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Subcellular Localization
and Signaling of Bruton’s
Tyrosine Kinase (Btk)

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In loving memory of my mother

Leontina Vallejo-Ospina

Sciences knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.

– Louis Pasteur, 1876 –
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ABSTRACT

Bruton's tyrosine kinase (Btk) is a non-receptor tyrosine kinase related to the Src family of kinases. Mutations in various parts of the gene have been shown to cause X-linked agammaglobulinemia (XLA), a primary immunodeficiency in humans, characterized by a defect in B-cell development. XLA patients lack B-cells and consequentially have very low levels of immunoglobulins in their serum. Thus, these patients suffer from an increased susceptibility mainly to extracellular bacterial infections.

The molecular mechanism(s) underlying Btk localization, activation and signaling are not fully understood. We analyzed the subcellular localization of Btk employing a recombinant chimeric Btk fused with the Green Fluorescent Protein (GFP) with subsequent analysis of images using digital confocal microscopy. Different biochemical protein analyses were also performed.

During this study we have found that Btk can translocate to the plasma membrane of living cells and play an important role as a potent inducer of cytoskeletal reorganization resulting in membrane ruffle formation. Moreover, we found that Btk can translocate to the nucleus and that Btk utilizes functional CRM-1 dependent nuclear export signal(s) to shuttle between the nucleus and the cytoplasm. We also found that Tec family kinases bind to caveolin-1, a major structural component of caveolae (rafts or microdomains) located in the plasma membrane. Finally, we demonstrate that Cbl acts as an E3-ubiquitin ligase for Btk and that ubiquitinated Btk is targeted for proteasomal degradation when Btk is expressed at high levels. Furthermore, upregulation of the small-ubiquitin-related-modifier (SUMO-1) downregulates Btk.

In conclusion, the subcellular localization of Btk has implications regarding cytoskeletal regulation and/or potential targets inside the nucleus, which may be of relevance for B-cell development and differentiation. Also, Cbl-dependent ubiquitination as well as sumoylation are likely to provide a deeper insight into the negative regulation of Btk-mediated cell signaling.

Keywords: B-cells, Bruton’s tyrosine kinase, X-linked agammaglobulinemia, pleckstrin homology domain, cytoskeleton, nucleocytoplasmic shuttling, caveolin-1, ubiquitin, SUMO-1.
LIST OF PUBLICATIONS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BH</td>
<td>Btk homology</td>
</tr>
<tr>
<td>BLNK</td>
<td>B-cell linker protein</td>
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<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>Cav-1</td>
<td>Caveolin-1</td>
</tr>
<tr>
<td>CRM1</td>
<td>Chromosome region maintenance 1</td>
</tr>
<tr>
<td>CT-B</td>
<td>Cholera toxin B</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>GEM</td>
<td>Glycolipid-enriched-microdomain</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol(1,4,5)-triphosphate</td>
</tr>
<tr>
<td>MoAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal(s)</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal(s)</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol(3,4,5)triphosphate</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13 acetate</td>
</tr>
<tr>
<td>PR</td>
<td>Prolin rich sequences</td>
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<tr>
<td>PTK</td>
<td>Protein Tyrosine Kinase</td>
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<tr>
<td>SH</td>
<td>Src homology</td>
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<tr>
<td>SUMO-1</td>
<td>Small-ubiquitin-related-modifier</td>
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<tr>
<td>TH</td>
<td>Tec homology</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>XID</td>
<td>X-linked immunodeficiency</td>
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<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
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INTRODUCTION

Signal transduction pathways and their relevance to the development of lymphocytes has become a major focus of a myriad of laboratories. There is considerable interest in the Tec family of kinases because many members of this family are of critical importance during antigen-receptor signaling and lymphocyte development. The first Tec kinase to be linked to antigen receptor signaling was Bruton’s tyrosine kinase (Btk), which represents the prototypic family member in this regard. Depending on intracellular localization and translocation, Btk is likely to participate in the formation of numerous types of complexes important in a variety of signaling events regulating B-cell functions.

X-linked agammaglobulinemia (XLA)

XLA was the first primary immunodeficiency described in 1952 by Dr. Ogden C. Bruton (Bruton, 1952). Mutations in every part of the human Btk gene have been shown to lead to XLA with the severity of the disease being independent of the mutation (Futatani et al., 1998; Sideras and Smith, 1995; Vihinen et al., 1997). XLA is characterized by an increased susceptibility to infections beginning most often during the first year of life when transferred maternal Ig has been catabolized. Analysis of serum demonstrates a pronounced decrease in the level of all Ig isotypes. IgA is usually undetectable (Ochs and Smith, 1996). There is a virtual absence of humoral response to recall antigens. B lymphocyte and plasma cell numbers are markedly decreased, whereas T lymphocytes subsets show a relative increase. The defect is caused by an arrest in B-cell differentiation (Figure 1), distinguishing XLA from most other Ig deficiencies. B lineage cells in all organs are affected, resulting in a reduced size of lymph nodes and tonsils.
XID is caused by a single point mutation in the pleckstrin homology domain of murine Btk, a conserved arginine in position 28 is changed by a cysteine residue (Rawlings et al., 1993; Thomas et al., 1993). XID is relatively a mild immunodeficiency compared with XLA (Rigley et al., 1989; Wicker and Scher, 1986). Detailed analysis of the immune defects in XID show that only the peritoneal CD5 positive cells as well as the IgM and IgG3 subfractions of antibodies are absent or markedly reduced. In XID mice, responses to type II T cell-independent antigens exemplified by the trinitrophenyl-derivated Ficoll, are absent and B-cells cannot form multicellular colonies in response to certain mitogens (Rigley et al., 1989; Wicker and Scher, 1986). However, responses to T cell-dependent antigens are normal.

