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Role of Bruton's tyrosine kinase in innate and adaptive immunity

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Summary

Btk is a cytoplasmic tyrosine kinase, which is mainly involved in B cell receptor signalling. Gene targeting experiments revealed that Btk is important for B cell development and function. However, Btk is not only expressed in B cells, but also in several other haematopoietic lineages except for T cells and plasma cells. Recently we found that Btk is involved in Toll-like receptor signalling. Toll-like receptors play an important role in innate immunity. They are highly expressed on mast cells, macrophages and dendritic cells, which are essential for the recognition and consequently for the elimination of microbial pathogens. Therefore Btk is an important protein expressed by immunocompetent cells of innate as well as adaptive immunity.

Introduction

Bruton's tyrosine kinase (Btk) is a cytoplasmic non-receptor tyrosine kinase that belongs to the Tec-kinase family. Tec-kinases (Btk, Bmx, Itk, Rlk, Tec) are largely expressed in cells of different lineages of

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the hematopoietic system. The essential role of Btk for B cell development and function became clear when it was found that mutations in the gene coding for Btk are responsible for X-linked agammaglobulinemia (XLA) in men [1], [2] and X-linked immunodeficiency (*Xid*) in mice [3],[4]. Although the phenotype of *Xid* is milder than that of XLA, both diseases are characterized by dramatic defects in B cell development and function resulting in a reduction of mature B cells in the peripheral blood as well as in secondary lymphoid organs accompanied by a severe reduction of serum immunoglobulin levels. Nevertheless, the expression of Btk is not restricted to B cells, it is also expressed in myeloid cells, like mast cells [5],[6], erythroid cells [6],[7],[8], platelets [9], monocytes/macrophages [6], [10] neutrophiles [11], dendritic cells (DCs) [12], in hematopoietic stem cells (HSC) and multipotent progenitors [13] as well as in primary neuronal cells [14]. Although btk mutations cause primarily severe B cell defects, functions of btk deficient or mutated myeloid cells are also affected (see below).

Tec-family kinases are characterized by an N-terminal pleckstrin homology (PH) domain which is able to bind to membrane phospholipids [15, 16] as well as proteins, like heterotrimeric G-Proteins [17-20], PKC-isoforms [21-24], Stat3 [22], F-actin [25] and Fas [26]. Additionally, Btk contains a Tec-homology-region (TH) implicated in the autoregulation of Tec-kinases, proline-rich (PR) Src-homology regions SH3 and SH2 necessary for interaction with other PR-rich sequences or for the binding to sequences harbouring phosphorylated tyrosine residues, and a C-terminal kinase domain (SH1) (reviewed in [27]). The multifaceted functions of Btk are realized due to its ability to form complexes with other proteins and lipids. This complex formation is a transient event in the process of signal transduction.

Btk and the adaptive immune system

Btk in B cell development and function

Mutations in a gene coding for Btk cause XLA in humans [1], [2] and *Xid* in mice [3, 4]. XLA is a severe inherited immunodeficiency disease, characterized by defective antibody responses and peripheral blood B cells, that are reduced in number and show an immature phenotype [28, 29]. Although Btk is expressed very early in B cell ontogeny [6, 10], the B cell defect appears only at the pre-B1a cell stage at which the development to mature B cells takes place (reviewed in [30]).

XLA becomes evident six to nine months after birth when the level of maternally-derived IgG decreases. Characteristically, affected males develop hypogammaglobulinemia and are highly susceptible to bacterial infections, which are the most common clinical manifestations. However, XLA patients have normal T cell functions and an intact cellular immunity.

More than 400 unique mutations in the Btk gene of XLA-patients have been identified that are scattered along the Btk gene [31]. In general, no correlation could be observed between the type of mutation and the phenotype of XLA patients [32]. Even in the same family identical mutations may lead to diverse clinical effects [33, 34].

