is diminished and activity is downregulated by SH2-containing inositol 5'-phosphatase (SHIP) (Bolland et al., 1998). The Btk PH domain also binds the corresponding cytosolic form, inositide 1,3,4,5-tetrakisphosphate (IP4) (Fukuda and Mikoshiba, 1997; Salim et al., 1996). Missense mutations in the PH domain, causing XLA, can either alter the PIP3 binding or the stability of the domain. The mutants affecting the PIP3 binding accumulate in the predicted PIP3 binding site, which is positively charged (Hyvönen and Saraste, 1997). To this group belongs the R28C mutation found in Xid mice, which provided the first evidence for the functional importance of the PH domain. The R28C mutation was later found in humans (Vihinen et al., 1997a). From a random mutagenesis screening of Btk, a gain-of-function substitution was found. This missense mutation was located in the PH domain and changed a glutamate at position 41 to a lysine. This mutant of Btk could drive fibroblast growth in soft agar and was accompanied by an increase in membrane association and tyrosine phosphorylation (Li et al., 1995).

The PH domain has also been shown to bind protein. The first molecules to be described in this context were the heterotrimeric G protein (Gβγ) (Tsukada et al., 1994), Gαq and Gα12 (Bence et al., 1997; Jiang et al., 1998). Other proteins such as F-actin (Yao et al., 1999), the transcription factor BAP-135/TFII-I (Novina et al., 1999; Yang and Desiderio, 1997) and CD95/Fas receptor (Vassilev et al., 1999), have also been demonstrated to interact with the PH domain. Most of these interactions cause an increase in the kinase activity and/or an increase in the activity of the interacting partner. PKC (Johannes et al., 1999; Yao et al., 1994), Bam11 (Kikuchi et al., 2000) and IBtk (Liu et al., 2001) have been reported to interact with the PH domain and negatively regulate the activity of Btk. Interestingly, mice deficient in the two PKCβ isoforms develop an immunodeficiency similar to the Xid phenotype (Leitges et al., 1996).

The TH domain

The Tec homology domain is approximately 80 amino acids, and is distinguished by having two regions. The N-terminal part, referred to the Btk motif, is a 27 residue stretch with sequence homology to Ras-GAP and binds a zinc ion, which interacts with three conserved cysteines and a histidine (Vihinen et al., 1994; Vihinen et al., 1997b). The Btk motif is essential for the binding of the Btk PH domain to G-protein (Jiang et al., 1998; Lowry and Huang, 2002; Ma and Huang, 1998) and stabilizing the PH domain (Hyvönen and Saraste, 1997). The C-terminal part possesses a proline rich region (PRR), which can bind the SH3 domain of the Src kinases Fyn, Hck and Lyn (Alexandropoulos et al., 1995; Cheng et al., 1994). However, a second important regulatory function might be an interaction of the TH and SH3 domains in Btk in an intramolecular as well as intermolecular fashion (Hansson et al., 2001a).

The SH3 domain

Src homology 3 (SH3) domains are known to interact with proline-rich regions with consensus sequence PxxP (Ren et al., 1993; Yu et al., 1994). The Btk SH3 domain is approximately 60 amino acids long and forms a globular structure (Hansson et al., 1998). Following activation of Btk, tyrosine 223 in the SH3 domain is autophosphorylated by Btk (Park et al., 1996). Mutations in the SH3 domain of the Src and c-Abl tyrosine kinase increase their transforming potential (Jackson et al., 1993; Seidel-Dugan et al., 1992). Deletion or mutation of the tyrosine 223 to a phenylalanine in the SH3 domain of Btk, increases the transforming potential of the Btk E41K mutant without affecting the kinase activity (Park et al., 1996). So far, missense mutations in the SH3 domain have not been found in XLA patients (Vihinen et al., 2001).

Since the SH3 domain is involved in protein-protein interactions it has also been shown to bind to several signaling molecules. The SH3 domain interacts with Wiskott-Aldrich syndrome protein (WASP), when defective, is associated with a hematopoietic disease affecting multiple blood cell lineages (Cory et al., 1996; Guinamard et al., 1998; Kinnon et al., 1997). In addition, the SH3 domain has been reported to bind p120 Cbl (Cory et al., 1995), EWS, Sam68, Vav (Guinamard et al., 1997), Syk (Morrogh et al., 1999) and the negative regulator Sab (Matsushita et al., 1998). Data show that the SH3 domain can also bind the proline rich region of the TH domain in an intra- or intermolecular fashion (Hansson et al., 2001b; Laederach et al., 2002; Okoh and Vihinen, 2002; Patel et al., 1997).

Recent data also show that deletion of the SH3 domain in Btk results in accumulation of the protein in the nucleus (Mohamed et al., 2000).

The SH2 domain

The SH2 domain was first identified in the retroviral oncoprotein v-Fsp as an approximately 100 amino acid region. A related sequence was later found in other proteins, including Src, leading to the introduction of the "Src homology" nomenclature and SH2 domain (SH1 being the catalytic kinase domain) (Czernilofsky et al., 1980; Sadowski et al., 1986). The function of an SH2 domain is to bind phosphorylated tyrosine residues (pY) (Moran et al., 1990). A stretch of eight amino acids in the SH2 domain, containing the amino acid FLVR motif, is essential for recognition of pY (Hidaka et al., 1991) and the sequence surrounding the pY influences specificity in binding the SH2 domain (Songyang et al., 1993). The SH2 domain in Btk has so far only shown to interact with the tyrosine phosphorylated adaptor protein B-cell linker/SH2-containing leukocyte protein of 65 kDa (BLNK/SLP-65) in B-cells (Hashimoto et al., 1999; Su et al., 1999). This interaction is required for the activation of phospholipase Cγ (PLCγ) (Su et al., 1999).