Mice bearing a homozygous mutation in the Btk gene (Btk−/−) have been generated replacing different parts of the gene (Hendriks et al., 1996; Kerner et al., 1995; Khan et al., 1995). Nevertheless, they all show the milder XID phenotype. It seems therefore that there are species-specific differences with regard to the function of Btk in human and mice. The ability of Tec to compensate for Btk may also explain phenotypic differences in XID mice compared with XLA patients (Ellmeier et al., 2000).

**Figure 1.** An overview of B cell differentiation and block in XLA patients. A partial block after pro-B cells and an almost complete block after pre-B cells result in reduced levels of B lymphocytes.

**X-linked Immunodeficiency (XID)**

Stem cell → Pro-B cell → Pre-B cell → B lymphocyte → Plasma cell
The *Btk* gene

*Btk* was identified in 1993 (Tsukuda *et al.*, 1993; Vetrie *et al.*, 1993). Simultaneously, two independent groups demonstrated that the *Btk* gene, encoding a novel cytoplasmic protein tyrosine kinase (PTK), was defective in XLA. The *Btk* gene was also the first cytoplasmic PTK implicated in a hereditary disease.

The *Btk* gene is localized in the 21.3-22 region of the long arm of the X chromosome (Xq). It is composed of 19 exons including a 5´ untranslated exon. Most exons are short, ranging between 55 and 217 nucleotides. Exon 19 is the longest, encompassing more than 500 nucleotides containing the end of the catalytic region of *Btk* as well as a 3´ untranslated sequence (Smith *et al.*, 1998; Smith and Notarangelo, 1997).

Expression of *Btk*

*Btk* is expressed in all hematopoietic cells including erythroid progenitors and myeloid cells (de Weers *et al.*, 1993; Lachance *et al.*, 2002; Smith *et al.*, 1994b; Vihinen and Smith, 1996). In spite of the demonstration that *Btk* is expressed outside the B-cell lineage there is no evidence indicating that a functional defect in non-B-cells contributes to the XLA phenotype. *Btk* is expressed from very early stages throughout B-cell development, but not *Btk* expression has been found in plasma cells (Figure 2) (Smith *et al.*, 1994a). Recently, it has been suggested that *Btk* dosage is dynamically regulated during B-cell development or functional responses. The earliest developmental stage, pro-B cells had a broader range of *Btk* expression than observed in cells of later stages. On the other hand, *Btk* levels are significantly lower in B-cells from peripheral sites, such as spleen, compared with marrow (Nisitani *et al.*, 2000).
Btk protein is composed of 659 amino acids with a total molecular weight of 77 KDa; the corresponding mRNA is normally found as a single species. (Rawlings et al., 1993; Smith et al., 1994b; Tsukuda et al., 1993). It belongs to the Tec kinases subfamily of Src-like kinase together with the homologs Tec, Itk, Bmx and Txk (Mano, 1999b; Smith et al., 2001; Yang et al., 2000). These kinases are distinguished by the presence of an N-terminal pleckstrin homology (PH) domain followed by a Tec homology TH, a Src-homology 3 (SH3), an SH2 domain and a C-terminal kinase domain (SH1) (Figure 3A). These unique features contribute to the regulation of these kinases through protein-protein and protein-lipid interaction and may help determine their varied functions in different signaling pathways. Numerous signaling molecules can interact with each of these domains (Takesono et al., 2002) (Figure 3B).

**Figure 2.** Expression pattern of Btk
The Tec family is the only tyrosine kinase family noticed to have a PH domain. The Btk-PH domain has 138 residues and has been crucial for elucidate the function of the domain (Vihinen et al., 1995b). The PH domain has been suggested to replace the function of myristoylation in membrane anchoring. Substitution of Btk-PH domain residue E41 by lysine was shown to increase phosphorylation on tyrosine residues and causes an increase in membrane targeting (Afar et al., 1996; Varnai et al., 1999). This could mean that Btk phosphorylation is linked to membrane interaction. Interestingly, expression of the constitutively activated Btk, E41K, blocks the development of follicular recirculating B-cells (Dingjan et al., 1998).