In B cells, where Btk is expressed continuously during B cell development from the late pro-B cell stage till the mature $IgD^{high}IgM^{low}$ stage, the absence of a functional Btk leads to a failure of several signal transduction pathways, regulating important physiological processes of the cell like apoptosis, growth, cell cycle, proliferation, and particularly antigen receptor mediated signal transduction [35]. B cells bearing a mutated

btk gene are hyporesponsive to BCR and several other transmembrane signals and do not respond to T cell-independent type II antigens. In those mice the serum IgM and IgG3 levels are dramatically reduced, whereas the IgG1, IgG2a and IgG2b concentrations are normal. The B cell number is reduced to approximately 50% [36]. Moreover, the B1 subpopulation of B cells, characterized by the expression of the surface marker CD5, is depleted in Btk-mutant mice [37, 38]. Btk -/- mice have a phenptype that cannot be distinguished from that of *Xid* mice [37, 39, 40]. Additionally, the phenotype of Btk-mutant mice is similar to mice bearing a mutation of components of the B cell signalosome (p85α, BLNK/SLP-65, and PLCγ2) [41, 42]. Recently a function of Btk as a tumor suppressor gene was suggested and this function was independent of Btk kinase activity [43]. Although Btk deficient animals do not develop tumors, the incidence of tumor development is higher in SLP-65/Btk double deficient mice, than in SLP-65 single knock animals. Moreover, overexpression of a constitutive active Btk mutant E41K in SLP-65/Btk double deficient mice prevented tumor development.

Activation of Btk by the B cell receptor (BCR)

One of the initial events that occur in response to engagement of the BCR is the activation of non-receptor protein tyrosine kinases (PTKs) like Syk, Lyn and Fyn, and the phosphorylation of the intracellular sequences of the BCR, so called immunoreceptor tyrosine-based activation motifs (ITAMs), by these kinases (reviewed in [42] and figure 1 A). This enables the cytoplasmic Src-family kinase Syk to bind to the phosphorylated ITAMs. Thereby Syk undergoes conformational changes leading to its catalytic activation. Syk plays a crucial role in the activation process of Btk, since in Syk -/- cells Btk activity is dramatically reduced [44]. Btk is phosphorylated by Src-kinases Lyn and/or Syk [45] at Y551 in the SH1 domain which increases dramatically Btk kinase activity [38, 46]. A second phosphorylation site is located in the SH3 domain in the Btk protein at Y223 which is autophosphorylated by Btk [47]. The phosphorylation on Y223 has only minor role in Btk catalytic activity but is important for the protein binding of proline-rich sequences. Recent findings suggest that the presence of the adaptor/scaffold protein BLNK/SLP-65, which binds to the Btk SH2 domain and is itself phosphorylated by Syk, is necessary for the Btk phosphorylation on Y551 by Src-family kinases [48] (figure 1 B).

Activated Syk is involved in tyrosine phosphorylation of adaptor proteins recruiting the PI3 kinase to the membrane. Activated PI3 kinase generates phosphatidylinositol-3,4,5-triphoshate (PIP_3) that binds to and activates PH-domain containing proteins. The fact that the PI3 kinase p85α -/- mice have a similar phenotype as the *Xid* mice implies the important role of PI3 kinase in Btk function [49, 50]. It is supposed that PI3 kinase products are necessary to recruit Btk to the plasma membrane. It was shown that the PH domain of Btk is sufficient to bind PIP_3 selectively *in vitro* [16, 51]. A mutation in the Btk gene causing *Xid* (R28C) abolishes the binding of Btk to inositol lipids *in vitro* [16, 51] and *in vivo* [52] preventing Btk activation. Another mutation E41K, also located in the PH domain, shows increased membrane association [52, 53] accompanied by transforming capabilities as a result of a constitutive Btk activation [53]. These findings demonstrate that the binding of Btk to the membrane is mediated by its PH domain and this is critical for its kinase activity.

