BLNK mediates Syk-dependent Btk activation

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Btk is a critical molecule in B cell antigen receptor (BCR)-coupled signaling, and its activity is regulated by Lyn and Syk. Although the molecular mechanism of Lyn-dependent Btk activation has been investigated, that of Syk-dependent Btk activation has remained unidentified. We have demonstrated that BLNK mediates Syk-dependent Btk activation. In a reconstitution cell system, coexpression of BLNK allows Syk to phosphorylate Btk on its tyrosine 551, leading to the enhancement of Btk activity. This phosphorylation depends on the interaction of Btk and BLNK by means of the Btk-Src homology 2 domain. The existence of such an activation mechanism is supported by the observation that the BCR-induced Btk phosphorylation and activation are significantly reduced in BLNK-deficient B cells as well as in Syk-deficient B cells. Although previous observations have identified the function of BLNK as the linker that integrates the action of Btk and Syk into downstream effectors such as phospholipase Cγ2, our present study indicates another function of BLNK that connects the activity of Syk to that of Btk.

The B cell antigen receptor (BCR) signal transduction is mediated by three distinct families of cytoplasmic protein tyrosine kinase (PTK). The Src-family PTKs such as Lyn are located immediately downstream of BCR, and their activation is followed by the activation of two other PTKs, Syk and Btk (1–4). It has been demonstrated that the activation of Btk depends, at least partially, on the activity of Lyn. Coexpression experiments in B cells and fibroblasts have demonstrated that Lyn transphosphorylates the tyrosine 551 residue (Y551) in the Btk catalytic domain, which is the critical residue for the enhancement of Btk catalytic activity (5, 6). Y551 in Btk is also phosphorylated after BCR crosslinking, which indicates that the phosphorylation of Y551 is also important for BCR signaling (6, 7). In addition to the Src-family PTKs, recent findings have indicated that Syk is also involved in Btk activation. A genetic dissection experiment on the DT40 B cell line demonstrated that the BCR-induced tyrosinephosphorylation of Btk is significantly reduced in Syk-deficient cells as well as in Lyn-deficient cells (7). Furthermore, Btk phosphorylation is almost completely eliminated in Lyn/Syk double-deficient cells (7). In Syk-deficient RBL-2H3 mast cells, Btk activation after Fcε receptor crosslinking is greatly diminished when compared with that in wild-type cells (8). These observations suggest that Syk contributes to Btk phosphorylation/activation in B cells and mast cells. However, in contrast to these observations in hematopoietic cells, previous studies with nonhematopoietic cell reconstitution systems have indicated that coexpression of Syk did not affect the tyrosinephosphorylation or the catalytic activity of Btk (5, 6). This discrepancy seems to suggest the requirement of a third, hematopoietic cell-specific molecule for Syk-dependent Btk phosphorylation and activation.

It has been found that a recently identified B cell specific adaptor molecule, BLNK/SLP-65/BASH (9–11), integrates the activities of Btk and Syk into downstream effectors such as phospholipase Cγ2 (PLCγ2) (12, 13). A previous study of ours suggested that PLCγ2 was fully activated on BLNK through the formation of a molecular complex including Btk, Syk, and BLNK (12). In view of previous observations that some part of Btk activation depends on Syk (7) and that both Btk (12, 14) and Syk (9, 11) interact with BLNK, it seems reasonable to test the possibility that BLNK could also mediate the functional association of Btk and Syk, which leads to Syk-dependent Btk activation.

In the study presented here, we provide evidence that, in the presence of BLNK, Syk phosphorylates Y551 of Btk, which results in its activation, and demonstrate that this phosphorylation requires the interaction of the Btk-SH2 domain and BLNK in a reconstituted cell system. In support of this observation, BLNK is shown to be required for BCR-induced Btk phosphorylation and activation in DT40 cells.