The catalytic domain

The largest domain in Btk is the catalytic domain. This domain is also referred to as the kinase domain or rarely as the SH1 domain. The kinase domain is highly conserved among protein tyrosine kinases. The common features of the kinase domain are that the approximately 250 amino acids are organized into two lobes with the ATP binding site and catalytic site in between. Characteristically, kinase domains also have conserved tyrosine residues located in a so-called activation loop close to the catalytic site. These tyrosine are phosphorylated upon activation (al-Obeidi et al., 1998). The phosphorylation that activates Btk takes place on tyrosine 551 (Y551) and is mediated by SFK (Mahajan et al., 1995; Rawlings et al., 1996). The crystal structure of the Btk kinase domain also shed some light into the role of tyrosine phosphorylation of the conserved residue in the kinase domain during activation (Mao et al., 2001).

The kinase domain in Btk has been shown to interact and phosphorylate the transcription factors BAP-135/TFII-I (Egloff and Desiderio, 2001) and STAT5A (Mahajan et al., 2001). In addition to its role as a catalytic domain, it can function as a protein-binding domain. The kinase domain contains a caveolin-binding motif that is conserved in the Tec family. Both Btk and Bmx were found to associate with caveolin *in vivo*, leading to

a reduction of Btk tyrosine phosphorylation (Vargas et al., 2002). The G protein βγ subunit has an enhanced effect on Btk activity when interacting with the catalytic domain (Lowry and Huang, 2002). In concert with the PH domain it binds the Fas cell death receptor, protecting the cells from Fas induced cell death or apoptosis (Vassilev et al., 1999).

Btk homologue in other species

Proteins homologues to Btk have also been found in non-mammalian species (Smith et al., 2001). The Btk protein in the fruit fly (*Drosophila melanogaster*) has been shown to be important for embryonic development and adult reproduction (Baba et al., 1999a; Guarnieri et al., 1998; Roulier et al., 1998). Btk homologues have also been identified in the skate fish *Raja eglanteria* (Haire et al., 1997), zebra fish *Brachydanio rerio* (Haire et al., 1998), Japanese pufferfish *Fugu rubripes* (AJ290422, Goode, D and Elgar, G. unpublished), chicken *Gallus gallus* (Takata and Kurosaki, 1996) and sea urchin *Anthocidaris crassispina* (Sakuma et al., 1997). These Btk homologues have not been analyzed further.

Kinases

Cellular responses such as cell survival, proliferation, differentiation and apoptosis or programmed cell death are dependent on intracellular signaling. Protein phosphorylation for cell signaling started with the finding that reversible phosphorylation can alter the activity of glycogen phosphorylase, almost 50 years ago (Krebs and Fischer, 1962). The first tyrosine phosphorylation was described in 1979 when the immunoprecipitate of polyoma T antigen contained phosphorylated tyrosine (Eckhart et al., 1979) and the protein tyrosine kinase activity was discovered to be associated with the translational product of the Rous sarcoma virus, *v-src* (Hunter and Sefton, 1980; Sefton et al., 1979). Molecules with tyrosine kinase activity are either receptor proteins (membrane bound) or cytoplasmic (intracellular or non-receptor) proteins (Hunter, 2000).

Btk in signal transduction

Activators

Btk can be activated by a large number of receptors. Of major interest is its involvement in B-cell receptor (BCR) signaling. Engagement of the BCR initiates a series of phosphorylation events and activation of proteins, with Btk as one of the targets for

phosphorylation and activation (Aoki et al., 1994; de Weers et al., 1994; Saouaf et al., 1994). Btk can also be activated through the high affinity IgE receptor (FcεR) on mast cells (Kawakami et al., 1994), B-cell co-stimulatory receptor CD19 (Kitanaka et al., 1998), B-cell CD72 (Venkataraman et al., 1998) as well as by gp130 (Matsuda et al., 1995) and the receptors for the interleukins IL-3 (Deng et al., 1998) and IL-5 (Sato et al., 1994). CD40 (Brunner et al., 2002), CD38 (Kikuchi et al., 1995), CD32 (Oda et al., 2000) and G-protein coupled receptors (GPCR) have also been demonstrated to activate Btk (Bence et al., 1997; Jiang et al., 1998; Langhans-Rajasekaran et al., 1995) Activation and translocation of Btk to the cell membrane have recently been shown when cells are stimulated with insulin, IGF-1, EGF, SDF-1α, anti-IgE and anti-IgM (Nore et al., 2000).

Activation mechanism

Just like other kinases, phosphorylation of the activation loop in the kinase domain is needed for activation. Results from several studies show that tyrosine 551 in the kinase domain is phosphorylated by SFKs, resulting in partial activation of Btk, which is followed by autophosphorylation of tyrosine 223 in the SH3 domain (Afar et al., 1996; Park et al., 1996; Rawlings et al., 1996). Phosphorylation of the two regulatory tyrosine residues, together with association of the PH domain with phosphoinositides in the cell membrane (Li et al., 1997), results in full activation of the protein (Figure5).