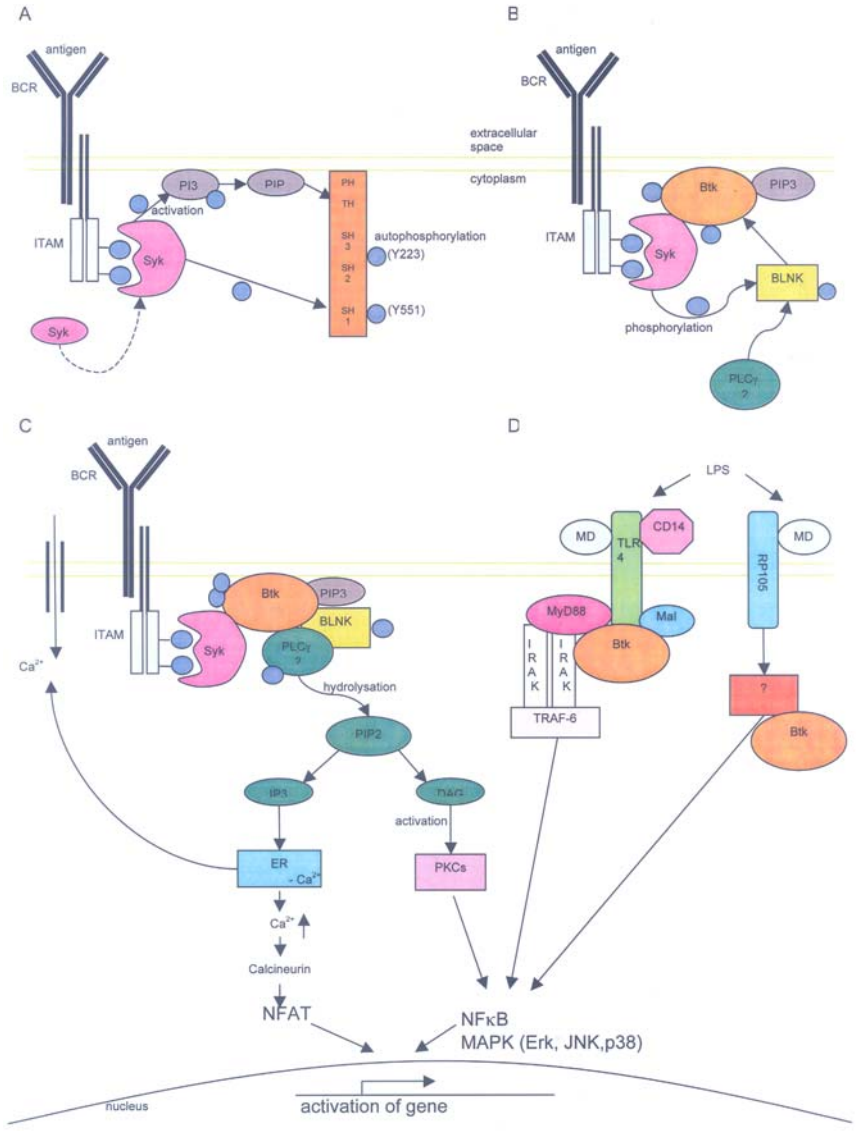

Figure 1

The phoshorylated scaffold protein termed B cell linker protein (BLNK; also called SLP-65 or BASH) binds to membrane targeted Btk and provides docking sites for a variety of downstream Btk targets, like Grb2, Vav, Nck and PLCγ2. The protein complex composed of Btk, PLCγ2 and BLNK facilitates Btk mediated PLCγ2 phosphorylation and activation [48, 54, 55] (figure 1 C). Activated PLCγ2 hydrolyses PIP_2 to produce IP3 and diaglycerol (DAG). IP3 in turn activates Ca^{2+} mobilization from