Materials and Methods

Cells and Antibodies. 293T cells were cultured in DMEM medium supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Wild-type and various mutant (Lyn-, Syk-, and BLNK-deficient) DT40 chicken B cells have already been described (15, 16). Anti-chicken IgM mAb M4, anti-T7 mAb, anti-Flag M2 mAb, and anti-phosphotyrosine mAb 4G10 were purchased from Southern Biotechnology Associates, Novagen, Sigma-Aldrich, and Upstate Biotechnology (Lake Placid, NY), respectively. Anti-Btk mAb 48-2H (17) and 43-3B (18) were previously described, as were rabbit anti-porcine Syk (15) and anti-chicken BLNK Ab (16).

cDNAs, Mutagenesis, and Transfection. Wild-type human Btk, kinase-inactive human Btk cDNA (K430R) in pEF-BOS vector [Btk(K−)] (18), and T7-tagged human Btk cDNA in pApuro vector (7) were previously described. Double point mutations of the substitution of tyrosine 551 by phenylalanine plus K430R [Btk(K−/Y551F)], and of arginine 307 by lysine [Src homology 2 (SH2) domain mutation] plus K430R [Btk(K−/SH2−)] were created by means of PCR and inserted into the pEF-BOS vector. Porcine Syk (19) and Flag-tagged BLNK (12) cDNA expression vectors have been described previously. The kinase-inactive porcine Syk cDNA in pApuro vector [Syk(K−)] was provided by Hirohei Yamamura (University of Kobe, Kobe, Japan). DNA transfection into 293T cells was described previously (18). T7-tagged human Btk cDNA was transfected into DT40 cells by electroporation at 260 V, 960 μF and selected in the presence of 0.5 μg/ml puromycine.

Immunoprecipitation and Immunoblotting. A total of 1×10^7 DT40 cells were stimulated with M4 (4 μg/ml) at 37°C for the indicated times. Cells were lysed in Nonidet P-40 lysis buffer [1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM sodium vanadate supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF)]. Cell lysates were incubated with the indicated antibodies and protein-A Sepharose and then analyzed by means of immunoblotting as described previously (18).

Abbreviations: BCR, B cell antigen receptor; PTK, protein tyrosine kinase; PLCγ2, phospholipase Cγ2; SH2, Src homology 2.

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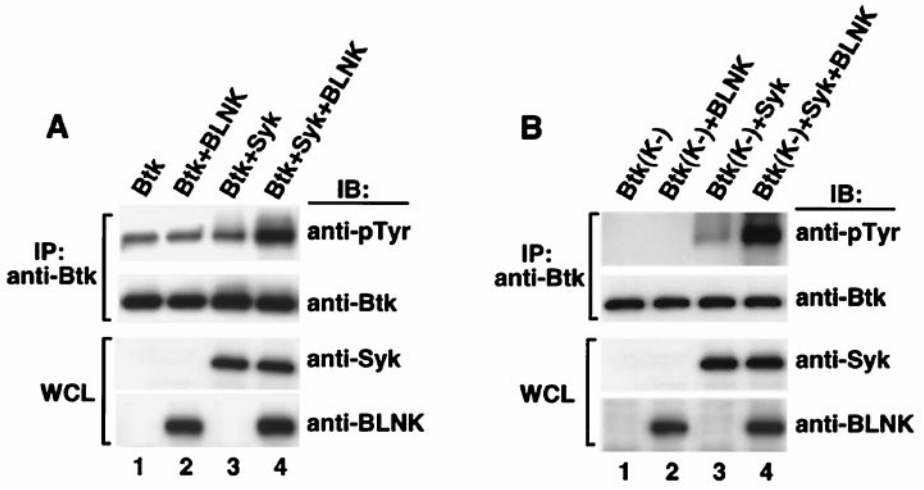

Fig. 1. BLNK mediates Syk-dependent Btk phosphorylation. cDNAs of Btk (*A*) or Btk(K−) (*B*) were cotransfected into 293T cells with the indicated combinations of Syk and/or BLNK. Btk was immunoprecipitated (IP) from cell lysates with anti-Btk mAb 48-2H, and the immune complexes were separated by 4–20% gradient SDS-PAGE gels, then immunoblotted (IB) with anti-phosphotyrosine (pTyr) mAb 4G10 (*Top*). The filter was reprobed with anti-Btk mAb 43-3B to confirm the equal amount of precipitated Btk (*Second Panel*). The whole cell lysates (WCL) were immunoblotted with the anti-Syk Ab (*Third Panel*) or the anti-BLNK Ab (*Bottom*) to detect the expression of each protein.

***In Vitro* Kinase Assay.** Anti-Btk or anti-T7 immunoprecipitates from cell lysates were washed twice with kinase buffer (10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 1 mM sodium vanadate). A total of 40 μl of kinase buffer and [γ-^{32}P]ATP (10 μCi) was added to each sample. The reaction was performed at 30°C for 5 min, then terminated by adding the sample buffer and heating at 100°C for 5 min. The autophosphorylated Btk was detected with autoradiography (18).

