Transphosphorylation of Bruton’s Tyrosine Kinase on Tyrosine 551 Is Critical for B Cell Antigen Receptor Function*

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Bruton's tyrosine kinase (Btk) is required for B cell development and B cell antigen receptor (BCR) function. Cross-linking of BCR induces phosphorylation of Btk at Tyr551 and Tyr223. However, the functional requirement of these phosphorylation for BCR signaling remains unclear. We demonstrate here that mutation of Tyr551, not Tyr223, abrogates the BCR-induced calcium mobilization. Not only Lyn, but also Syk was required for tyrosine phosphorylation of Btk in BCR signaling. These results suggest that transphosphorylation of Btk on Tyr551 is essential for BCR function and that this phosphorylation is mediated through the concerted actions of Lyn and Syk.

The B cell antigen receptor (BCR) is composed of surface immunoglobulin noncovalently associated with a pair of Igα/Igβ disulfide-linked heterodimers, which are essential for signal transduction. Stimulation of the BCR induces the enzymatic activity and tyrosine phosphorylation of three distinct families of nonreceptor cytoplasmic protein tyrosine kinases (PTKs), the Src family, Syk, and Btk. The Src family kinases are rapidly activated after BCR engagement, and their activation correlates with the initial tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif on the BCR (1). During this event, the Src family kinases activate Lyn and Syk downstream of Src family kinases. Utilizing co-overexpression system in fibroblasts and COS cells, it has been demonstrated that Lyn transphosphorylates Btk on Tyr551 in the catalytic domain, a site homologous to the Src family kinase consensus autophosphorylation site (6, 7). This results in a 5–10-fold increase in Btk enzymatic activity (7). The increase in activity also leads to increased autophosphorylation at Tyr223 in the SH3 domain of Btk (8). The identical phosphopeptides were generated after cross-linking of the BCR, indicating that these sites are also tyrosine phosphorylated in B cells (7). Although the importance of phosphorylation of Tyr551 and Tyr223 for fibroblast transformation has been examined (8, 9), functional significance of phosphorylation of Tyr551 and Tyr223 of Btk in BCR signaling remains elusive.

To genetically define the functional relationship among Lyn, Syk, and Btk in BCR signaling, we established each PTK-deficient DT40 B cells (10, 11). Our previous results have shown that the BCR-induced calcium mobilization is abrogated in Btk-deficient DT40 cells and that the loss of Btk does not significantly affect the activation of Lyn and Syk in BCR signaling (11). Here we show that BCR-induced tyrosine phosphorylation of Btk is abolished in Lyn/Syk double-deficient DT40 cells, suggesting that Btk acts downstream of Lyn and/or Syk in BCR signaling. Moreover, this phosphorylation is partially inhibited in Lyn- or Syk-deficient cells, indicating contribution of both Lyn and Syk to Btk phosphorylation. The Btk Y223F mutant was able to restore the BCR-induced calcium mobilization, whereas the Y551F mutant could not. Thus, these results suggest that phosphorylation of Tyr551 of Btk through Lyn and Syk is essential for BCR signaling.

EXPERIMENTAL PROCEDURES

Cells, Antisera, and DNA Transfection—DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. Anti-chicken IgM mAb M4 and anti-Phosphotyrosine mAb (4G10) and anti-T7 mAb were obtained from Upstate Biotechnology, Inc. and Novagen, respectively. T7-tagged and mutant Btk cDNAs were created by polymerase chain reaction, and the resulting constructs were confirmed by DNA sequencing. These cDNAs were cloned into pApuro expression vector (10). For DNA transfection into DT40 cells, DNA was linearized, electroporated, and selected in the presence of puromycin (0.5 μg/ml). The expression of Btk was analyzed by Western blotting.

Fluorescence and Immunoblot Analysis—DT40 cells were stimulated by mAb M4 for indicated time. Cells were solubilized in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM monobenzimidazole, and 0.2 mM sodium vanadate supplemented with protease inhibitors described previously (10). Cell lysates were sequentially incubated (1 h at 4 °C for each incubation with Ab and protein A-Sepharose). For immunoblotting, samples were separated on SDS-PAGE and transferred to nitrocellulose membrane (Amersham Corp.). Filters were incubated with mAb 4G10 or anti-T7 mAb. After washing, filters were developed using a sheep anti-mouse IgG Ab conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL).

