Constitutive membrane association potentiates activation of Bruton tyrosine kinase

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Mutations in the nonreceptor tyrosine kinase Btk result in the B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice. Genetic and biochemical evidence implicates Btk as a key component of several B cell signaling pathways. Activation of Btk by a point mutation (E41K) within the PH domain (Btk*) results in fibroblast transformation and is correlated with increased membrane localization of Btk. When wild type Btk is activated by coexpression with Lyn, the tyrosine phosphorylated pool of Btk is highly enriched in the membrane fraction. To determine whether membrane association is sufficient to activate Btk, we targeted Btk to the plasma membrane using a series of fusion proteins including GagBtk, CD16Btk and CD4Btk. Constitutive membrane association greatly enhanced the ability of Btk to transform Rat2 fibroblasts in the presence of high levels of Src activity. All membrane targeted forms of Btk were highly tyrosine phosphorylated. Transformation required membrane localization, Btk kinase activity, transphosphorylation by Src family kinases, and an intact SH2 domain but not the PH or SH3 domains. These data suggest that membrane localization is a critical early step in Btk activation.

Keywords: Btk; pleckstrin homology domain; membrane targeting; transformation

Introduction

Recruitment of cytosolic signaling molecules to the plasma membrane is a critical step in the initiation of intracellular signaling. Autophosphorylation of receptor tyrosine kinases creates docking sites to which cytoplasmic signaling proteins bind either directly (Kazlauskas *et al.*, 1989) or via adapter molecules such as Grb2 (Downward, 1994). Signaling through receptors without intrinsic enzymatic activity relies on the activity of constitutively and/or inducibly bound kinases (Ihle *et al.*, 1995; Chan *et al.*, 1994; Cambier *et al.*, 1994). Some nonreceptor tyrosine kinases (NRTKs) also communicate with the plasma membrane and elicit their function independent of cell surface receptors (Resh, 1994). Other cytosolic proteins interact with membrane associated signaling molecules such as Ras which are downstream of receptors in signal transduc-

tion pathways (Vojtek *et al.*, 1993). In many cases, cytosolic signal transducers can be artificially activated by the introduction of membrane targeting motifs such as lipid addition signals, retroviral Gag sequences, or the extracellular and transmembrane domains of cell surface proteins (Stokoe *et al.*, 1994; Leever *et al.*, 1994; Aronheim *et al.*, 1994; Quilliam *et al.*, 1994; Klippel *et al.*, 1996; Burgering *et al.*, 1995; Kolanus *et al.*, 1993; Sakai *et al.*, 1995).

Btk is a member of the Btk/Tec family of NRTKs (Rawlings and Witte, 1995). Like Src family kinases, Btk contains Src homology (SH) SH1, SH2 and SH3 domains, but Btk exhibits several distinct structural features which distinguish these two kinase families. Btk lacks both the amino-terminal myristylation signal which targets Src to the membrane and the carboxyl-terminal negative regulatory tyrosine which inhibits Src activity. The amino-terminal region of Btk/Tec family kinases contains a pleckstrin homology (PH) domain and a Tec homology (TH) domain (Haslam *et al.*, 1993; Mayer *et al.*, 1993; Vihinen *et al.*, 1994; Rawlings *et al.*, 1993).

Loss of Btk activity results in the immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993; Rawlings *et al.*, 1993; Thomas *et al.*, 1993). These diseases are characterized by defects in B cell development and function. Genetic and biochemical analyses indicate that Btk is a component of the signal transduction pathways utilized by the B cell antigen receptor (BCR), IL-5R, IL-6R, IL-10R, CD38, and FcεRI (Wicker and Scher, 1986; Hinshelwood *et al.*, 1995; Saouf *et al.*, 1994; Aoki *et al.*, 1994; de Weers *et al.*, 1994; Koike *et al.*, 1995; Sato *et al.*, 1994; Hitoshi *et al.*, 1993; Matsuda *et al.*, 1995; Santos-Argumedo *et al.*, 1995; Go *et al.*, 1990; Kawakami *et al.*, 1994). Activation of Btk by either receptor crosslinking or a point mutation E41K in the PH domain (Btk*) is dependent on Src family kinases (Rawlings *et al.*, 1996; Afar *et al.*, 1996; Park *et al.*, 1996; Saouf *et al.*, 1994; Mahajan *et al.*, 1995) and is correlated with increased tyrosine phosphorylation (Rawlings *et al.*, 1996; Li *et al.*, 1995; Mahajan *et al.*, 1995; Hinshelwood *et al.*, 1995; Aoki *et al.*, 1994; de Weers *et al.*, 1994; Saouf *et al.*, 1994; Sato *et al.*, 1994; Kawakami *et al.*, 1994; Matsuda *et al.*, 1995) and translocation to the plasma membrane (Li *et al.*, 1995; Kawakami *et al.*, 1994).