Results

BLNK Mediates Syk-Dependent Btk Phosphorylation in Nonhematopoietic Reconstituted Cells. By using a reconstituted system in 293T cells, we evaluated the possibility that BLNK might mediate Syk-dependent Btk phosphorylation. Although even the expression of Btk alone resulted in some Btk phosphorylation, probably because of its spontaneous autophosphorylation in these cells (Fig. 1*A*, lane 1, *Top*), this phosphorylation did not increase by cotransfecting either BLNK or Syk (lanes 2 or 3). In contrast, coexpression of three molecules, Btk, Syk, and BLNK, significantly enhanced the tyrosinephosphorylation of Btk (lane 4). This Btk phosphorylation was further assessed by using a kinase-inactive Btk [Btk(K−)] construct that did not exhibit any base line-level autophosphorylation or transphosphorylation by endogenous PTKs in 293T cells (Fig. 1*B*, lane 1, *Top*). When Btk(K−) was expressed with Syk only, its phosphorylation was very faint (lane 3), which seemed to be consistent with a previous report that coexpression of Btk and Syk in nonhematopoietic cells did not induce Btk phosphorylation (5, 6). In contrast, the coexpression of Btk, Syk, and BLNK resulted in a drastic enhancement of the tyrosinephosphorylation of Btk (lane 4). Because no tyrosinephosphorylation of Btk was observed when Btk was coexpressed with BLNK only (lane 2) or with BLNK and kinase-inactive Syk (data not shown), this observation does not mean that BLNK can mediate Btk phosphorylation by endogenous PTK(s) in 293T cells, but rather that Btk is effectively phosphorylated by Syk in the presence of BLNK. We obtained essentially the same results by using COS7 cells instead of 293T cells (data not shown).

Interaction of BLNK and Btk-SH2 Domain Is Required for Syk-Dependent Btk Phosphorylation. It has been reported that Syk is the kinase responsible for the tyrosinephosphorylation of BLNK (ref. 9, and Fig. 2*A*, *Third Panel*). Furthermore, we previously reported that the phosphorylation of BLNK subsequently provides the docking site for the Btk-SH2 domain that enhances the binding of Btk and BLNK, whereas the interaction of the Btk-SH3 domain and BLNK also seems to partially contribute to the association of these two molecules (12). Actually, in the 293T reconstituted cells used in our experiment, Syk-dependent phosphorylation of BLNK significantly potentiated the association of Btk with BLNK (Fig. 2*A*, compare lanes 1 and 2, *Top*), and this association was reduced by introducing a mutation in the Btk-SH2 domain (compare lanes 2 and 3). Because our previous observation demonstrated that the proper binding of Btk and BLNK by means of the Btk-SH2 domain is necessary for the appropriate interaction of Btk and its effectors, such as PLCγ2 (12), we next assessed the possibility that the interaction of Btk and BLNK by means of the Btk-SH2 domain might be also important for Syk-dependent Btk phosphorylation. As shown in Fig. 2*B*, SH2-mutated Btk(K−) was only very weakly phosphorylated by Syk (lane 3, *Top*) when compared with the significant phosphorylation of Btk(K−) (lane 2), which suggests that the interaction of the Btk-SH2 domain and phosphorylated BLNK is necessary for Syk-dependent Btk phosphorylation.

Syk Phosphorylates Tyrosine 551 of Btk and Leads to the Enhancement of Btk Activity in the Presence of BLNK. It has been well established that the enhancement of PTK catalytic activity is generally associated with the phosphorylation of specific tyrosine residue(s) in the activation loop of the catalytic domain (20). In the case of Btk, Lyn-dependent phosphorylation of the tyrosine 551 residue (Y551) in the catalytic domain results in a drastic increase in Btk catalytic activity, implying that the phosphorylation of Y551 plays a key role in the regulation of Btk activity (6). If Syk, as well as Lyn, functions as a positive regulator of Btk, Y551 in Btk would also be the Syk-dependent phosphorylation site. To examine the Btk phosphorylation site generated by Syk, the tyrosinephosphorylation of Btk(K−/Y551F) was compared with that of Btk(K−) in 293T cells coexpressing Syk and BLNK. As shown in Fig. 3*A*, the Syk-dependent phosphorylation of Btk(K−/Y551F) was very weak (lane 2) when compared with the prominent phosphorylation of Btk(K−) (lane 3). This result indicates that the major phosphorylation site generated by Syk is Y551 of Btk.