the intracellular stores by binding to the IP3 receptors of the endoplasmatic reticulum (ER) [56], whereas DAG activates protein kinase C (PKC) isoforms [41]. The resulting depletion of Ca^{2+} stored within the ER lumen drives the slow activation of store-operated channels in the plasma membrane, mediating the so called capacitative Ca^{2+} entry. Analyses of Btk mutants Y223F and Y551F showed, that the transphosphorylation at Y551 by Src kinases is essential for calcium mobilization upon BCR engagement, whereas autophosphorylation at Y223 had no influence on this process [45]. Recently, a second pathway of Btk mediated activation of PLCγ2 and PI3K was shown, involving phosphatidylinositol-4-phosphate 5 kinases (PIP5Ks), the enzymes that synthesize PtIns-4,5-P_2 (PIP_2) [57]. In response to BCR activation Btk binds to PIP5Ks and shuttles them to the membrane to synthesize local PtIns-4,5-P_2 – the substrate required by both the Btk activator PI3K and the Btk target PLCγ2.

Interestingly, it was shown that *in vivo* Btk autophosphorylation at Y223 is not required for Btk function, except for the regulation of λ light chain usage. Furthermore, during B cell development, Btk function is partially independent of its catalytic activity, probably by acting as an adapter molecule [58].

Targets of Btk activation

Studies using the *Xid*-mouse model [3, 4] or btk gene targeted mice [1, 37, 39] revealed that Btk is involved in a wide array of signaling pathways that are essential for the maintenance of peripheral blood B cell numbers and for proliferation, survival and responsiveness of B cells.

One of such signalling cascades in which Btk is involved is the already discussed Ca^{2+} induced pathway. Ca^{2+} elevation leads to the activation of the calmodulindependent protein kinase II and the calmodulin-activated serine/threonine phosphatase calcineurin. Calcineurin dephosphorylates NFAT transcription factors and enables them to translocate into the nucleus where they activate gene transcription. In Btk deficient cells the NFAT-activity is reduced due to an inefficient activation of PLCγ2, preventing the elevation of Ca^{2+} upon BCR stimulation since the production of IP3 is reduced [59-61]. Activation of PLCγ2 and the production of DAG are also involved in the activation of a second transcription factor in response to antigen receptor stimulation in B cells – namely NF-κB [61]. Consequently, Btk deficient B cells show impaired NF-κB activation [62-64].

Another transcription factor – TFII-I – is also regulated by Btk [65]. TFII-I is a ubiquitously expressed multifunctional transcription factor. In resting cells, TFII-I is retained in the cytosol by binding to Btk. Upon BCR cross-linking TFII-I becomes phosphorylated in a Btk independent manner, translocates into the nucleus and regulates gene transcription.

Several reports indicate that Btk is also involved in the activation of MAP kinases. Btk is required for the activation of ERK2 [42], JNK, and p38 [66]. MAP kinases are essential mediators of survival and apoptotic signals.

Btk becomes activated by a large array of receptors

The BCR is not the only receptor, by which Btk is activated in B cells. Btk is able to interact with several receptors located on the B cell surface [35]. Moreover, since Btk is

not exclusively expressed in B cells but also in myeloid lineages, it can also interact with and be activated by other cell type specific receptors.

Btk has been shown to be activated by G-protein coupled receptors and binds to βγ, Gαq and Gα12 subunit of the heterotrimeric G-proteins [17-20]. This association increases the Btk kinase activity [17]. Since overexpression of Tec in NIH3T3 cells resulted in the activation of RhoA, a role of Btk in regulation of Rho GTPases was suggested [67].

Btk was also found to be activated and colocalized with actin fibers upon IgE receptor stimulation on mast cells [25]. Therefore it was assumed that Btk is involved in the reorganization of the cytoskeleton upon receptor stimulation.

In addition, in DT40 chicken B cells and human B cell line NALM-6-UMI Btk is constitutively associated with Fas [26] – a member of the tumor necrosis factor (TNF) receptor family and a regulator of apoptosis in several cell types. The Fas-Btk interaction involves the PH domain of Btk and is independent of receptor ligation.

Btk can also be activated by cytokines and their appropriate cytokine receptors. In B cells, IL-6 was shown to activate Btk and Tec via Jak family kinases. Jak1 associates with Btk and directly phosphorylates it [68].