Several lines of evidence suggest that PH domains play a role in signal-dependent membrane association of proteins (Hemmings 1997). The Btk PH domain has been shown to bind *in vitro* to Gβγ, protein kinase C,

phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P₃) and the inositol phosphates IP₄, IP₅ and IP₆ (Touhara *et al.*, 1994; Tsukada *et al.*, 1994; Yao *et al.*, 1994; Salim *et al.*, 1996; Fukuda *et al.*, 1996). As these molecules are found at membrane proximal sites, these interactions may serve to target Btk to the membrane. Interestingly, mutations in the PH domain which result in XLA and xid abolish the binding of Btk to PI(3,4,5)P₃, IP₄, IP₅ and IP₆ (Salim *et al.*, 1996; Fukuda *et al.*, 1996). The PH domains of other molecules have also been implicated in membrane association.

Phosphatidylinositol-4,5-diphosphate (PIP₂) and many inositol phosphates bind *in vitro* to the PH domains of several proteins including PLC δ , dynamin, β -spectrin, pleckstrin, RasGAP and others (Harlan *et al.*, 1994; Lemmon *et al.*, 1995; Salim *et al.*, 1996; Ferguson *et al.*, 1995; Wang and Shaw, 1995; Fukuda *et al.*, 1996). The coupling of insulin receptor substrate (IRS) to the insulin receptor is mediated by the PH domain of IRS (Yenush *et al.*, 1996). The kinase activity of Akt is directly regulated by binding of phosphatidylinositol-3,4-bisphosphate to its PH domain (Franke *et al.*, 1997). Finally, the spectrin PH domain has been shown to target a heterologous protein to the plasma membrane (Wang *et al.*, 1996).

To determine whether membrane association is sufficient to activate Btk, we generated several membrane targeted forms of Btk. Constitutive membrane association greatly enhanced the activity of Btk as measured by its ability to transform fibroblasts expressing an activated Src allele. Transformation required Btk kinase activity, transphosphorylation of Btk by Src and an intact SH2 domain but not the PH or SH3 domains. These data suggest that membrane association is a critical early step in Btk activation.

Results

Tyrosine phosphorylated Btk is found predominantly in the membrane

Activation of Btk by Fc ϵ RI crosslinking or the E41K mutation results in the translocation of a small fraction of Btk protein to the plasma membrane (Kawakami *et al.*, 1994; Li *et al.*, 1995). In addition, activation signals result in an increase in the level of phosphotyrosine on Btk (Li *et al.*, 1995; Rawlings *et al.*, 1996; Mahajan *et al.*, 1995; Hinshelwood *et al.*, 1995; Aoki *et al.*, 1994; de Weers *et al.*, 1994; Saouf *et al.*, 1994; Kawakami *et al.*, 1994; Sato *et al.*, 1994; Matsuda *et al.*, 1995). These observations suggest that membrane targeting and tyrosine phosphorylation are tightly integrated steps in the activation of Btk. To address this issue, we employed a vaccinia virus system in which coexpression of Btk and Lyn in NIH3T3 cells results in increased tyrosine phosphorylation of Btk and increased Btk *in vitro* kinase activity (Rawlings *et al.*, 1996). The level of tyrosine phosphorylation on cytosolic and membrane associated Btk was compared (Figure 1). While Btk protein remained predominantly cytosolic when coexpressed with Lyn, the majority of tyrosine phosphorylated Btk was membrane associated (Figure 1, Lane 2). As previously described (Rawlings *et al.*, 1996), phosphorylation of Btk required the kinase activity of both Btk (Figure 1, Lane 4) and Lyn (Figure 1, Lane

1) as well as the presence of Y551 in Btk (Figure 1, Lane 3).