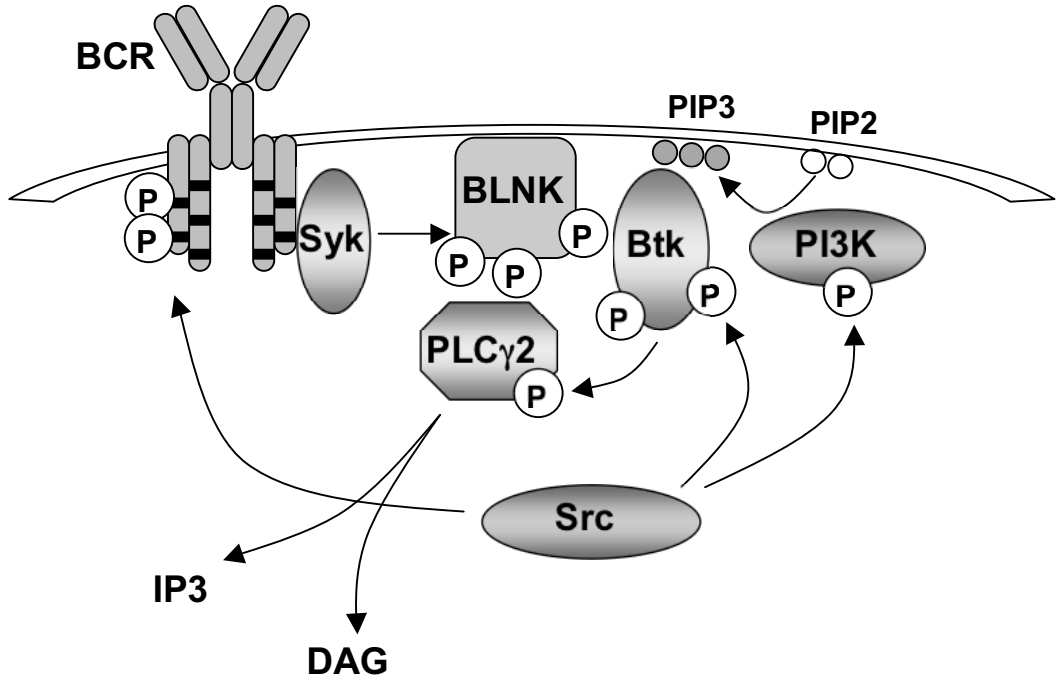

Figure 5. A schematic model of the activated BCR signalosome.

In the inactive state, Src kinases form a closed conformation with intramolecular interactions. Multidomain constructs of Btk can also undergo these kind of interactions, which could affect activity (Laederach et al., 2002). However, a recent report of the inactive, non-phosphorylated full-length Btk structure, indicated a linear arrangement of the domains in Btk and not as in the Src molecule (Marquez et al., 2003).

The BCR signalosome

As described previously, Btk associates with a large number of proteins. Probably the best documented downstream function of Btk is the activation of PLCγ and the subsequential regulation of intracellular Ca^{2+} release downstream of the B-cell receptors (BCR). The first step in antigen signaling is the engagement of B-cell receptor following activation of SFK and subsequent tyrosine phosphorylation of PI3K and the BCR, the latter leading to interaction and activation of Syk. Activated Syk kinase phosphorylates the downstream target BLNK/SLP-65 (Fu et al., 1998), enabling a complex of BLNK/SLP-65, PLCγ, Vav and Grb2 to form in B-cells. Activated PI3K produces PIP3, the principal lipid ligand for Btk. PIP3 targeted Btk is fully activated by phosphorylation of SFK (Kurosaki et al., 1994) and associates with BLNK/SLP-65 through its SH2 domain (Hashimoto et al., 1999). Tyrosine phosphorylated BLNK/SLP-65 recruits Btk and PLCγ2 and brings them close to each other so that Btk can activate PLCγ2 by phosphorylation (Watanabe et al., 2001). Formation of this signaling complex is critical for the activation of PLCγ2, which catalyses the hydrolysis of PIP2 to the second messengers IP3 and DAG.

IP3 functions as a major mediator of Ca^{2+} mobilization from cytoplasmic storage vesicles inside the cell, while DAG is a well established activator of protein kinase C (PKC) (Kurosaki and Tsukada, 2000). IP3 binds to the ion channel IP3-receptor in the membrane of intracellular Ca^{2+} stores in the endoplasmic reticulum, causing a release of Ca^{2+} to the cytoplasm. The Btk deficient DT40 chicken B-cell line exhibits a reduction in PLCγ2 phosphorylation upon BCR stimulation and a consequent failure to mobilize Ca^{2+} and generate IP3 (Takata and Kurosaki, 1996). Overexpression of any of the Tec family members can overcome this defect (Fluckiger et al., 1998; Tomlinson et al., 1999). This suggests that PLCγ2 is a general target for Tec kinases. While Itk physically interacts with PLCγ2 (Perez-Villar and Kanner, 1999), Btk and maybe other Tec family kinases phosphorylate PLCγ2, causing its activation (Rodriguez et al., 2001; Watanabe et al.,

2001). Supporting these findings, BCR stimulated B-cells from XLA patients did not induce Ca^{2+} mobilization (Genevier and Callard, 1997).

Activation of Akt (or protein kinase B, PKB) by the BCR in B-cells, is regulated by Btk and Syk (Craxton et al., 1999), and in mast cells, Akt positively regulates IL-2 transcription (Kitaura et al., 2000). Btk has been shown to interact with Akt and induce its phosphorylation (Lindvall and Islam, 2002).