Biochemical analyses demonstrate that the PH domain of Btk preferentially binds to PtdIns(3,4,5)P3 and Ins(1,3,4,5)P4 (Kojima et al., 1997; Rameh et al., 1997; Salim et al., 1996). Individual point mutations within the Btk-PH domain can be classified as either structural or functional in the light of the three-dimensional structure and biochemical data. All functional mutations cluster into the positively charged end of the molecule around the predicted

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**Btk PH Domain**

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binding site for phosphatidylinositol lipids. It is likely that these mutations inactivate the Btk pathway in cell signaling capacity by reducing its affinity for insositol phosphates, which cause a failure in translocation of the kinase to the cell membrane (Hyvonen and Saraste, 1997; Yang et al., 2001). Recently, PtdIns(3,4,5)P3 binding of the PH domain was found to increase Btk kinase activity in vitro (Saito et al., 2001). This conserved domain can also bind to proteins, including heterotrimeric G-proteins subunits, isoforms of protein kinase C (PKC), transcription factors, F-actin, Vav, Fas, and focal adhesion kinase (FAK) (Jiang et al., 1998; Johannes et al., 1999; Ma and Huang, 1998; Qiu and Kung, 2000). Two recently identified inhibitors of Btk kinase activity, I-Btk and Bam11, interact with the PH domain of Btk (Kikuchi et al., 2000; Liu et al., 2001).

**TH (Btk homology and Prolin rich) Domain**

The Btk homology (BH) motif is characterized by a zinc-binding sequence with homology to Ras-GAP and is needed to maintain the structural integrity of the PH domain (Smith et al., 2001). The Btk motif has been shown to be essential for the binding of Btk PH domain to Gβγ (Jiang et al., 1998; Touhara et al., 1994). Tec kinases also possess one or two proline-rich sequences (PR), which were first shown to be binding partners for the SH3 domains of the Src kinases Fyn, Hck and Lyn (Alexandropoulos et al., 1995; Cheng et al., 1994). However, a second important regulatory function for the PR region has been suggested by demonstrating an intramolecular interaction between the PR and SH3 domains of Itk (Andreotti et al., 1997; Brazin et al., 2000; Laederach et al., 2002).

**Btk SH3 Domain**

The function of the Btk SH3 is unclear. Interaction with the proline-rich c-Cbl proto-oncogene has been identified (Cory et al., 1995). In addition, interaction of Btk SH3 with Cbl-b positively regulates Btk-mediated activation of PLCγ2 in B-cells (Yasuda et al., 2002). Other signaling molecules such as Sam68, Vav, EWS, WASP and Syk have been also described as targets for Btk-SH3
domain (Craxton et al., 1999; Guinamard et al., 1997; Morrogh et al., 1999).
There are no known missense mutations in this domain leading to XLA (Mattsson et al., 1996; Vihinen, 1996; Vihinen et al., 2001; Zhu et al., 1994).
Other mutations of the SH3 domain cause an increase in the transforming activity of Btk, which is consistent with an inhibitory interaction with the PR domain (Afar et al., 1996; Park et al., 1996). For Btk, autophosphorylation of a tyrosine residue (Y223) in the SH3 domain (Park et al., 1996) can change the affinity of the SH3 domain for certain binding partners and may relieve the interaction between the PR and SH3 domains (Morrogh et al., 1999). Btk activity is negatively regulated by SH3-binding protein of Sab (Matsushita et al., 1998; Wiltshire et al., 2002; Yamadori et al., 1999).

**SH2 Domain**

The SH2 domain is a protein interaction domain conserved among many signaling molecules that bind to phosphorylated tyrosine residues in the context of specific peptide sequences (Pawson and Gish, 1992). Most of the XLA-causing Btk-SH2 domain mutations disrupt pY peptide binding sites, but there are also some structural mutations (Haire et al., 1997; Saffran et al., 1994; Vihinen et al., 1995a; Vorechovsky et al., 1995). It has been reported that the proper binding of Btk to the B-cell linker protein (BLNK) by means of Btk-SH2 domain is necessary for the appropriate interaction of Btk and its effectors, such as PLCγ2 and Syk (Baba et al., 2001; Hashimoto et al., 1999).

**Btk Kinase Domain**

The catalytic domain of Btk consists of about 250 residues and a long linker that connects it to the SH2 domain. The catalytic region, which extends from residue 383 to the carboxyl-terminus, contains two sequence motifs (DLAARN, 521-526 and PVRW, 560-563) that distinguish protein-tyrosine kinases from protein-serine/threonine kinases. A putative autophosphorylation site is present at Y551, but like Itk and Tec, Btk differs from members of the Src kinase family in its lack of a negative regulatory phosphorylation site corresponding to Y527 of Src (Bjorge et al., 2000; Desiderio and Siliciano,
1994; Vetrie et al., 1993; Wahl et al., 1997). Phosphorylation at Y551 of Btk is mediated through the concerted actions of Src family protein kinases and is essential for BCR function (Kurosaki and Kurosaki, 1997; Mahajan et al., 1995).