In vitro kinase assay, the immunoprecipitates were washed with 20 mM Heps, pH 8, and 150 mM NaCl after washing with lysin buffer. Added to each sample was 50 μl of kinase buffer (20 mM Heps, pH 8, 10 mM magnesium acetate, 10 mM MnCl2) in the absence or presence of ATP (1 μM). Recombinant glutathione S-transferase fusion protein containing a cytoplasmic domain of mouse Igα (glutathione S-transferase/Igα) was made and used as an exogenous substrate (6). The reactions were allowed at 30 °C for 10 min and terminated by the addition of sample buffer.

Calcium and Phosphoinositide Analysis—For calcium analysis, cells (5 × 106) were resuspended in phosphate-buffered saline containing 20 mM Heps, pH 7.2, 5 mM glucose, 0.25% bovine serum albumin, and 1 mM CaCl2, and loaded with 3 μM Fura-2/AM at 37 °C for 45 min. Cells were washed twice and adjusted to 106 cells/ml with continuous monitoring of fluorescence spectrophotometer (model F-2000; Hitachi) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium level were done as described (12).

For phosphoinositide analysis, cells (106) were labeled with myo-[3H]inositol (10 μCi/ml, 105 Ci/mmol) for 4–5 h in inositol-free RPMI 1640 supplemented with 10% dazolized fetal calf serum, then stimulated in the presence of 10 mM LiCl with mAb M4. The soluble inositol phosphates were extracted with trichloroacetic acid at indicated time.

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1 The abbreviations used are: BCR, B cell antigen receptor; PTK, protein tyrosine kinase; Ab, antibody; mAb, monoclonal antibody; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; IP3, inositol 1,4,5-trisphosphate.
points, and applied to AG1-X8 (formate form) ion exchange columns (Bio-Rad) preequilibrated with 0.1 M formic acid. The columns were washed with 10 ml of H2O and 10 ml of 60 mM ammonium formate, 5 mM sodium tetraborate. Elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M).

RESULTS

We have used a genetic approach to determine the requirements for Btk tyrosine phosphorylation following BCR engagement. We utilized three mutants of the chicken B cell line DT40, generated by inactivation of either lyn, syk, or both genes by homologous recombination (10, 11). Since the Ab raised against chicken Btk does not immunoprecipitate efficiently, we expressed an epitope-tagged version of Btk (designated T7-Btk) into wild type and these mutant DT40 cells. Clones expressing similar levels of T7-Btk in these deficient DT40 cells were selected. These clones were lysed prior to and following BCR ligation, and T7-Btk was immunoprecipitated with anti-T7 mAb. As shown in Fig. 1, Btk was inducibly tyrosine-phosphorylated following BCR stimulation in wild type DT40 cells, consistent with previous reports (5, 13–15). In contrast to wild type cells, Lyn/Syk double-deficient DT40 cells failed to exhibit any Btk tyrosine phosphorylation following BCR ligation, indicating requirement of Lyn and/or Syk for the BCR-induced tyrosine phosphorylation of Btk. In Lyn-deficient DT40 cells, tyrosine phosphorylation of Btk at 1 and 3 min after BCR ligation was significantly reduced, whereas this phosphorylation at 10 min reached almost the same level as that in wild type cells. Compared with Lyn-deficient DT40 cells, Syk-deficient cells showed a complementary time course of the BCR-induced phosphorylation of Btk; phosphorylation of Btk was only observed at 1 and 3 min after BCR stimulation. These data suggest that either Lyn or Syk alone is capable of phosphorylating Btk at least to some extent and that the concerted actions of Lyn and Syk are required for full phosphorylation of Btk in BCR signaling.