Effects of calcium

The divalent cation calcium (Ca^{2+}), is used by cells to regulate many of their activities. It activates a wide range of Ca^{2+} sensitive regulatory enzymes. For example, transcription factors that control gene expression and that are activated through the Ca^{2+}-binding protein Calcineurin, respond not only to a certain Ca^{2+} concentration, but also to particular oscillation frequencies (Dolmetsch et al., 1998; Li et al., 1998). The serine/threonine phosphatase Calcineurin dephosphorylates the protein nuclear factor of activated T-cells (NFAT) (Good et al., 1997), resulting in activation and nuclear entrance where it promotes transcription of the IL-2 receptor gene (Beals et al., 1997; Jain et al., 1993). Calmodulin is another Ca^{2+}-binding protein that serves as a regulatory subunit to other enzymes when bound to Ca^{2+}. Calmodulin regulates proteins involved in cyclic nucleotide metabolism, cytoskeleton regulation, Ca^{2+} transport, protein phosphorylation and protein dephosphorylation, resulting in muscle contraction, secretion, metabolism, neuronal excitability and proliferation (Clapham, 1995).

Regulation of transcription

In B-cells lacking Btk, it was found that when activated through the BCR the cells went into apoptosis, indicating a role of survival in B-cells (Anderson et al., 1996). Two research groups independently reported activation of the nuclear factor kappa B (NF-κB) through Btk after BCR stimulation (Bajpai et al., 2000; Petro et al., 2000). NF-κB is an important factor regulating genes involved in cell proliferation and survival (Khan, 2001). Investigation using B-cells showed that BLNK and PLCγ2 were necessary for activation of NF-κB (Petro and Khan, 2001; Tan et al., 2001). Btk was demonstrated to activate *bcl-x* gene promoter through PLCγ2 and NF-κB (Petro et al., 2002). The Bcl-X_L protein is the long isoform of the B-cell cll (chronic lymphocytic leukemia)/lymphoma 2 (Bcl-2) family

of anti-apoptotic proteins (Hussein et al., 2003). Activation of NF-κB results in transcription of other genes (Chen et al., 2001).

It has been found that Btk also can activate other transcription factors. A protein of 135 kDa was identified to interact with the PH domain of Btk in activated B-cells and named BAP-135 (Yang and Desiderio, 1997). BAP-135 binds to DNA and it was later found to be the ubiquitously expressed transcription factor TFII-I (Perez Jurado et al., 1998). Activation of BAP-135/TFII-I by Btk induces nuclear accumulation of BAP-135/TFII-I driving transcription (Novina et al., 1999).

Btk has been shown to associate with the transcription factor Bright (B cell regulator of immunoglobulin heavy chain transcription). Together with Bright, Btk was found in the nucleus of activated cells (Webb et al., 2000). Association of Btk is necessary for Bright to bind DNA (Webb, 2001). At the time, Btk had also been shown to shuttle between the cytoplasm and the nucleus (Mohamed et al., 2000).

Cytoskeletal regulation

Btk is not only involved in the activation of PLCγ, regulation of calcium mobilization and activation of transcription. Studies have shown that Btk also can be involved in cytoskeleton regulation. The first indications that Btk may contribute to actin cytoskeletal regulation came from work with the *Drosophila* TFK Btk29A (formerly Src29A or Tec29A), which is required for growth of ring canals (actin-based intracellular bridges between nurse cells and oocyte). A similar phenotype is also observed in the Drosophila lacking the Src homolog Src64, which interacts with Btk29A and regulates its localization (Guarnieri et al., 1998; Roulier et al., 1998).

In mammalian cells, Btk has been shown to interact with Vav and Wiskott-Aldrich syndrome protein (WASP), two molecules involved in actin reorganization. Vav is a member of the guanine nucleotide exchange factors (GEF) that regulate the exchange of GDP to GTP to activate the Rho family members of small GTPases, which are key components in actin reorganization (Bustelo, 2001). The Wiskott-Aldrich syndrome is associated with defective actin cytoskeleton regulation. WASP can serve as a substrate for Btk (Baba et al., 1999b; Guinamard et al., 1998) and activated Cdc42 interacts with WASP, facilitating the activation of Arf2/3 complex, which nucleates new actin filaments (Snapper and Rosen, 1999). F-actin has been shown to interact with the PH domain of Btk (Yao et al., 1999). Stimulation of cells with different stimuli leads to translocation of Btk to membrane ruffles, regions of the cell membrane formed by actin polymerization. It was also shown that Btk induces the formation of lamellipodia through Rac1 and Cdc42 (Nore

et al., 2000). The mitogen activated protein kinase (MAPK) JNK (Janus kinase) is activated by Rho members, resulting in gene transcription. Activation of Btk by the Fcε receptor on mast cells, mediates JNK activation through Rac1 but also PLCγ2 following transcription of the IL-2 gene in mast cells (Hata et al., 1998; Inabe et al., 2002; Kawakami et al., 1997).