**Mechanisms of Btk activation**

BCR cross-linking triggers a cascade of signaling events that culminate in the tyrosine phosphorylation of many PTKs, including the B-cell Tec family member Btk (Desiderio, 1997; Satterthwaite et al., 1998). A major biochemical landmark following BCR ligation or RTKs stimulation in eukaryotic cells in addition to the generation of second messengers, inositol(1,4,5)-triphosphate (IP₃) and diacylglycerol (DAG), is the increase in the intracellular levels phosphatidylinositol(3,4,5)triphosphate (PIP3). PIP3 is generated through PI3-kinase-dependent phosphorylation of PIP2, the major lipid in unstimulated cells (Mohamed et al., 1999; Satterthwaite and Witte, 2000). PIP3, as well as other lipids, bind efficiently to the PH domain of many proteins including Btk itselft (Rameh et al., 1997; Salim et al., 1996). This binding assists in the recruitment of Btk to the plasma membrane of the cell where it subsequently becomes activated.

A recent model to explain Btk activation after BCR cross-linking has been described (Kurosaki, 1999) (Figure 4). Both, Btk and Syk are phosphorylated by activated-Lyn in the activation loop (Y551 of Btk and Y519 of Syk). Activated Syk phosphorylates the recently identified B-cell specific adaptor molecule, BLNK/SLP-65 (Fu et al., 1998) and thereby brings BLNK to the plasma membrane fraction, eventually leading to co-localization of Syk, BLNK, and Btk. The membrane-associated Btk might be further recruited to phosphorylated BLNK with the help of the Btk SH2 domain. Then, phosphorylated BLNK brings PLCγ2 into close proximity of a Syk-BLNK-Btk complex and thereby facilitates the tyrosine phosphorylation and subsequent activation of PLCγ2.

Another Btk activation mechanism implicates that BLNK for mediating Syk-dependent activation. Activated Syk phosphorylates BLNK, and subsequent
the recruitment of Btk to phosphorylated BLNK enables Syk to phosphorylate Btk on its Y551 (Baba et al., 2001).

Engagement of the BCR and FcR are not the only direct way leading to the activation of Tec family kinases. Btk is also activated by a wide variety of other receptors (Kawakami et al., 1999), including FcεRI, G-protein-coupled receptors (GPCR), interleukin (IL)-3, IL5 and IL-6 (Kawakami et al., 1994; Reth and Wienands, 1997). In addition, cross-linking of the costimulatory surface molecule CD19 on B-cells with a monoclonal antibody (MoAb) induces tyrosine phosphorylation of Btk (Kitanaka et al., 1998).

![Figure 4. Model for Btk activation upon BCR engagement](image)

**Consequences of Btk activation**

Given the multi-modular structure of the Btk protein and the multitude of signaling molecules that can interact with it, it is anticipated that this kinase triggers multiple signal pathways and generates pleiotropic effects (Figure 5).
**Ca²⁺ influx**

Recent work has defined calcium flux following PLCγ2 phosphorylation and activation as a downstream biochemical signal from the BCR that is Btk dependent (Tomlinson et al., 2001). PLCγ2 is also regulated by other tyrosine kinases including Src family members and Syk (Mano, 1999a; Pillai and Moran, 2002; Rawlings, 1999). In a chicken B-cell lymphoma cell line deficient in Btk expression, there is a failure to sufficiently activate PLCγ2 and a lack of IP3 and DAG products previously shown to be associated with regulation of the early release of calcium from intracellular stores (Takata and Kurosaki, 1996). Consistent with this finding, human B-cells from XLA patients do not induce calcium flux in response to BCR-engagement. Ectopic expression of wild type Btk, Tec or Itk restores Ca²⁺ mobilization in these cells (Scharenberg et al., 1998; Tomlinson et al., 1999), indicating the presence of an *in vivo* “Tec kinases → PLCγ2 → calcium flux” signaling mechanism. It is, however, not yet known whether PLCγ2 is a direct cellular substrate of Btk.

**Cytoskeleton**

Reorganization of the actin cytoskeleton is critical for efficient antigen receptor signaling, during which multiple signaling molecules converge at the plasma membrane to form complexes required for transmitting signals to the nucleus (Acuto and Cantrell, 2000). Several emerging lines of evidence suggest that Tec kinases not only regulate PLCγ2 but also contribute to regulation of actin cytoskeletal rearrangements in response to antigen receptor and other signaling pathways (Takesono et al., 2002).

In mammalian cells, interactions between the actin cytoskeleton and Tec kinases have been suggested according to several experiments. Using a GST-PH domain fusion protein, F-actin was shown to directly associate with basic residues in the N-terminal PH domain of Btk (Yao et al., 1999). In platelets, Btk localizes to the cytoskeleton upon activation of the thrombin receptor (Mukhopadhyay et al., 2001). This effect is regulated by αIIbβ3 and depends on PI3K activity (Cantrell, 2001). Btk is also important for signaling
via the collagen receptor glycoprotein VI (GPVI) in platelets (Quek et al., 1998).