Alternative strategies target Btk to the plasma membrane

These results suggest that constitutive membrane association may be sufficient to activate Btk. To address this hypothesis, a panel of fusion proteins was constructed to target Btk to the cell surface (Figure 2). Three alternative approaches were employed (see Materials and methods section for details). Each of the strategies has been previously used to direct heterologous proteins to membranes (Burgering *et al.*, 1995; Kolanus *et al.*, 1993; Sakai *et al.*, 1995; Chu *et al.*, 1994). GagBtk consisted of the first 236 amino acids of the Abelson leukemia virus Gag protein (Reddy *et al.*, 1983) fused to the entire Btk molecule. CD16Btk encoded a transmembrane protein composed of the extracellular domain of CD16, the transmembrane domain of CD7 and the entire Btk molecule as the intracellular portion (Kolanus *et al.*, 1993; Sakai *et al.*, 1995). CD4Btk was constructed as an alternative transmembrane fusion protein in which the last 14 amino acids of CD4 are replaced by the full length Btk molecule (Jasin *et al.*, 1991). All three proteins have similar *in vitro* kinase activity to wild type Btk and Btk* (data not shown).

To confirm that these fusion proteins were present in the membrane fraction, these proteins were expressed

Figure 1 Tyrosine-phosphorylated Btk is membrane associated. NIH3T3 cells were coinfectd with recombinant Btk and Lyn vaccinia viruses for 6 h, and fractionated by hypotonic lysis, Dounce homogenization, and differential centrifugation. Btk was immunoprecipitated from the membrane (left) and cytosolic (right) fractions with an anti-Btk antibody and detected by immunoblotting with either an anti-phosphotyrosine antibody (4G10) or an anti-Btk antibody. Lane 1, kinase inactive Lyn(K275G) and Btk; Lane 2, Lyn and Btk; Lane 3, Lyn and Btk(Y551F); Lane 4, Lyn and kinase inactive Btk(K430R)

in Rat2 cells expressing Src(E378G) (see below) by retroviral infection and their subcellular localization was examined. As previously described, wild type Btk protein is cytosolic, while a small fraction of the activated allele Btk* is membrane associated (Figure 2, Li *et al.*, 1995). All the modified alleles of Btk accumulated in the membrane fraction, as predicted (Figure 2). Cytoplasmic contamination in the membrane fraction was monitored by reprobing the blot with an antibody against the cytosolic protein p120GAP (Li *et al.*, 1995). GAP was present exclusively in the cytosolic fraction (Figure 2).

Alternative membrane localization strategies activate Btk for transformation behavior but are not sufficient to replace an activation signal from Src

Transformation of fibroblasts has been a convenient assay to identify both activating mutations in and upstream regulators of Btk (Li *et al.*, 1995; Afar *et al.*, 1996). Rat2 cells provide a useful model for studying the regulation of Btk by Src family kinases. Neither the

activated Src allele, Src(E378G), nor Btk* alone can transform Rat2 cells (Levy *et al.*, 1986; Afar *et al.*, 1996). When coexpressed, however, these two molecules send a potent transforming signal (Afar *et al.*, 1996). Btk*, but not Src(E378G), is highly tyrosine phosphorylated in these transformed cells, suggesting that Src(E378G) acts upstream of and provides an activation signal for Btk (Afar *et al.*, 1996).