Downregulation of Btk activity

The negative regulation of Btk is thought to take place in different ways. Activated lipid phosphatase SH2-containing inositol 5'-phosphatase (SHIP), prevents the recruitment and activation of Tec family members following PLCγ activation by converting the PH domain binding substrate PIP3 to PIP2 (Bolland et al., 1998; Scharenberg et al., 1998). Similar effects were also seen on Itk, the Btk family member in one T-cell line designated Jurkat, where the inositol 3'-phosphatase PTEN is mutated (Shan et al., 2000). Certain proteins exhibit a negative regulatory effect on Btk. The inhibitor of Btk (IBtk) and Bam11 binds to the PH domain and Sab interacts with the SH3 domain in Btk. The interaction of these proteins interferes with Btk activation and Btk-dependent downstream events like Ca^{2+} mobilization (Kikuchi et al., 2000; Liu et al., 2001; Yamadori et al., 1999). Exactly how these proteins inhibit Btk activity is still not known. Association of different PKC isoforms with the PH domain of Btk has also been known to decrease Btk kinase activity (Johannes et al., 1999; Yao et al., 1994). Recently, it was shown that, in B-cells and mast cells, PKCβ phosphorylated serine 180 in the TH domain of Btk. This alters the membrane localization and presumably also its activity (Kang et al., 2001). PKC is activated by the second messenger diacylglycerol (DAG) that is produced by PLCγ, and which is activated by Btk. This mechanism provides an autoinhibitory function that turns off the enzymatic activity of Btk following PLCγ activation. A third way to inhibit Btk activity is to dephosphorylate tyrosines 223 and 551 in Btk. The specific phosphatase or phosphatases for these tyrosines is not yet known although there is an indication that SHP-1 might be involved in this process (Maeda et al., 1999).

Two chemical compounds have been developed to inhibit Btk catalytic activity for studies *in vitro*. LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)propenamide] and the quinone epoxide antibiotic, terric acid, inhibit the enzymatic function of Btk (Mahajan et al., 1999), while terric acid also blocked the interaction with PKC (Kawakami et al., 1999).

AIMS OF THE PRESENT STUDY

Btk was identified to be the crucial for proper B-cell development, and mutations in the gene cause XLA in humans. Understanding the regulation, functions and structure of the protein has proceeded rapidly from the time the gene was discovered with about 500 articles in the subject. Phosphorylation and protein-protein interactions are key events for protein function, where the individual domains play a central role.
To extend the known knowledge we focused on the function of the different domains.

The specific aims were:

- To study how XLA-causing mutations affect the function and structure of Btk domains
- To study activation mechanisms of Btk
- To investigate the tyrosine 223 phosphorylation site in the SH3 domain of Tec family members
- To try to identify new constitutively active forms of Btk

COMMENTS ON METHODOLOGY

Circular dichroism analysis

Circular dichroism spectroscopy (CD) is a technique valuable for analyzing the composition of the secondary structural elements of a protein in solution. The CD is a phenomenon that results when chromophores in an asymmetrical environment are exposed to polarized light. In proteins the major optically active groups are the amide bonds of the peptide backbone and the aromatic side chains. Proteins or polypeptides have regions where the peptide chromophores are in highly ordered forms, such as the secondary structures α-helices and β-sheets. These regions form characteristic CD spectra. Data are normally collected from a sample exposed to light of between 195 and 260nm and processed with different computer programs, using different calculation methods (Greenfield, 1996).

Transfections

In the articles, different protocols for transferring genes into cells (transfection) have been used. From the original calcium phosphate precipitation (Chen and Okayama, 1987), to the cation lipid FuGene6 (Roche) and the easy and inexpensive polyethylene imine (PEI) reagent. PEI cation has a high buffering capacity, which leads to lysosomal swelling and eventual disruption and release of the PEI/nucleic acid complex into the cytoplasm (Boussif et al., 1995). DNA is mixed with a ratio of 2 μg with 1.2 μl of 0.1 M of 25 kDa PEI in water.

Green fluorescent protein

The green fluorescent protein (GFP) contributes to the luminescent light of the jellyfish *Aequorea victoria*. The GFP protein is a unique molecule of light-emitting proteins because it does not need any cofactors or substrates for the generation of light. In jellyfish, GFP is activated by an indirect energy transfer from another bioluminescent protein, the calcium dependent aequorin. Purified GFP or expressed in cells can be activated by the energy transfer from ultraviolet light exposure (Misteli and Spector, 1997). The gene for GFP was cloned in 1991 (Prasher et al., 1992), and the 27 kDa protein is today used in several applications for biological research.

The human *BTK* was cloned in frame with the *gfp* gene. Transfected into cell lines, the Btk-GFP fusion protein can be studied for localization in the cell with fluorescent microscopy. It was also tested for kinase activity to verify its functionality (Nore et al., 2000). Full length Btk or the PH domain of Btk fused to GFP have successfully been used in several articles (Bäckesjö et al., 2002; Bobe et al., 2001; Hamman et al., 2002; Heinonen et al., 2002; Kikuchi et al., 2000; Lindvall and Islam, 2002; Marshall et al., 2001; Mohamed et al., 2000; Nore et al., 2000; Varnai et al., 1999).

Cell proliferation assay

WST-1 reagent from Roche was used to determine the proliferation and viability of cells. WST-1 is a colorimetric reagent that is based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases to formazan in viable cells. WST-1 is mixed with the cells and incubated for 30 minutes to 4 hours depending on cell line. The absorbance is measured at 450 nm.