**Figure 5.** The signals transmitted by Btk

**Cell Survival**

B-cell activation through the surface IgM molecule induces the transcription of the \( Bcl-x_L \) gene, coding for a potent anti-apoptotic factor. In XID B-cells, however, IgM stimulation fails to activate the \( Bcl-x_L \) gene, and instead induces apoptosis (Anderson et al., 1996). Furthermore, XID B-cells present a defect in cyclin induction after activation with sIg, leading to defects in B-cell cycle entry (Brorson et al., 1997). More importantly, forced expression of Bcl-x\(_{L}\) partly rescues the immune defect in XID mice (Solvason et al., 1998). In addition, Btk suppresses the Fas-mediated apoptotic signal via the disruption of the FAS-FADD interaction in chicken B-cells (Vassilev et al., 1999).
Regulation of Apoptosis

It remains unclear whether Btk functions as pro- or anti-apoptotic factor in vivo. The latter possibility has been discussed in the previous section. In contrast, Btk may be indispensable to the radiation-mediated apoptosis mechanism in B-cells (Uckun et al., 1996). Similarly, the chicken B-lymphoma cell line, DT40, has been shown to undergo apoptosis in response to radiation. However, targeted mutation of the Btk gene, make these cells refractory to radiation, a resistance that was not achieved by disruption of the Lyn or Syk genes. In addition, Kawakami et al. demonstrated that mast cells prepared from XID or Btk−/− mice are resistant to the apoptosis triggered by cytokine deprivation (Kawakami et al., 1997). It was shown that Btk activates JNK in a Ras-dependent manner (Deng et al., 1998). These data lend credit for support a pro-apoptotic role for Btk. It is also possible that Btk has a dual function in apoptosis, with the fate of Btk-driven apoptosis signaling being context-sensitive and cell type-dependent (Islam and Smith, 2000).

Transcriptional activation

There are evidences that also support direct interplay between Tec kinases and transcription factors (Takessono et al., 2002). Yang and Desiderio (Yang and Desiderio, 1997) have attempted to purify tyrosine-phosphorylated proteins associated with Btk in activated B-cells. Molecular cloning of one such protein, pp135, revealed it to be identical to a DNA binding protein, later shown to be TFII-I (Grueneberg et al., 1997), an ubiquitously expressed multifunctional transcriptional factor. TFII-I/BAP-135 associates with the PH domain (probably also with the TH domain) of Btk, and becomes tyrosine-phosphorylated in vivo. Importantly, co-expression of Btk with TFII-I/BAP-135 induces the activation as well as nuclear import of the latter (Novina et al., 1999). TFII-I can bind to several promotor elements including the initiator (Inr) elements found in the promotor regions of VpreB, TdT and λ5. TFII-I was also shown to drive the expression of c-fos proto-oncogene.
Recent reports have nicely delineated the role of NFkB activation following BCR cross-linking and using Btk-deficient-cells, the involvement of Btk in this pathway was clearly demonstrated (Bajpai et al., 2000; Petro and Khan, 2001; Petro et al., 2000). Furthermore, interaction of Btk with the transcription factor, Bright, which has the ability to up-regulate Ig transcription in mature B-cell lines, has been described (Herrscher et al., 1995; Webb, 2001; Webb et al., 1998; Webb et al., 2000).

A growing body of evidence suggests that Btk signaling is propagated through the MAP kinase pathway (Kawakami et al., 1998; Lewis et al., 1998). This is supported by two recent papers showing that induction of cytokine gene expression by Btk in activated mast cells is mediated via the stress-activated protein kinases, JNK/SAPK (Hata et al., 1998a; Hata et al., 1998b). Btk activation has been also found to induce phosphorylation and transcription of the proto-oncogene c-Jun in response to receptor cross-linking in mast cells and the exposure of B-cells to ionizing radiation (Goodman et al., 1998; Hata et al., 1998a).
AIMS OF THE PRESENT STUDY

Since the discovery of the \textit{Btk} gene, much progress has been made in the elucidation of the structure, regulation, and function of the protein. Understanding the various mechanisms behind subcellular localization of Btk has been a target of this study. Localization of proteins to different cell compartments is often brought about by protein-protein interaction domains. Another major class of subcellular targeting domains binds specifically to lipid ligands in the cell membrane.

The specific aims have been:

- To visualize the subcellular localization of Btk using GFP chimeras of native and mutated forms of Btk.
- To analyze the role of Btk in regulating cell motility.
- To search for potential partners of Btk by sequence and biochemical analyses.
- To analyze the nature of Btk degradation in lymphoid and adherent cells.
COMMENTS ON METHODOLOGY

All the methods used in this thesis are described in detail in separate papers. Therefore, only certain general comments regarding the use of some of the methods are given below.

Use of the GFP

The green fluorescent protein (GFP) is responsible for the green bioluminescence from the jellyfish *Aequorea victoria*. The chromophore itself is formed by a Ser65-Tyr66-Gly67 tripeptide (Misteli and Spector, 1997; Tsien, 1998). Mutational analysis of this and other regions of the protein have produced “improved” GFP isoforms, one of which emits blue light instead green (Tyr66-His, Tyr145-Phe) (Heim and Tsien, 1996), a red shifted variant, and another isoform significantly brighter than the wild-type (Ser65-Thr, Phe64-Leu) (Cormack *et al*., 1996). GFP detection does not require fixation or permeabilization of cells. GFP is already established as an invaluable tool for many branches of biological research. In particular, GFP has been used to monitor protein localization and to visualize dynamic cellular events.