When expressed in Rat2 cells in the absence of Src(E378G), none of the membrane localized forms of Btk produced growth in soft agar (data not shown) indicating that membrane targeting was not sufficient to replace the activation signal from Src. In order to determine whether membrane localized forms of Btk could synergize with Src, agar assays were performed with Rat2 cells coexpressing Src(E378G) and the Btk fusion proteins. All membrane targeted alleles of Btk transformed these cells (Figure 3). GagBtk and CD16Btk gave rise to numerous large colonies (Figure 3). CD4Btk accumulated to a lower level than the other fusion proteins (Figure 4) which likely accounts for the decreased number of colonies formed

Figure 2 Different strategies to target Btk to the plasma membrane. The relative position of Btk, Btk*, GagBtk, GagMyr(-)Btk, Cd16Btk, and CD4Btk to the plasma membrane are diagrammed. Their domain structures represented as follows: SH1, the kinase domain; SH2 and SH3 the Src homology 2 and 3 domains; PH, the pleckstrin homology domain; TH, the Tec homology domain; Gag, the first 236 amino acids of the MuLV Gag protein; CD16, the CD16 extracellular domain and CD7 transmembrane domain; CD4, the CD4 extracellular and transmembrane domains. These Btk alleles were introduced into Rat2 cells expressing Src(E378G) by retroviral infection. Cells expressing different alleles of Btk were fractionated by hypotonic lysis, Dounce homogenization and differential centrifugation (Li *et al.*, 1995). Fractions were subject to SDS-PAGE and immunoblotted with an anti-Btk antibody or anti-GAP antibody

Figure 3 Soft agar colony formation by cells expressing Btk, Btk* and membrane targeted alleles of Btk. Rat2 cells expressing Src(E378G) were infected with the following Btk retroviruses of equivalent titer: Btk, Btk*, GagBtk, CD16Btk, CD4Btk. Three days after infection, 5×10^3 cells were plated into soft agar in medium containing 20% fetal calf serum in 6 cm plate. Colonies larger than 0.5 mm in diameter were counted 20 days after plating at the same time the picture was taken. The numbers presented are averages of duplicate plates from one assay and are representative of two independent experiments

by cells expressing CD4Btk relative to the other transforming alleles.

Activation of Btk by receptor crosslinking, over-expression with Src kinases, or the Btk* mutation is correlated with increased tyrosine phosphorylation of Btk (Figure 4, Li *et al.*, 1995; Rawlings *et al.*, 1996; Mahajan *et al.*, 1995; Hinshelwood *et al.*, 1995; Aoki *et al.*, 1994; de Weers *et al.*, 1994; Saouf *et al.*, 1994; Kawakami *et al.*, 1994; Sato *et al.*, 1994; Matsuda *et al.*, 1995). This is a result of both transphosphorylation by Src family kinases at Y551 and autophosphorylation at Y223 (Rawlings *et al.*, 1996; Park *et al.*, 1996). As predicted from these data, GagBtk, CD16Btk and CD4Btk (Figure 4) were all highly tyrosine phosphorylated when coexpressed in Rat2 cells with Src(E378G). Phosphorylation is required for signaling by membrane targeted Btk, as mutations which prevent transphosphorylation by Src (Y551F) or inactive Btk kinase activity (K430R) inhibited GagBtk mediated transformation (Figure 5).

Activation of GagBtk requires the myristylation signal

It is possible that the Gag, CD16, and CD4 fusion products impart a property other than membrane association which is responsible for the activation of Btk. The Gag sequence contains a myristylation signal that is crucial for plasma membrane localization (Henderson *et al.*, 1983). To evaluate the importance of

this motif for GagBtk mediated transformation, the critical Gly residue at position two of GagBtk (Henderson *et al.*, 1983) was mutated to Ala. The subcellular localization, transformation activity, and tyrosine phosphorylation of this myristylation defective mutant, GagMyr(–)Btk, and GagBtk were compared. GagMyr(–)Btk demonstrated reduced membrane association (Figure 2) and did not transform Rat2 cells expressing Src(E378G) (Figure 5) indicating that myristylation is required for GagBtk transformation activity. The residual membrane localization is most likely due to association with intracellular membranes, since a similar portion of this Gag protein directs GagMyr(–)LacZ fusion protein solely to perinuclear structures as measured by immunofluorescence (Jones *et al.*, 1990). GagMyr(–)Btk was tyrosine phosphorylated (Figure 4), indicating that tyrosine phosphorylation of Btk is not in itself sufficient for transformation. These data also imply that targeting to the appropriate membrane is necessary for downstream signaling events.