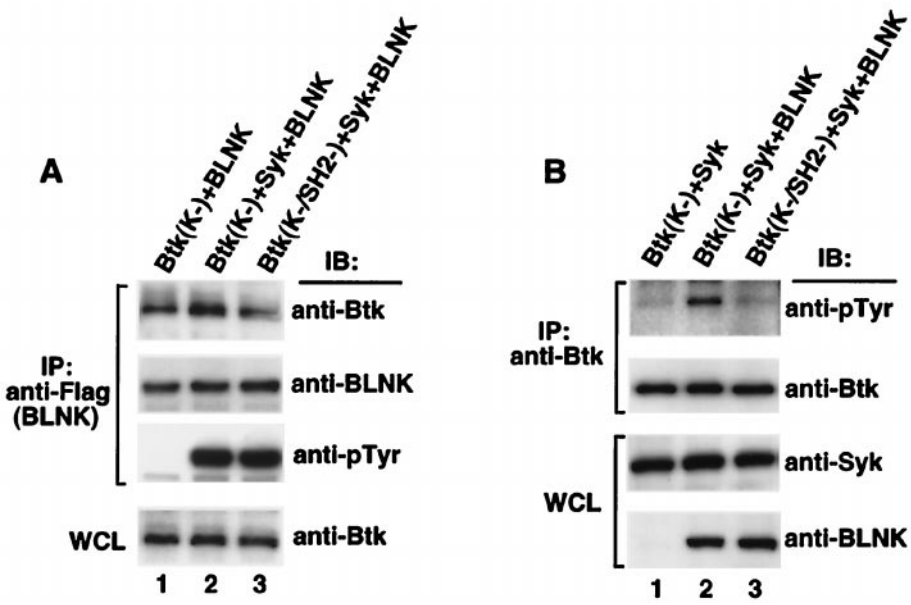

Fig. 2. Syk-dependent Btk phosphorylation requires the interaction of BLNK and Btk-SH2 domain. cDNAs of Btk [Btk(K−) or Btk(K−/SH2−)] were cotransfected into 293T cells with the indicated combinations of Syk and/or BLNK. (*A*) Assessments of the coprecipitation of Btk and BLNK were performed by tagging BLNK with Flag sequence as described in our previous report (12). Flag-tagged BLNK was immunoprecipitated from cell lysates with anti-Flag mAb M2. Immune complexes were immunoblotted with anti-Btk mAb 43-3B for detecting the coprecipitation of Btk (*Top*), followed by reprobing with anti-BLNK Ab (*Second Panel*). The Syk-dependent tyrosinephosphorylation of BLNK was detected by immunoblotting with anti-pTyr mAb 4G10 (*Third Panel*). Equal expression levels of Btk, Syk, and BLNK in each experiment were confirmed by immunoblotting the whole cell lysates with anti-Btk mAb 43-3B (*Bottom*), anti-Syk Ab and anti-BLNK Ab (not shown). (*B*) Btk was immunoprecipitated from cell lysates with anti-Btk mAb 48-2H, and the tyrosinephosphorylation was evaluated by anti-pTyr mAb 4G10 (*Top*). The filter was reprobed with anti-Btk mAb 43-3B to confirm the equal amount of precipitated Btk (*Second Panel*). The whole cell lysates were immunoblotted with the anti-Syk Ab (*Third Panel*) or the anti-BLNK Ab (*Bottom*).

To examine whether the Syk-dependent Btk phosphorylation actually enhances Btk activity, Btk was expressed with Syk and/or BLNK in 293T cells, and its catalytic activity was evaluated by *in vitro* kinase assay. As shown in Fig. 3*B*, Btk activity was approximately 2.5 times enhanced by the coexpression of both Syk and BLNK (compare lanes 1 and 3), whereas Btk activity was not significantly increased by the presence of Syk alone (lane 2). Taken together, these observations indicate that Syk can act as the positive regulator of Btk in the presence of BLNK.