It has been demonstrated recently that transphosphorylation of Btk at Tyr551 results in increased its enzymatic activity, leading to autophasphorylation of a second tyrosine Tyr223 in a fibroblast system (8). To determine that this sequential phosphorylation of Btk occurs also after BCR stimulation, we transfected Y551F and Y223F mutants of Btk into Btk-deficient DT40 cells (Fig. 2A). Stimulation of BCR did not enhance tyrosine phosphorylation of Btk(Y551F) (Fig. 3). This result suggests the possibility that Tyr551 of Btk is only target of Lyn/Syk-dependent phosphorylation after BCR stimulation in DT40 cells. Alternatively, phosphorylation at Tyr551 is a critical step for subsequent phosphorylation of Btk in BCR signaling context. In contrast to Btk(Y551F), Btk(Y223F) showed increased tyrosine phosphorylation upon BCR stimulation, although this phosphorylation was only observed at 1 min after receptor cross-linking (Fig. 3). Thus, these results implicate that a primary target of tyrosine through concerted actions of Lyn and Syk is Tyr551 of Btk in BCR signaling, leading to phosphorylation of Tyr223. Since Btk(Y223F) did not show significant tyrosine phosphorylation at 3 and 10 min after receptor stimulation, our finding also suggests that phosphorylation of Tyr223 is required for sustained Btk phosphorylation in BCR signaling.

Utilizing Btk-deficient DT40 cells expressing Btk(Y551F) and Btk(Y223F), we analyzed the effects of these mutations on BCR signaling. Wild type and kinase-negative T7-Btk were also transfected into Btk-deficient DT40 cells as a positive and a negative control, respectively (Fig. 2A). To determine whether these mutations affect tyrosine kinase activity, Btk immunoprecipitates were used for in vitro kinase assays with glutathione S-transferase ligands as exogenous substrate. Btk(R525Q) exhibited no kinase activity, indicating that the immunoprecipitates are largely free of contaminating tyrosine kinases. When the extent of tyrosine phosphorylation is normalized to the amount of protein present in each kinase assay, both Btk(Y551F) and Btk(Y223F) had similar transphosphorylation activity compared with wild type Btk (Fig. 2B). We showed previously that the BCR-induced PLC-γ2 activation is
pressed Syk itself may not be fully activated in heterologous systems. To test this possibility, we transfected T7-Btk into Syk-deficient cells expressing Syk mutant in which Tyr\textsuperscript{518}/Tyr\textsuperscript{519} is exchanged to Phe\textsuperscript{518}/Phe\textsuperscript{519} in this mutant DT40 cell, the BCR-induced tyrosine phosphorylation of Btk was similar to that in Syk-deficient DT40 cells (data not shown), implicating that the requirement of Syk is due to up-regulated Syk through transphosphorylation of Tyr\textsuperscript{518}/Tyr\textsuperscript{519}. It is also possible that Btk may not be a direct substrate of Syk. Assuming that another PTK acts downstream of Syk in BCR signaling, our data might be accounted for by the involvement of this Syk-regulated PTK in tyrosine phosphorylation of Btk.

It has been reported that two tyrosines, Tyr\textsuperscript{551} and Tyr\textsuperscript{223}, are phosphorylated after cross-linking of BCR. Btk(Y551F) exhibited no tyrosine phosphorylation upon BCR cross-linking, whereas Btk(Y223F) showed an increase of tyrosine phosphorylation at 1 min after receptor stimulation, suggesting that Tyr\textsuperscript{551} phosphorylation is a prerequisite for subsequent phosphorylation of Btk in BCR signaling events. Thus, these results support the previous contention that phosphorylation of Btk at Tyr\textsuperscript{551} is followed by its autophosphorylation at Tyr\textsuperscript{223} (7, 8). Since Btk(Y223F) did not show significant tyrosine phosphorylation at 3 and 10 min after receptor stimulation, phosphorylation of Tyr\textsuperscript{223} appears to be required for sustained Btk phosphorylation. Recent crystallographic analysis of Itk (Btk/Tec family PTK expressed in T cells) may provide insights into the mechanism for phosphorylation of Tyr\textsuperscript{223} of Btk (17). Based on this analysis, the proline-rich domain adjacent to the SH3 domain of Btk/Tec family kinases contains an SH3 ligand (Fig. 2A), allowing intramolecular interaction. Interestingly, Tyr\textsuperscript{223} of Btk is located within the interface of this interaction. Thus, phosphorylation of Tyr\textsuperscript{223} may disrupt this intramolecular interaction, thereby changing the conformation of Btk. This conformational change might be required for sustained phosphorylation of Btk in BCR signaling.