The PH domain is not required for GagBtk mediated transformation

An intact PH domain is required for the function of wild type Btk *in vivo* (Rawlings *et al.*, 1993; Thomas *et al.*, 1993; Vinihen *et al.*, 1995) and for the transforming activity of Btk* (Li *et al.*, 1995). As previously discussed, mounting biochemical evidence

Figure 4 Increased tyrosine phosphorylation of membrane targeted alleles of Btk. Rat2 cells expressing Src(E378G) were infected with Btk retroviruses of equivalent titer expressing: (a) Btk, Btk*, GagBtk, GagMyr(-)Btk, CD16Btk, and CD4Btk or (b) Btk, GagBtk, GagBtk(R307K), GagBtk(K430R) and GagBtk(Y551), GagBtk(ΔSH3), GagBtk(R28C), GagBtkΔPH. Three days after infection, 10^7 cells were lysed and Btk proteins were immunoprecipitated with anti-Btk serum. Immunoprecipitated samples were analysed by immunoblotting with anti-phosphotyrosine antibody (4G10) or affinity purified anti-Btk antibody. The positions of the Btk proteins are indicated by arrows

supports the hypothesis that PH domain containing proteins may be recruited to the plasma membrane via interaction with membrane proximal phospholipids or proteins (Hemmings, 1997; Harlan *et al.*, 1994; Lemmon *et al.*, 1995; Ferguson *et al.*, 1995; Wang and Shaw, 1995; Salim *et al.*, 1996; Fukuda *et al.*, 1996; Wang *et al.*, 1996; Touhara *et al.*, 1994; Tsukada *et al.*, 1994; Yao *et al.*, 1994; Yenush *et al.*, 1996). GagBtk proteins with PH domain mutations were used to determine if the Btk PH domain serves as essential signaling function in addition to membrane localization. Neither the mutation which results in murine xid (GagBtk(R28C)) nor complete deletion of the PH domain (GagBtkΔPH) blocked GagBtk mediated transformation (Figure 5). Single mutants Btk(R28C) or BtkΔPH had no transforming activity (data not shown; Li *et al.*, 1995). These observations indicate that constitutive membrane association alleviates the requirement for PH domain function in fibroblast transformation.

The SH2 but not the SH3 domain is essential for GagBtk mediated transformation

Once targeted to the membrane and activated by Src, it is likely that Btk interacts with downstream signaling molecules to elicit its function. To further clarify the domains of Btk required for these signaling events, the effect of mutations in the SH2 and SH3 domains of GagBtk was investigated. The mutation R307K, which prevents the binding of the Btk SH2 domain to phosphorylated tyrosine residues, completely blocked the transformation activity of both Btk* and GagBtk in Rat2 cells expressing

Src(E378G) (Figure 5, data not shown). GagBtk(R307K) retained a high level of tyrosine phosphorylation, indicating that the SH2 domain dependent event occurs downstream of the interaction with Src (Figure 4). Consistent with results previously described for a similar mutation in Btk*, removal of the SH3 domain did not impair GagBtk mediated transformation. These data indicate that the Btk SH2 but not the SH3 domain is essential for GagBtk downstream signaling and transformation of rat fibroblasts.

Discussion

Membrane association is a critical step in Btk activation

Plasma membrane association plays a critical role in the activation of many cytosolic kinases. We demonstrate here that activated Btk molecules are located predominantly in the membrane fraction. In addition, we show that constitutive membrane targeting potentiates Btk activity in a fibroblast transformation assay, the only currently available cell culture model system for Btk activation. Once targeted to the membrane, Btk requires kinase activity, transphosphorylation by Src family kinases, and an intact SH2 domain in order to signal. Taken together with previous data (Li *et al.*, 1995; Rawlings *et al.*, 1996; Park *et al.*, 1996; Mahajan *et al.*, 1995; Saouaf *et al.*, 1994; Kawakami *et al.*, 1994), these results support the following working model for Btk activation. Cell surface receptor crosslinking results in both the translocation of a fraction of Btk to the