BLNK Is Required for BCR-Induced Btk Phosphorylation and Activation. It has been shown that the BCR-induced tyrosinephosphorylation of Btk is greatly reduced in Syk-deficient DT40 cells (7). If Syk-dependent Btk phosphorylation is mediated by BLNK in B cells, BCR-induced Btk phosphorylation and activation in BLNK-deficient DT40 cells can be expected to be also reduced, similarly to that in Syk-deficient cells. Because anti-Btk antibodies, which were currently available, did not immunoprecipitate the chicken endogenous Btk efficiently, accurate assessments of the Btk phosphorylation and activation were performed by expressing T7-tagged Btk stably in wild-type and mutant (BLNK-deficient and Syk-deficient) DT40 cells as described in a previous reports (7). Following BCR crosslinking, T7-Btk was immunoprecipitated by anti-T7 mAb to evaluate its tyrosinephosphorylation. In contrast to the prominent Btk phosphorylation in wild-type cells, BLNK-deficient DT40 cells exhibited no enhancement of Btk phosphorylation after BCR crosslinking, similar to that observed in Syk-deficient cells (Fig. 4*A*). Consistent with the absence of induced Btk phosphorylation in BLNK-deficient cells, no significant increase in Btk activity was observed in BLNK-deficient cells after BCR crosslinking (Fig. 4*B*). The reduction of Btk phosphorylation in BLNK-deficient cells could not be the result of the altered Syk activity in these cells because similar levels of Syk phosphorylation (Fig. 4*C*) and activation (data not shown) were observed in wild-type and BLNK-deficient cells. These observations in DT40 cells seem to agree with the previous results for 293T reconstituted cells, which indicates a role for BLNK in Btk activation.

Discussion

In this study, we demonstrated that BLNK mediates the Syk-dependent tyrosinephosphorylation and activation of Btk in a reconstitution cell system and, in apparent support of this observation, that BLNK is required for BCR-induced tyrosinephosphorylation and subsequent activation of Btk in DT40 B cells.

Previous observations have identified the function of BLNK as the linker that integrates the actions of Btk and Syk into downstream effector molecules (9–13). It has been demonstrated that the Syk-dependent phosphorylation of BLNK makes the interaction of BLNK with Btk (12, 14) and PLCγ2 (9) possible by means of their SH2 domains, which presumably enables Btk, Syk, and PLCγ2 to colocalize on BLNK so that PLCγ2 can be fully activated by Btk and Syk (12, 13). Our present study provides evidence for another function of BLNK, that is, to connect the activities of Syk and Btk.

Although our data indicate that Btk serves as a cellular substrate for Syk, so far the substrate specificity of Syk has been only partially identified. A chemical peptide library strategy suggested that Syk has a preference for acidic residue(s) at the −1 or +1 position from the targeted tyrosine residue (21). *In vitro* experiments have demonstrated that Syk phosphorylates PLCγ1 at the tyrosine residues 771 and 783 (22) and c-Cbl at 700, 731, and 774 (23), with some of these residues also being the sites for phosphorylation by Src-family PTKs. Considering these previous observations, the flanking amino acid sequence of Y551 of Btk (DDEYTSS), in which the −3 to −1 positions from