The Btk-GFP fusion protein

A fusion between Btk gene and GFP was produced by standard recombinant DNA techniques and was introduced into mammalian cells by transient transfection. The fate of the resulting protein was followed using confocal microscopy. Importantly, Btk-GFP fusion protein was carefully tested for its functionality; the large GFP-tag of 27 KDa did not affect the Btk kinase activity. In parallel, gain and loss mutations of Btk were also cloned into GFP construct and were included in this study as controls.
Confocal Microscopy and Imaging

Confocal microscopy (CM) has become a powerful tool for studying both the localization of cellular components and microorganisms, with the use of target-specific fluorescent probes and labeled antibodies in fixed and living cells. CM permits the visualization of fluorescence in a single plane of focus, creating a vastly sharper image. The sharper images can be obtained either by a laser-based pinhole confocal microscopy or by digital deconvolution (digital confocal) of CCD camera images (Figure 6) (Sheppard and Shotton, 1997). The images were acquired and analyzed using the image processing software Slide Book (Intelligent Imaging Innovations Inc, Denver, Colorado, USA). Out-of-focus information was removed through a mathematical process called deconvolution, which uses knowledge of collection optics to compute and subtract away the portion of the image that is out of focus. Images were post-processed and mounted in Adobe Photoshop 5.0 (Adobe Systems Inc, San Jose, California, USA).
Figure 6. Schematic diagram of a digital confocal microscopy imaging system
RESULTS AND DISCUSSION

Btk-GFP membrane translocation (Paper I)

We have visualized and analyzed the translocation of Btk–GFP fusion protein with scanning electron microscopy, immuno–electron–microscopy and confocal microscopy. Many of these data show that the redistribution of Btk could be modulated according to the external stimuli (serum starvation, growth factors and receptor–mediated stimuli), and was visualized through the different patterns of localization observed: diffuse pattern, patchy or punctuate pattern and the formation of membranes ruffles. The latter pattern of localization has capture our attention and we have considered possible interaction of Btk with some members of the cytoskeleton such as F–actin. The data obtained clearly shows that Btk co-localizes with this cytoskeletal protein (Figure.7) (Nore et al., 2000).

Figure 7. Btk-GFP visualization in membrane ruffles. (A) Untransfected cells. (B) Cells transiently transfected with Btk-GFP. Cells were serum stimulated during 1 hr before the F-actin staining with rhodamine-phalloidin.

To evaluate whether the activity of Btk contributed to the formation of ruffles or lamellipodia, a constitutively active version of Btk was used, E41K or Btk*. Accordingly, transient transfection of Btk*-GFP expression plasmids resulted in pronounced induction of lamellipodia, compared to mock or Btk-GFP wild type. Co-transfection studies using the Btk and Btk* together with the
dominant negative form of Rac and Cdc42 confirmed the role of Btk in the cytoskeletal reorganization.

The first suggestion that Tec kinases may contribute to actin cytoskeletal regulation came from studies of Drosophila Tec family kinase Tec 29 (corresponding to mammalian Btk), which is required for growth of ring canals (actin-bridges between nurse cells and the oocyte) (Guarnieri et al., 1998; Roulier et al., 1998). Further contributions of the Tec kinases to actin cytoskeletal regulation have been suggested from interactions of the kinases with Vav and WASP, two molecules involved in actin reorganization. Vav family members are guanine nucleotide exchange factors (GEFs) that facilitate the exchange of GDP for GTP to activate members of the Rho GTPase family such as Rho, Rac, and Cdc42, which are involved in actin cytoskeletal reorganization (Abe et al., 2000; Bustelo, 2001; Guinamard et al., 1998).

**Btk ruffle formation is PI3K dependent (Paper I)**

PI3K stimulation by e.g. growth factors can induce phosphorylation of Btk and PI3K inhibitors impair this process. Direct PI3K activation is sufficient to disrupt epithelial polarization and induce cell motility and invasion (Tapon and Hall, 1997). PI3K inhibition also disrupts actin structures, suggesting that activation of PI3K by Cdc42 and Rac1 alters actin organization, leading to increased motility and invasiveness. According to this and our present results, we suggest that Btk has an intrinsic ruffle–inducing capability, which could be potentiated by PI3K activation, and that the small GTPases act downstream of Btk (Figure 8) (Nore et al., 2000).