Figure 5 Effect of Gag or Btk domain mutations on GagBtk transformation. Rat2 cells expressing Src(E378G) were infected with Btk retroviruses of equivalent titer expressing: GagBtk, GagMyr(-)Btk, GagBtk(R28C), GagBtkΔPH, GagBtkΔSH3, GagBtk(R307K), GagBtk(K430R), and GagBtk(Y551). Three days after infection, 5×10^3 cells were plated into soft agar in medium containing 20% fetal calf serum in 6 cm plate. Colonies larger than 0.5 mm diameter were counted 20 days after plating at the same time the picture was taken. The numbers presented are averages of duplicate plates from one assay and are representative of two independent experiments

plasma membrane and the phosphorylation of Btk by a two step mechanism. Transphosphorylation by Src family kinases is followed by Btk autophosphorylation. The temporal order of tyrosine phosphorylation and membrane association in a physiological setting remains to be determined.

The PH domain as a membrane targeting motif

Several lines of evidence indicate that membrane association of Btk is PH domain mediated. PH domain mutations result in both xid in mice and XLA in humans (Vihinen *et al.*, 1995; Rawlings *et al.*, 1993; Thomas *et al.*, 1993), demonstrating the importance of this motif for Btk function. The observation that the myristylated Gag sequences alleviate the requirement for the PH domain suggests that the PH domain serves to facilitate membrane association of Btk. This is supported by the finding that the spectrin PH domain is sufficient to target a reporter gene to the plasma membrane (Wang *et al.*, 1996).

Several potential PH domain ligands are located at membrane proximal sites and may serve as docking sites for Btk. The phospholipids and inositol phosphates $PI(3,4,5)P_3$, IP_4 , IP_5 and IP_6 bind to the Btk PH domain (Salim *et al.*, 1996; Fukuda *et al.*, 1996). These interactions are disrupted by PH domain point mutations found in XLA and xid (Salim *et al.*, 1996; Fukuda *et al.*, 1996). Recent findings from our laboratory demonstrating that PI3 kinase and Btk interact both genetically and biochemically (Zuomei Li *et al.*, manuscript in preparation) indicate that the PI3 kinase product $PI(3,4,5)P_3$ is likely to be an important physiological ligand for the Btk PH domain.

Protein/protein interactions between the Btk PH domain and membrane associated signaling molecules may also be important for Btk membrane targeting and activation. The $\beta\gamma$ subunits of heterotrimeric G proteins can interact with the PH domain of Btk (Tsukada *et al.*, 1994; Touhara *et al.*, 1994), and their overexpression has been shown to increase Btk kinase activity (Langhans-Rajasekaran *et al.*, 1995). The Btk PH domain can also bind to PKC isoforms (Yao *et al.*, 1994). PKC β knockout mice have a strikingly similar phenotype to Btk deficient mice, indicating that these two molecules may be components of the same signaling pathway (Leitges *et al.*, 1996).

The Btk PH domain may have functions other than membrane targeting. The E41K mutation results in a small increase in the transforming activity and tyrosine phosphorylation of GagBtk (data not shown). In addition, while only a small fraction of Btk* is membrane associated, it transforms almost as well as GagBtk and CD16Btk which are present exclusively in the membrane fraction. The E41K mutation may induce conformational changes which make Btk more accessible to Src family kinases or less accessible to downregulatory phosphatases. Alternatively, it might affect binding of the PH domain to a ligand capable of regulating Btk activity or its downstream signaling functions. While these PH domain functions can enhance Btk activity they are clearly not required for signaling by membrane associated forms of Btk, as a complete deletion of the PH domain from GagBtk does not block transformation.