Additionally, it was shown that Btk is also involved in the control of integrin-mediated adhesion of pre-B cells involving cytoskeletal reorganization and integrin clustering [69], necessary for migration, recirculation and homing of B cells.

Recently we could show that human Btk is phosphorylated and activated not only by BCR- but also by CD40 stimulation [70, 71].

Furthermore, Btk deficient B cells show a reduced proliferation potential upon LPS stimulation [37, 72]. LPS signals are recognized by TLR4 [73] and RP105 [74, 75] on mouse B cells. We showed that Btk associates with the intracellular domains of several TLRs and Btk activity is increased upon LPS induced TLR4 stimulation [76]. Additionally, the importance of Btk for different signalling pathways of mast cells and macrophages, like degranulation, Ca^{2+} mobilization, NO- and TNFα production, which are induced by LPS or FcεR stimulation, was shown [77, 78].

Btk and the innate immune system

The innate immune response in vertebrates is the first line of defense against invading microorganisms. The main players in innate immunity are phagocytes such as neutrophils, macrophages and dendritic cells. Until recently, the manner in which vertebrates respond to pathogens was obscure. It is now clear that a family of proteins, the Toll-like receptors (TLRs) (reviewed in [79]), contribute to the signal transduction induced by many pathogen-associated molecular patterns (PAMPs) – conserved motifs predominantly found in microorganisms but not in vertebrates. Stimulation of TLR causes an immediate defensive response, including the production of an array of antimicrobial peptides and cytokines.

I. Role of Btk in macrophages

Macrophages are large phagocytic mononuclear cells. They develop like DCs from bone marrow derived myeloid precursor cells. Macrophages play an important role in innate immunity, as they recognize the foreign pathogen by a number of pathogen

recognition receptors (PRRs) followed by phagocytosis. Macrophage effector functions lead to the production of inflammatory cytokines like TNF-α and to nitric oxide (NO) release. Since Btk is expressed in macrophages [80, 81] a function of Btk in those cells was suggested. Btk is activated upon LPS treatment of macrophages [76] leading to increased TNF-α secretion [82]. *Xid* mice-derived macrophages produce less NO [83], TNF-α and IL1β [78], but secrete higher amounts of IL12 [83]. The reduced NO production correlates with the decrease of inducible NO-synthase expression in these cells [83]. The impaired macrophage function in Btk mutant mice could be one explanation for the delayed cure from microfilaria [84].

II. Role of Btk in mast cells

Mast cells are particularly involved in the initiation of allergic reaction. Mast cells are mainly activated by cross-linking of the high affinity IgE receptor (FcεRI), leading to degranulation accompanied by release of mediators, like histamines and leukotrienes, and by cytokine secretion. Btk is expressed in mast cells and its expression is upregulated upon mast cell activation [5]. Btk deficient mast cells develop normal [85, 86]. Like in B cells, in mast cells Btk is involved in the regulation of JNK1, JNK2 and p38 [66]. Since JNK activity is necessary for the induction of gene transcription of TNF-α, IL-2 [87] and other cytokines via activation of the AP-1 transcription factor, Btk deficient mast cells are consequently characterized by impaired proinflammatory cytokine secretion like IL-12, TNF-α and IL-6 [87]. In addition, in Btk deficient mast cells phosphorylation of PKB/Akt, PKCβ1 and PLCγ2 is impaired, therefore the generation of IP3 and intracellular Ca^{2+} elevation are reduced [85, 86]. Thus, these findings suggest an impaired FcεRI induced function of Btk mutant mast cells.

III. Role of Btk in DCs

DCs are bone marrow derived leukocytes and constitute the most potent antigen presenting cells. Immature DCs are able to recognize and to capture foreign antigens, they mature and migrate to secondary lymphoid structures to present antigenic peptides on the relevant MHC molecules. DCs are the central players in activation of naïve CD4+ T cells and to drive them into TH1 or TH2 lineages. The expression of Btk was detected in DCs [12]. Nevertheless, no influence of Btk on DC differentiation, maturation and antigen presentation could be detected [12]. Since Btk is the only member of Tec family kinase detected in DCs a phenotype of Btk mutant DCs is expected. To elucidate finally the function of Btk in DCs more studies are required.