**Nuclear localization of Btk (Paper II)**

In this work we have extended our initial observations regarding the intracellular distribution of Btk to include events taking place in the nucleus. Our results show that Btk can indeed translocate to the nucleus of cells (Mohamed et al., 2000; Nore et al., 2000). This nucleocytoplasmic shuttling is sensitive to the exportin 1/CRM1-inactivating drug, leptomycin B, indicating that Btk utilizes functional nuclear export signals (NES) (Figure 9).
Figure 8. Model to explain the involvement of Btk in cytoskeleton reorganization.
The machinery used by Btk to get into the nucleus is enigmatic, and whether it is mediated by specific NLS remains to be established. In this respect, there is a short basic region containing a cluster of positively charged residues in the PH domain that may correspond to an NLS region. Our analyses, however, indicate that this region is not necessary for Btk nuclear translocation, since deletion of the Btk-PH domain (ΔPH1) leads to an equal redistribution of Btk within the nucleus and cytoplasm in the majority of transfected cells. We also demonstrate that the nuclear accumulation of ΔPH1 is dependent on the expression of Src family kinases.

Figure 9. Btk-GFP nuclear accumulation is an exportin/CRM-1 dependent mechanism. Cells were evaluated before (A) and after (B) Leptomycin B addition.

Although we do not yet know the exact mechanism of the nuclear localization of Btk, our findings indicate that the nucleocytoplasmic shuttling of Btk may have implications regarding potential targets inside the nucleus, which may be critical in gene regulation during B-cell development and differentiation. This hypothesis may be supported by reports that show direct connections between Tec kinases and transcription factors. In particular, interactions and phosphorylation with BAP-135/TFII-I, Bright, STAT3 and STAT5 have been reported, as was mentioned previously in the introduction section (Saharinen et al., 1997; Tsai et al., 2000; Webb et al., 2000; Yang and Desiderio, 1997). Furthermore it is also now clear that Rlk/Txk and Itk can traffic to the nucleus (Debnath et al., 1999; Perez-Villar et al., 2001). The nuclear localization of
Rlk is required to induce INF-γ expression in the Jurkat T cell line (Kashiwakura et al., 1999). A recent study extended these findings to show that Rlk directly binds to DNA to stimulate expression of INF-γ, suggesting novel roles for Tec kinases in the nucleus (Takeba et al., 2002).

**Btk localization in caveolae (Paper III)**

The *in vivo* interaction of two members of the Tec family kinases, Btk and Bmx, with the membrane-organizing coat protein, caveolin-1 was described (Vargas et al., 2002). We present evidence that such interaction is mediated through the catalytic domain of Btk. Furthermore, phosphorylation as well as kinase activity of Btk were neither necessary nor required for its functional interaction with caveolin-1.

We have shown here that caveolin-1 down-regulates tyrosine phosphorylation of Btk. In addition, a caveolin-1 scaffolding peptide but not a control peptide was sufficient to entirely inhibit the *in vitro* autokinase kinase activity of Btk. These data are consistent with the general idea that caveolins may function as negative regulators inhibiting the activity of many signaling proteins (Okamoto et al., 1998; Park et al., 2000; Razani et al., 2000; Zhang et al., 2000; Zundel et al., 2000).

The expression of caveolin in lymphocytes has been controversial for years (Hatanaka et al., 1998; Scherer et al., 1997). In this paper we describe our unexpected finding regarding expression of caveolin-1 in splenocytes, purified splenic B-lymphocytes and B-cell depleted splenic cells. Moreover, in these cells, caveolin-1 was unexpectedly found to be constitutively tyrosine phosphorylated. It is intriguing to learn that caveolin-1 is present and tyrosine phosphorylated in splenic B-lymphocytes. In light of the recent report describing immunologic synapses in B-cells (Batista, 2001), it will be of considerable interest to determine whether caveolae structures exist in resting B-cells and change form following antigen presentation.

Localization of Btk and Itk within microdomains or lipid rafts has recently been demonstrated (Guo et al., 2000; Woods, 2001). However, the existing confusion regarding the relationship between caveolae and rafts has yet to be resolved. We speculate that rafts and/or caveolae binding recruits Btk, where
its phosphorylation states can be dictated by nearby resident kinases and phosphatases, resulting in a cascade of downstream signaling events. Localization to microdomains, whether it is caveolae or rafts, could itself be a prerequisite for proper signaling of Tec family kinases. It will be of considerable interest to understand the biological as well as functional implication(s) of Btk and caveolin-1 interaction.

**Btk degradation (Paper IV)**

The mechanism of Btk degradation was characterized. Our data show that the expression levels of Btk in pre-B-cells and mature B-cells are strongly reduced in the presence of proteasome-specific inhibitors and/or the protein kinase C-activating phorbol ester, PMA. These results are consistent with previous data reporting that proteasome inhibitors reduce the steady-state levels of certain proteins, such as, Luciferase, β-galactosidase and Fas ligand (Deroo and Archer, 2002; Tanimoto and Kizaki, 2002). In stark contrast, Btk is ubiquitinated and targeted for proteasomal degradation when expressed at very high levels. Degradation of Btk was independent of its tyrosine phosphorylation status.