The SH2 domain plays a critical role in Btk signaling

The SH2 domain mutation R307K impairs the ability of both GagBtk and Btk* to transform Rat2 cells expressing Src(E378G), indicating that the SH2 domain is required for Btk signaling in fibroblasts. A crucial role for the SH2 domain is further supported by

several observations in other cell systems. An SH2 mutant of Btk cannot rescue signaling defects in a Btk deficient chicken B cell line (Takata *et al.*, 1996), and the capacity of Btk to maintain a sustained increase in intracellular calcium is SH2 dependent (Zuomei Li *et al.*, manuscript in preparation). In addition, mutations in the SH2 domain have been identified in XLA patients (Saffran *et al.*, 1994; Ochs HD and Smith CI, 1996). The R307K mutation does not prevent Btk* transformation of NIH3T3 cells, however (Li *et al.*, 1995). This discrepancy may result from cellular changes in NIH3T3 cells which compensate for the lack of Btk SH2 domain function.

Because GagBtk(K307R) remains highly tyrosine-phosphorylated, yet lacks transformation activity, the Btk SH2 domain is most likely required for signaling events downstream of activation by Src. Three proteins whose tyrosine phosphorylation depends on the SH2 domain of Btk have recently been identified: pp70 (Afar *et al.*, 1996), BAP-135 (Yang and Desiderio, 1997) and PLC γ 2 (Takada, 1996; Zuomei Li *et al.*, manuscript in preparation). The clarification of the role of these and other molecules which interact with the Btk SH2 domain or require the SH2 domain for activation will be an important step in the understanding of Btk signaling pathways.

Materials and methods

Cell culture gene transfer and soft agar assay

NIH3T3 cells, Rat2 cells and Rat2 cell expressing Src(E378G) were grown in DMEM media supplemented with 5% fetal calf serum (FCS). Recombinant Btk retroviral cDNAs were constructed by inserting the wild type and mutant Btk cDNAs into the pSR α MSVTKneo vector (Muller *et al.*, 1991). Helper-free retroviral stocks were prepared by transient hyperexpression (Muller *et al.*, 1991) using 293T cells to increase the viral titer (Pear *et al.*, 1993). The titer of the viruses were measured by their ability to confer neomycin resistance to NIH3T3 cells ($\sim 10^6$ colony forming unit/ml). These titered retroviruses were used to infect Rat2 or Rat2 cells with Src(E378G). Recombinant Btk and Lyn vaccinia viruses were generated as described (Rawlings *et al.*, 1996). NIH3T3 cells were infected with vaccinia viruses for 6 h before they were harvested for analysis.

Colony forming ability was measured by an agar assay as described (Lugo *et al.*, 1989) using Rat2 cells. Briefly, 5×10^3 cells infected with different Btk viruses were plated in Iscoves media containing 20% FCS and 0.3% noble agar on an agar bed with 0.6% noble agar and media in each 6 cm plate. Colonies were counted (more than 0.5 mm in diameter) 2 weeks after plating.

Constructs

The Gag sequence was generated by polymerase chain reactin (PCR) using the following primer pairs: 5'-ATTGCGGCCGCACCATGGGCCAGACTGTTACC-3' and 5'-AATCCATGGGTAAAGGTCAGAAGAGGA-3' and Gag-Abl (MuLV) as the template. The PCR product was subcloned into the TA cloning vector (Invitrogen). GagBtk and GagBtk(R28C) were created by insertion of Gag in front of the Btk coding sequence (wildtype or the R28C mutant respectively) at the *Nco*I site in the pBluescriptSK(-) vector (Stratagene). The myristylation mutant, GagMyr(-)Btk, was created similarly using a

different 5' primer: 5'-ATTGCGGCCGCACCATGGGCCAGACTGTTACC-3', that changes codon 2 from Gly to Ala. GagBtk and GagBtk(R28C) were then subcloned in to the pSR α MSVTKneo vector (Muller *et al.*, 1991). GagBtk(R307K), GagBtk(K430R), GagBtk(Y551F), and GagBtk(Δ 204–263) were created by replacing a *Hind*III fragment of GagBtk with equivalent fragments containing the indicated Btk mutations (Li *et al.*, 1995) in pSR α MSVTKneo. Btk(Δ PH) was created in two steps. First, the 5' portion was amplified by PCR using primers 5'-GGACCATGGACCTGGTACAGAAA-3' and 5'-GCTGAAAAAGGTCGTGGC-3' and Btk as the template. Then a *Hind*III fragment of this PCR product was ligated to Btk at the *Hind*III site within Btk. Gag(Δ PH) was created by inserting the Gag sequence in front of Btk(Δ PH), similar to GagBtk.