RESULTS AND DISCUSSION

Paper I

Src homology 2 (SH2) domains are important for protein-protein interactions, with the feature to recognize and bind phosphorylated tyrosine (pY) residues. Twenty missense mutations causing XLA had been found in the SH2 domain when the analysis took place. Today, 25 XLA-causing amino acid substitutions have been identified. We wanted to study how six of these missense mutations affect the structural and functional properties of the protein. The six mutations are: G302E, R307G, Y334S, L358F, Y361C, H362Q, and one nonpatient mutation C337S, which enhances protein stability. In this study we also identified a novel missense mutation H362R in the SH2 domain, causing classical XLA.

The SH2 domains were expressed as glutathione S-transferase (GST) fusion proteins, and the GST-part was later removed by thrombin digestion. Expression of the mutant SH2-GST was only possible at the lower temperatures, 16 and 22°C, with reduced levels and altered solubility. The recombinant SH2 domain did to some extent aggregate. The mutant C337 to serine was created to remove the only cysteine in the SH2 domain that could form disulfide bridges with other cysteines (Darby and Creighton, 1995). The protein yield and solubility was the same as with the native SH2 domain. Furthermore, it had no tendency to dimerize. The SH2 polypeptides were tested for the ability to bind phosphotyrosine. Only the native SH2 domain retained the binding capacity to the pY-column, while the mutants showed binding of less than 10 percent (Table I). The Circular dichroism (CD) spectrum showed the typical pattern of an $\alpha+\beta$ protein, which is what SH2 domains are known to be (Greenfield, 1996). Comparing the native SH2 domain spectra with the mutant domain, it was clearly visible that the native Btk and the R307G were identical. Y361C had minor changes in the conformation, while the other mutations differed greatly from the wild type.

Together, these results suggest that mutation of the conserved arginine in the FLVR motif that is essential for pY binding (Hidaka et al., 1991), alters the affinity for pY binding and not the structural conformation. In the model Y361 is located adjacent to the hydrophobic pY-binding site. The mutant Y361C did not bind to pY and the CD spectra indicated a minor change in the secondary structure. Thus, these mutants could be described as a functional mutations or structural-function mutation in the case of Y361C. The other five XLA-causing mutations have major changes in the CD analysis

Table I. Solubility and pY-binding assays of the Btk SH2 domain

SH2 Protein	Relative Solubility of SH2 Proteins		Protein Bound to pY-Sepharose (percentage ± SD)
	SH2-GST fusion	Digested with thrombin	
Native SH2	+++++	+++++	100 ± 1.2
G302E[b]	+++	++	13 ± 2.6
R307G[b]	+++++	++++	4.2 ± 0.1
Y334S[b]	+++	+	9.0 ± 2.0
L358F[b]	++++	+++	4.8 ± 0.6
Y361C[b]	++	++	1.0 ± 0.7
H362Q[b]	+++	+	1.5 ± 1.8
C337S[c]	—	+++++	137 ± 0.6

[a] The long-term solubility on a time-scale from several hours (+) to weeks (+++++) of the GST-fused or thrombin-digested SH2 domains.
[b] SH2 domain containing missense mutation causing XLA.
[c] C337S was not made as a GST-fusion construct.

in combination with an altered phosphotyrosine binding, indicating that these are structural mutations.

Mutation of arginine 307 in the SH2 domain of Btk has been shown to negatively regulate the sustained increase of intracellular calcium and phosphorylation of PLCγ following B-cell receptor stimulation (Fluckiger et al., 1998). Collectively, these data demonstrate that interaction of pY with the SH2 domain of Btk is necessary for proper B-cell differentiation.

Paper II

In an attempt to study activation mechanisms, we found that the tyrosine kinase c-Abl could phosphorylate Btk. Cellular Abl (c-Abl) has an SH3, SH2 and a kinase domain, just like Btk and Src. In addition, like Src c-Abl carries an amino terminal myristoylation signal, but contrary to Src, also a large carboxy terminal domain with various functions, such as a trimeric nuclear localization signal (NLS), a nuclear export signal (NES) and protein-protein interaction regions. The NLS and NES motifs are essential for the shuttling of c-Abl between the cytoplasmic compartment and the nucleus, to regulate the cell in growth and apoptosis depending on the localization (Smith and Mayer, 2002). The native c-Abl is normally not phosphorylated and tightly regulated with a low catalytic activity (Pendergast et al., 1991).

We utilized a form of c-Abl with two mutations, P242E and P249E, which deregulate the protein (Barila and Superti-Furga, 1998). This constitutively active form was used in co-transfection assays with Btk in human embryonic kidney 293T cells. This showed that in addition to an activated form of Src, which was known to phosphorylate Btk, c-Abl also has this capacity. To demonstrate that the effect of c-Abl was direct and not through Src, which is present in 293T cell, we mixed Btk and c-Abl in an *in vitro* kinase assay. When Btk autophosphorylation was blocked with the inhibitor LFM-A13, it was evident that c-Abl could directly phosphorylate Btk. By using the tyrosine 223 and 551 mutants in Btk, we clearly saw that it was the Y223 that c-Abl phosphorylated and not the Y551 which is the target of SFKs (Figure 6). We also found that the sequence surrounding tyrosine 223 had similarities with other known c-Abl substrates. Moreover, with immunoprecipitation followed by western blot analysis and confocal microscopy, we could demonstrate interaction and colocalization of Btk and c-Abl.