Btk and Toll-like receptors

To achieve more insight into the function of Btk we performed a Yeast-two-hybrid screen to search for Btk interaction partners. Among several potentially interacting proteins we identified the receptor TLR8, a member of the TLR family [76]. The interaction of TLR8 with Btk was analysed in more detail. We identified the TLR8-Btk interaction domain comprising the intracellular TIR-domain, which is common and highly conserved in all TLRs. Additionally interaction studies revealed, that Btk interacts not only with TLR8, but also with other members of the TLR-family, namely TLRs 4, 6, and 9. We demonstrated that Btk is a member of the multiprotein complex

which is recruited to the TLR TIR domain (the TLR4 was studied) upon LPS stimulation. Btk does not only specifically interact with the TIR domain, but also with other proteins within the multiprotein complex, like Mal, MyD88 and IRAK [76] (figure 1 D). Moreover, we could also show, that the overexpression of a dominant negative Btk mutation prevents LPS induced NF-κB activation [76].

TLRs are expressed mainly on mast cells, macrophages and dendritic cells, but also on B cells. They recognize microbial particles and activate signalling pathways resulting in the induction of the immune response directed against the pathogen.

A second study using human Btk-deficient mononuclear cells from XLA patients also showed that Btk is involved in TLR signalling and responsible for LPS induced TNF-α production. Moreover, overexpression of Btk led to an increase of TNF-α production due to the stabilization of TNF-α mRNA via the 3' untranslated region [82].

TLR on B cells

Several recent studies have suggested a role of TLRs in the stimulatory effects by PAMPs on human B cells [88, 89]. In a recent study the expression as well as the function of TLRs on human B cells has been extensively and systematically investigated [90] showing that TLRs 1, 6, 7, 8, 9, and 10 are expressed on human B cells. On mouse B cells TLR4 is expressed and responsible for LPS signalling [74, 75]. The highest expression was observed for TLRs 9 and 10, and their expression was further increased in activated germinal center B cells. It is supposed, that in addition to their role as antibody-producing cells during the adaptive immune response, B cells may also respond to pathogens in a non-specific manner associated with the innate branch of the response. Two recent studies have directly explored the collaboration between TLR and the BCR in activating B cells [91, 92]. In these studies it was shown that stimulation of TLR9 by bacterial DNA potentiates BCR-induced B cell activation. In addition, TLR9 also cooperates together with the CD40 receptor to induce IL-12 secretion by activated B cells, which in turn resulted in an increased IFNγ production by in that way polarized TH1 cells [93].

Another pathogen recognition receptor, namely RP105, is expressed on B cells and cooperates with TLR4 in recognition of LPS [74, 75]. Its extracellular domain is very similar to Drosophila Toll, however, its intracellular domain is without similarity to other TLRs. Its expression is largely restricted to B cells and macrophages [94]. Stimulation of B cells by LPS enhances their antigen-presenting capacity and is associated with increased B cell proliferation and antibody secretion [95, 96]. As already mentioned, the response of Btk deficient B cells to LPS is impaired [37, 72]. Stimulation of the RP105 receptor on murine B cells with anti-RP105 antibodies leads not only to increased proliferation of these cells, but also protects them from apoptosis. B cells from *Xid* mice failed to respond to RP105 stimulation indicating the involvement of Btk in RP105 dependent signalling [97]. Interestingly, like for BCR signalling activation, a requirement of an intact PI3K was also described for the LPS response of mouse B cells [98]. If the PI3K activity induced by LPS treatment of B cells is necessary for Btk activation and membrane recruitment has still to be elucidated. The molecular mechanisms leading to the activation of Btk via TLR triggering as well as the signal transduction pathways from the receptor into the cell initiated by an active Btk remain to be resolved.

Taken together, these findings indicate a general role of Btk in innate and adaptive immunity signalling.

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