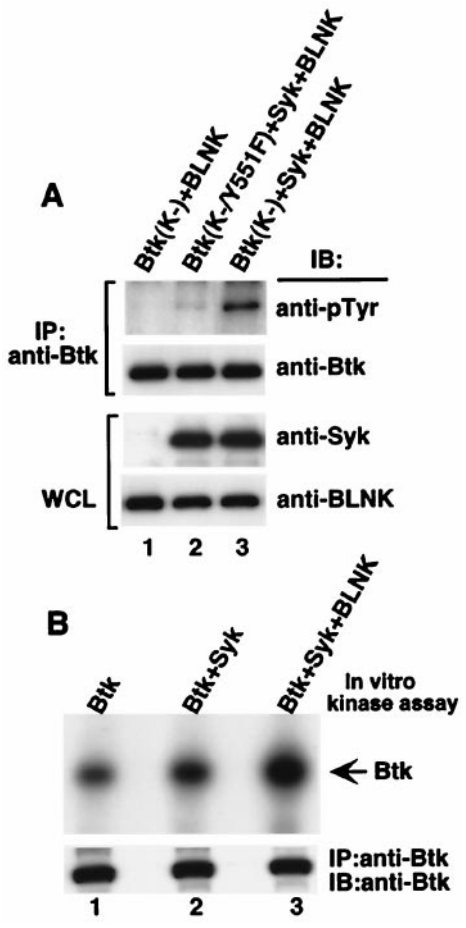

Fig. 3. Syk phosphorylates Y551 of Btk and enhances Btk activity in the presence of BLNK. (*A*) cDNAs of Btk [Btk(K−) or Btk(K−/Y551F)] were cotransfected with Syk and/or BLNK into 293T cells. Btk was immunoprecipitated with anti-Btk mAb 48-2H, and immunoblotted with anti-pTyr mAb 4G10 (*Top*), followed by reprobing with anti-Btk mAb 43–3B (*Second Panel*). Expression levels of Syk or BLNK were evaluated by immunoblotting the whole cell lysates with the anti-Syk Ab (*Third Panel*) or the anti-BLNK Ab (*Bottom*). (*B*) Btk expressed in 293T cells with or without Syk and BLNK was immunoprecipitated with anti-Btk mAb 48-2H, and an *in vitro* kinase assay was carried out as described in *Materials and Methods* (*Top*). The second panel indicates the equality of Btk protein in immunoprecipitates.

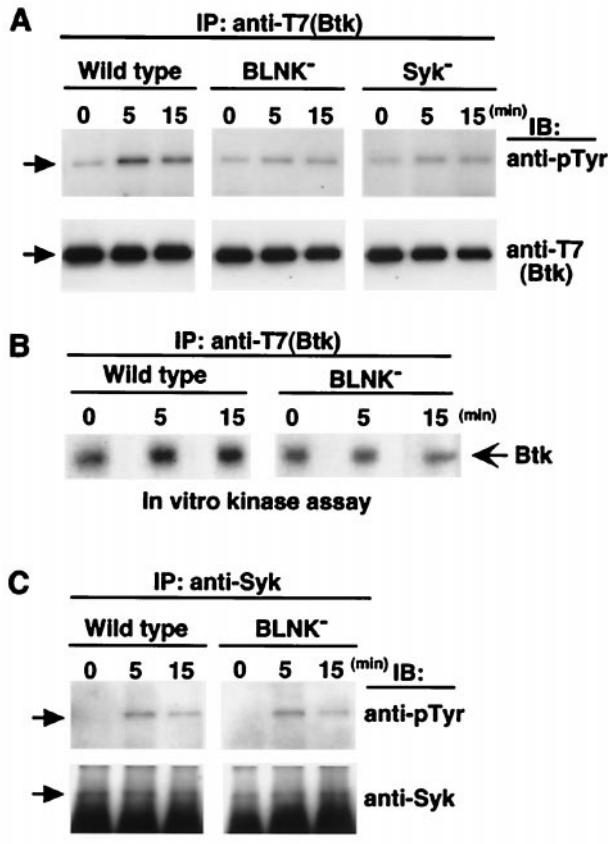

Fig. 4. BCR-induced tyrosinephosphorylation and activation of Btk are reduced in BLNK-deficient DT40 cells. Wild-type or mutant (BLNK-deficient and Syk-deficient) DT40 cells expressing T7-Btk were stimulated with anti-chicken IgM mAb M4 (4 μg/ml) for the indicated periods. Lysates were immunoprecipitated with the anti-T7 mAb (*A*) or the anti-Syk Ab (*C*), and then immunoblotted with anti-pTyr mAb 4G10 (*A* and *C*, *Top*), followed by reprobing with the anti-T7 mAb (*A*, *Bottom*) or the anti-Syk Ab (*C*, *Bottom*). (*B*) After stimulation of wild-type and BLNK-deficient DT40 cells with mAb M4, T7-Btk was immunoprecipitated with the anti-T7 mAb, and an *in vitro* kinase assay was carried out as described in *Materials and Methods*. The equality of Btk protein in immunoprecipitates was confirmed by anti-Btk immunoblotting (data not shown).

Y551 are occupied by acidic residues, appears to be preferred by Syk as well as Src-family PTKs. Consistent with this notion, our data show that Syk phosphorylates Y551 of Btk in the presence of BLNK and, accordingly, potentiates Btk activity, which suggests that Syk functions as a positive regulator of Btk in a BLNK-dependent manner.