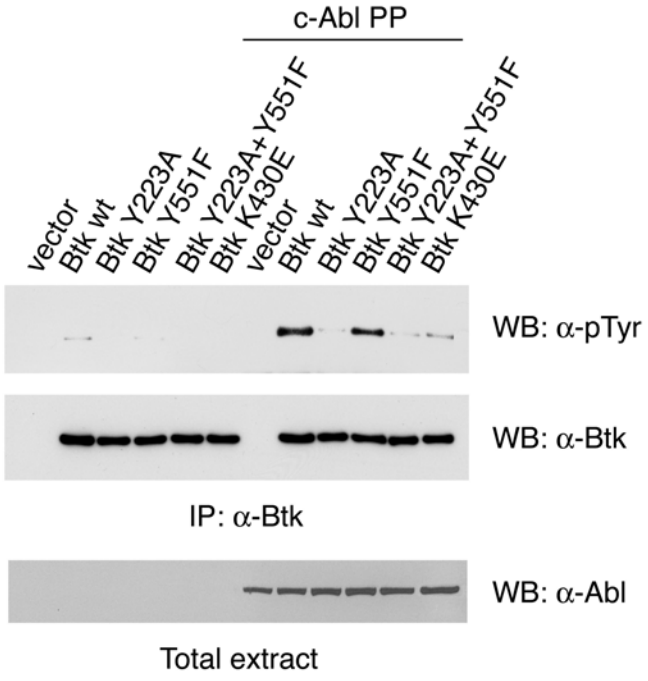

Figure 6. A Western blot analysis, showing tyrosine phosphorylation of Btk.

The function of the phosphorylated tyrosine 223 is not fully understood. However, purified Y223-phosphorylated SH3 domain, has been shown to interact with c-Cbl, but loses the binding with WASP and instead acquires a high affinity for activated Syk (Morrogh et al., 1999). Moreover, a Y223F mutant Btk expressed in B lymphoma, lacking Btk, had an altered Btk and PLCγ2 phosphorylation after B-cell receptor stimulation, and IP3 generation was reduced compared to cells expressing the wild type Btk (Kurosaki and

Kurosaki, 1997). Furthermore, it has been demonstrated that mutating Y223, or deleting the SH3 domain, in combination with the E41K activating Btk mutation, enhance transformation of fibroblasts, suggesting a negative regulatory role of the Y223 or the SH3 domain (Park et al., 1996).

Paper III

Btk can autophosphorylate the tyrosine 223 in the SH3 domain of Btk (Park et al., 1996). We wanted to identify a reliable substrate and examine the specificity of Tec family of tyrosine kinases. *In vitro* kinase (IVK) assays with purified, recombinant SH3 domains of Btk, Itk, Tec, Bmx, Grb2 and amphiphysin, were utilized to study the phosphorylation. Immunoprecipitated, endogenous Btk from a B lymphocyte cell line (Ramos) was subjected to an IVK assay with the SH3 domain of different proteins including Tec family members. The experiment showed that Btk preferentially phosphorylates its own SH3 domain, but also the SH3 domain of Bmx and to a much lower extent Itk. This was consistent with the achieved result when recombinant human full length Btk was used with the same conditions. The similar experiment using Itk from the T-cell line Molt-4, was preformed and demonstrated that the SH3 domain of Itk and Btk are good substrates for Itk. In cells expressing both Btk and Itk, such as mast cells, it is possible that they can regulate each other. Endogenous Tec protein from Ramos cells could phosphorylate the SH3 domain of Tec, Btk and to a reduced level Bmx and Itk. Since Tec has the ability to phosphorylate all the different SH3 domain of its family but not the SH3 domain of amphiphysin, and Btk mainly phosphorylates its own SH3 domain and Bmx, this suggests that Tec has broader substrate specificity, maybe related to its wider tissue expression pattern.

In order to characterize the phosphorylation site in the SH3 domains, we applied the two-dimensional tryptic phosphopeptide mapping using Hunter thin layer electrophoresis and chromatography to separate short phosphopeptides on thin layer chromatography cellulose plates (van der Geer and Hunter, 1994) followed by radio-Edman sequencing (Blume-Jensen et al., 1995). Comparing the radio-Edman sequencing results with a computer generated trypsin digest map, the phosphorylated tyrosine could be identified. In summary, we show that the Y223 in Btk was phosphorylated, confirming earlier studies (Park et al., 1996), and the Y180 in Itk, Y206 in Tec and both Y215 and Y223 in Bmx were phosphorylated (Figure 7). With these results we can exclude the possibility of Y225 in Btk and the conserved Y182 in Itk to be major phosphorylation sites. The Y180 in Itk corresponds to the Y223 in Btk. In Bmx, the phosphorylated Y215 corresponds to the

conserved Y223 in Btk. However, the Y223 in Bmx is not conserved among the TFKs. The Tec phosphorylation site Y206 is not the corresponding Btk Y223. Even if the tyrosine 215 in Tec is conserved in all SH3 domains, phosphorylation has not been reported at any of these sites (Figure 7). Moreover, with our data we can exclude the conserved Y188 (Y223 in Btk) in Tec as a target for phosphorylation by the TFKs. However, this site could possibly be a target for other tyrosine kinases.

Figure 7. Sequence alignments indicating phosphorylation sites (black box) in the Tec family SH3 domains.

From the existing structures, we found that the Y206 in the Tec SH3 domain is positioned at the same side as Y223 in Btk. The SH3 models show also that the pY site is located on the same surface. This argues for that these conserved tyrosines define a common binding surface for a specific interaction. Different Tec kinases are expressed in the same cell type, such as Btk and Tec in B-cells and Itk and Txk in T-cells. Interestingly, the Btk and Tec double knockout mice show a more severe B-cell deficiency than the Xid phenotype (Ellmeier et al., 2000). Similar effect has been shown in the Itk and Txk double knockout animal, where the phenotype is more severe compared to the Itk deficient mice (Liao and Littman, 1995; Liu et al., 1998; Schaeffer et al., 1999).