Furthermore, we present evidence that Cbl induces degradation of Btk in an ubiquitination-dependent manner suggesting an important role for Cbl ubiquitin ligase activity in the regulation of Btk. Interestingly, the non-receptor tyrosine kinase Syk has been shown to be a target of Cbl-mediated ubiquitination upon B-cell receptor stimulation (Rao et al., 2001). With regard to Btk localization, we observed that treatment of cells with proteasome inhibitors induces strong accumulation of Btk in a distinct microtubule-dependent perinuclear compartment, presumably aggresomes (Figure 10). The physiological significance of recruiting Btk to aggresomes is not clear. Nevertheless, this result is reminiscent of that observed in another study where the small heat shock protein, HSP27, was found to be recruited into aggresomes after exposure to proteasome inhibitors (Ito et al., 2002). This result is consistent with the general idea that aggresome formation is a cellular response to accumulation of missfolded proteins (Johnston et al., 1998).
We also show that small-ubiquitin-related-modifier (SUMO-1), downregulates Btk. It has been reported that SUMO-1 and its homologs can be conjugated to a large number of cellular proteins (Pichler and Melchior, 2002). Our results, however, indicate that Btk itself is not sumoylated, even though it harbors a classic SUMO-1 target site. Thus, it is possible that SUMO-1 functions in different ways depending on its substrate. Taken together, our findings indicate that the fate of the Btk protein is dictated by its expression levels through both ubiquitin and ubiquitin-related activities.

**Figure 10.** Proteasome inhibitors induce strong accumulation of Btk aggresome-like structures. Confocal analysis of 293T cells transiently transfected with Btk-GFP. (A) Non treated cells (B) after treatment with MG-132.
CLOSING REMARKS AND PERSPECTIVES

The spatial and temporal regulation of biochemical reactions in eukaryotic cells is achieved by a high degree of compartmentalization. Each protein is part of a functional biochemical network and all proteins, or the corresponding messenger within a particular network are at least once in their lifetime localized close to each other, within (or at) a particular organelle or compartment. This facilitates interactions and yet allows the crosstalk of different pathways. Exchange of information between different organelles, and of proteins between networks, is essential for the proper function of the cell as an entity and is achieved by the active transport of material.

In the present study subcellular localization of Btk was examined. Analysis of Btk localization can provide important clues to its function. We have observed that Btk localizes to specific subcellular compartments such as cytosol, plasma membrane, nucleus, cytoskeletal filaments, microtubule-related structures (MTOCs, aggresomes). This versatile capacity to localize in different compartments of the cell enables this kinase to likely participate in different cellular responses related to nuclear signaling, cellular structure, migration, adhesion, and cell division among others (Table 1).

<table>
<thead>
<tr>
<th>Btk localization</th>
<th>Potential function</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td><strong>Plasma membrane</strong>&lt;br&gt;Actin cytoskeleton, caveolae</td>
<td>Early signaling events, migration, adhesion and spreading cell; cell-cell contact, antigen presentation. Gene expression.</td>
<td>Phosphoinositide dependent. Presumably the dominant activity of Btk.</td>
</tr>
<tr>
<td><strong>Cytoplasmic</strong>&lt;br&gt;Microtubules, endosomes, Golgi</td>
<td>Protein traffic; endosomal membrane transport and regulation.</td>
<td>Most of cytosolic Btk may be a “reservoir” of protein.</td>
</tr>
<tr>
<td><strong>Nuclear</strong></td>
<td>Gene expression?</td>
<td>Src dependent.</td>
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Table 1. Subcellular locations of Btk
The Btk plasma membrane targeting was found to be a PI3K dependent mechanism. Membrane association of Btk is in part due to the property of the Btk-PH domain, which can bind to phospholipids with a high affinity. Deletion of the PH domain (ΔPH) in the Btk protein compromises the ability of Btk to translocate to the plasma membrane. However, Btk translocation to the plasma membrane can also be mediated through its catalytic domain (SH1), which is a target for caveolin-1. It will be very important to resolve the controversy between rafts, microdomains and caveolae in order to understand better the role of Btk in response to pre-BCR and BCR engagement. A fundamental question is the biochemical composition of the rafts themselves and the relationship between raft structures, BCR signaling, accumulation of proteins and antigen presentation.

The significance of Btk nuclear localization remains to be determined. Limited information exists with respect to Btk function at this intracellular location, and in particular, the specific substrates with which Btk may interact. Thus, the identification of Btk targets at this subcellular domain is an essential starting point for elucidating the biological functions of Tec family kinases localized in these regions.

Although this study has been useful in identifying potentially important players in the Btk regulation process, other experiments will be required to address how these factors are normally utilized within the cell to modulate Btk kinase activity. Further analyses addressing the regulation of these factors will be critical for determining of the physiological regulatory processes of Btk kinase activation. The specific localization of Btk to the plasma membrane, cytoskeleton, nucleus and microtubule-related structures illustrates that Btk is a complex signaling molecule with a number of potentially important cellular functions. Subcellular compartmentalization of Btk has the potential to facilitate its interaction with phosphorylated proteins, cellular phosphatases and signaling molecules involved in distinct signaling complexes.
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REFERENCES


architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J*, **17**, 5309-5320.


Hashimoto, S., Iwamatsu, A., Ishiiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T. and Tsukada, S. (1999) Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK-