The precise molecular mechanism by which Syk effectively phosphorylates Btk in the presence of BLNK remains unclear. However, the results obtained with the 293T reconstitution system indicate that the interaction of the Btk-SH2 domain and phosphorylated BLNK makes it easy for Btk to be phosphorylated by Syk. This observation concerning Btk and Syk seems to be similar to the previous finding that the proper binding of Btk and BLNK by means of the Btk-SH2 domain is indispensable for the Btk-dependent activation of PLCγ2, although the interaction of Btk and BLNK is also partially mediated by the Btk-SH3 domain (12). The SH2 domain-dependent interaction of Btk with BLNK may induce a conformational change in Btk that enables it to serve as the Syk substrate, or alternatively may prompt the appropriate colocalization of Btk and Syk on BLNK, which provides Syk with easy access to Btk. It should be noted here that, despite the apparent coprecipitation of Btk and BLNK (Fig. 2*A*), we have not managed to observe the coprecipitation of Syk and BLNK even in the 293T reconstitution system. This result, which contradicts that of others' reports (9, 11), is probably because the binding of Syk and BLNK is merely transient or is not able to bear under the conditions of our experiments.

Not only B cells but also other hematopoietic cells, including T cells, mast cells, as well as platelets, express Btk/Tec family PTKs (Btk, Itk) (24), Syk/ZAP-70 family PTKs (25) and hematopoietic adaptor molecules [BLNK, LAT, SLP-76 (26) or CLNK/MIST (27, 28)]. Because recent studies have shown that adaptors such as SLP-76 and LAT can complement the function of BLNK (29, 30), it is tempting to assume that a functional role similar to that in Syk-dependent Btk activation may be found among these homologous adaptor molecules. Because it has been shown that Syk is required for Btk activation also in mast cells (8) that express LAT and SLP-76, but not BLNK, it is possible that these adaptor molecules are involved in Syk-dependent Btk activation in these cells. In addition, activation of Itk, a T cell homologue of Btk, was reported to be defective in

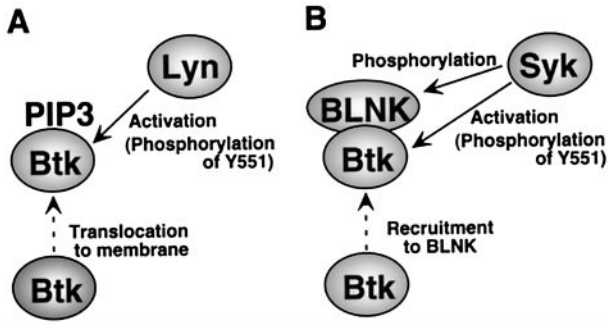

Fig. 5. Proposed mechanism of Btk activation. (*A*) It has been proposed that BCR crosslinking promotes the activation of Lyn and the translocation of Btk to membrane-localized phosphatidylinositol-3,4,5-trisphosphate. This translocation puts Btk in close proximity with Lyn, which subsequently phosphorylates Btk on its tyrosine 551. (*B*) We propose another Btk activation mechanism that the activated Syk phosphorylates BLNK, and then the recruitment of Btk to the phosphorylated BLNK enables Syk to phosphorylate Btk on its tyrosine 551.

ZAP-70 or LAT-deficient T cell lines after CD3 crosslinking (31, 32), which suggests the requirement of ZAP-70 and LAT for Itk activation. These observations raise the possibility that activation of Btk/Tec family PTKs may be generally regulated by both Syk/ZAP-70 kinase and hematopoietic adaptor molecules.

Several observations have suggested that the membrane translocation of Btk is a critical step in its activation. It has been postulated that the translocation of Btk is mediated by the interaction between its pleckstrin homology domain and membrane-localized phosphatidylinositol-3,4,5-trisphosphate, which is generated by phosphatidylinositol 3-kinase activated after BCR crosslinking. This interaction seems to put Btk in close proximity with Src-family PTKs and promote its activation through the tyrosinephosphorylation of Y551 by the latter (refs. 2–4, and Fig. 5*A*). BLNK has also been reported to translocate to the membrane fraction as the result of an unknown mechanism (9, 29). The interaction of Btk and BLNK may further promote their translocation to specific membrane fractions and allow them to interact with activated Syk bound to the immunoreceptor tyrosine-based activation motif of BCR, resulting in Btk phosphorylation and activation by Syk (Fig. 5*B*). It should be noted that, although the reduction of Btk phosphorylation and activation in BLNK-deficient DT40 cells seems to be consistent with the observation that BLNK mediates Btk phosphorylation by Syk in 293T reconstituted cells, it is also possible to postulate that BLNK promotes the membrane translocation of Btk, which may then enhance the access of Btk to Lyn, not only to Syk. Further experiments are needed for an accurate assessment of the contribution of BLNK to Syk-dependent and Lyn-dependent Btk activation.

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