Paper IV

The pleckstrin homology (PH) domain is necessary for the native Btk protein to target the phospholipids in the cell membrane. It binds specifically to PIP3. From a random mutagenesis scheme, a gain-of-function, E41K, mutation was identified in the PH domain of the Btk protein. We were interested in whether amino acids in the neighbouring area of this mutation had similar gain-of-function effects as E41K. The E41K mutation enhances the positive charge of the lipid binding region, so in a systematic approach, we changed the glutamic acid 41, 45 and the aspartic acid 43 to lysine or arginine. These Btk mutants were fused with GFP, and studied in the human HeLa cervical carcinoma cell line. The typical cytoplasmic distribution of Btk-GFP seen in other cell lines was observed with the wild

type protein, while the PH domain mutants showed microspike structure formation at the cell membrane, induced with different strength depending on mutation, except for D43R, which showed a unique pattern not detected with the other mutants. In Cos-7 cells, similar structures were observed with Btk E41K and active Rif and Arf6 (Brown et al., 2001; Ellis and Mellor, 2000; Nore et al., 2000). Dominant negative form of Arf6 or Rif siRNA did, however, not block the Btk E41K induced membrane protrusions.

The Btk PH domain mutants were further analyzed in the IL-5 dependent pro B-cell, Y16. The Btk-GFP distribution of all mutants in Y16 cells, where evenly distributed in the cytoplasmic compartment. Increased acidifications of some of the proliferating mutants draw our attention to the study of cell proliferation. With the WST-1 reagent we studied how much 10^4 cells /100µl proliferated in normal RPMI growth medium. The E41K, D43R and the double mutant E41K+E45R indicated a faster proliferation by the colorimetric measurements compared to the wild type and the other mutants. Y16 cells with these mutants could also grow in the absence of IL-5 (Figure 8). Indicating a transforming capacity. Interestingly, since the E41K and the D43R mutant enhance proliferation and E45 mutants do not, this might suggest that the E45 residue is located too far away from the region of lipid binding. Moreover, since the E41+E45 double mutants have an increased proliferative effect, whereas the E45 single mutant does not, the overall structural conformation of the PH domain with an E45 mutation is probably not altered. The enlarged electrostatic polarization that E41K, and maybe D43R, contribute with seems to increase the affinity for PIP2 and PIP3.

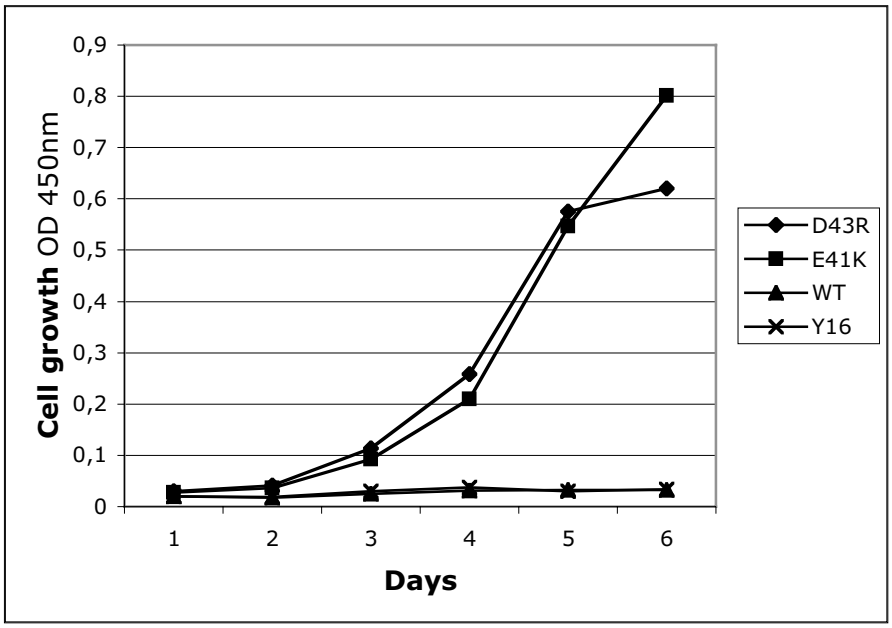

Figure 8. Growth curve of Y16 cell lines expressing different forms of Btk-GFP in absence of IL-5.

CLOSING REMARKS

Molecular functions of Btk have been studied in this thesis. Three of the five domains in Btk have been analyzed to answer different questions, such as: How does an XLA-causing mutation affect the SH2 domain? or How can we make a constitutive active form of Btk? Questions have been answered, but new ones were brought up and these need to be solved with future experiments. For example the mechanism behind the formation of microspike formation in HeLa and which proteins are involved in the transformation of Y16 cells with constitutive active forms of Btk. In order to understand the mechanism of regulation, it would be necessary to find out more about the structural constrains and which phosphatase, or phosphatases, that are involved in the down regulation of Btk activity. Phosphorylation on the tyrosine 551 in the regulatory loop is critical for the activation of Btk. Exactly how the phosphorylated tyrosine 223 regulates Btk needs to be thoroughly investigated.

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