A PCR generated (with primers 5'-ACCATGGACTC-CAGGATCCAG-3' and 5'-CGCCATGGTCTTCTTCTCAC TGAGG-3') murine CD4 fragment corresponding to the carboxyl terminal of CD4 was ligated back to CD4 to generate pSR α MSVCD4TKneo with an introduction of a *Not*I restriction site at the 3' end. This CD4 construct lacks 14 amino acids at its carboxyl terminus. Btk was subcloned in frame with CD4 into pSR α MSVCD4TKneo sequentially as a *Not*I–*Hind*III fragment and then a *Hind*III fragment to produce the final pSR α MSVCD4BtkTKneo construct.

CD16Btk was generated by ligation of the *Xba*I fragment from CD16:7:Syk (Kolanus *et al.*, 1993; Sakai *et al.*, 1995) into pBluescript, followed by in flame replacement of Syk with the *Nco*I/*Spe*I fragment of Btk. The resulting fragment encoding the CD16 extracellular domain, the CD7 transmembrane domain and the full length Btk protein was subcloned into the pSR α MSVTKneo vector by ligation into the *Not*I site.

Protein analysis

Total cell lysates were prepared by lysing 10^6 cells in 200 μ l boiling $2 \times$ sample buffer (2% SDS, 0.1 M Tris pH 6.8, 20% glycerol, 5% β -mercaptoethanol, 0.1% bromophenol blue). Immunoprecipitation and immunoblotting were done as described (Konopka and Witte, 1985). Briefly, 10^7 cells were lysed on the plate with boiling cell lysis buffer (1% Triton X-100, 10 mM phosphate buffer pH 7.0, 150 mM NaCl, 500 μ M sodium vanadate) plus 1% SDS. The cell lysates were diluted tenfold with cell lysis buffer and clarified by ultracentrifugation at 100 000 g for 30 min at 4°C. The supernatants were incubated with 10 μ l anti-Btk antibody at 4°C for 2 h. The samples were mixed with 100 μ l protein A Sepharose beads (suspended in five beads volume of cell lysis buffer) in the cold room for 1 h on a nutator and were washed three times with cell lysis buffer. For immunoblotting, immunoprecipitates or cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose filter. The filter was blotted with 5% skim milk in 150 mM NaCl, 10 mM Tris pH 7.5 and 500 μ M sodium vanadate for 1 h, except for anti-phosphotyrosine blotting, which was blocked with 6% BSA in 150 mM NaCl, 10 mM Tris pH 7.5, and 500 μ M sodium vanadate at 37°C for 1 h. The filter was then sequentially blotted with primary antibodies (anti-Btk amino-terminal (Tsukada *et al.*, 1993), 1:500 dilution; anti-GAP (Transduction Laboratories), 1:250 dilution, anti-phosphotyrosine (4G10), 1:1000 dilution) and secondary antibodies (horse-radish peroxidase (HRP) conjugated goat-anti-rabbit or goat-anti-mouse (BioRad), 1:5000 dilution). Proteins were visualized by the ECL technique (Amersham).

Subcellular fractionation

Subcellular fractionation was performed as described (Li *et al.*, 1995). Briefly, 10^7 cells expressing Btk alleles were scraped into 1 ml hypotonic lysis buffer (1 mM EDTA,

1 mM EGTA, 10 mM β -glycerophosphate, 1 mM sodium vanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 40 ng/ml PMSF) and were incubated on ice for 30 min. Cells were then Dounce homogenized (30 strokes). Cell lysates were loaded onto a 1M sucrose cushion and spun at 1600 g for 10 min. Post nuclei supernatant were spun at 100 000 g for 30 min. The supernatant contained the cytosolic fraction. The precipitate containing the membrane fraction was washed with the hypotonic lysis buffer twice and resuspended in hypotonic lysis buffer containing 1% SDS in the same volume as the cytosolic fraction. It was designated the membrane fraction.

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