Our functional data clearly indicate that Tyr\textsuperscript{551} is essential for BCR signaling, whereas Tyr\textsuperscript{223} is dispensable for BCR-induced PLC-\gamma activation. Since phosphorylation of Tyr\textsuperscript{551} was already reported to increase the kinase activity of Btk with 5–10-fold (7), one of the consequence of phosphorylation of Tyr\textsuperscript{551} is increased kinase activity upon BCR cross-linking. Although we carried out in vitro kinase assay on Btk immunoprecipitates in wild type DT40 cells, the BCR-induced activation of its in vitro kinase activity could not be reproducibly observed. Previous reports suggest that the magnitude of the BCR-induced activation of Btk is significantly smaller than that of maximum Btk phosphorylation by Lyn in heterologous systems (6, 7). Indeed, in our hand, this 5–10-fold activation of Btk in COS cells could be reproducibly detected. Thus, the most likely explanation is that the increase of Btk enzymatic activity through phosphorylation of Tyr\textsuperscript{551} in DT40 cells is too small for our detection system.

In the case of Syk, it has been demonstrated that in addition to recruitment of Syk to phosphorylated Igα/Igβ, phosphorylation at tyrosines (Tyr\textsuperscript{518} and/or Tyr\textsuperscript{519}) within its activation loop is critical for BCR signal transduction (16). In this report, we show that phosphorylation of Tyr\textsuperscript{518} on Btk activation loop is also an obligatory mechanism for its participation in BCR signaling. Thus, cytoplasmic PTK cascade through phosphorylation of tyrosine located in the activation loop may be one of the general mechanisms for cytoplasmic signal transduction.

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FIG. 4. Functional restoration of BCR signaling by Btk(Y223F) and Btk(Y551F). Calcium mobilization (A), IP\textsubscript{3} generation (B), and tyrosine phosphorylation of PLC-\gamma2 (C). C, Btk-deficient DT40 cells expressing these mutants were stimulated for 3 min with M4 (0.4 \mu M). Cells were lysed in 1% Nonidet P-40 lysis buffer and immunoprecipitated with anti-PLC-\gamma2 Ab. Immunoprecipitates were loaded on 6% SDS-PAGE, and the blotted membrane was incubated with mAb 4G10. After the filter was stripped, the same blot was reprobed with anti-PLC-\gamma2 Ab \( pY \), phosphotyrosine.

abrogated in Btk-deficient DT40 cells, leading to loss of calcium mobilization (11). Thus, we examined whether these mutations were able to restore these defects or not. As shown in Fig. 4A, DT40 cells expressing Btk(Y223F) exhibited normal calcium mobilization, whereas Btk(Y551F) was able to mobilize only small amount of calcium upon receptor cross-linking. Consistent with these data, cross-linking of BCR on DT40 cells expressing Btk(Y223F) stimulated inositol 1,4,5-trisphosphate (IP\textsubscript{3}) production and tyrosine phosphorylation of PLC-\gamma2, although these parameters were lower than wild type Btk (Fig. 4, B and C). This might reflect the lower expression level of Btk(Y223F) than wild type Btk (Fig. 2B). The BCR-induced IP\textsubscript{3} production and tyrosine phosphorylation of PLC-\gamma2 in DT40 cells expressing Btk(Y551F) was essentially the same as those in DT40 cells expressing Btk(R525Q) (Fig. 4 and data not shown). These results demonstrate that phosphorylation of Tyr\textsuperscript{551}, not Tyr\textsuperscript{223}, is essential for BCR signaling.

DISCUSSION

In Lyn-deficient DT40 cells, BCR-induced tyrosine phosphorylation of Btk was significantly inhibited at 1 and 3 min after stimulation (Fig. 1), indicating that Btk phosphorylation is mediated by Lyn in BCR signaling in these early time points. However, at 10 min after stimulation, this phosphorylation reached almost the same level in wild type cells, suggesting that tyrosine phosphorylation of Btk at 10 min after BCR stimulation is independent of Lyn. In contrast to Lyn-deficient DT40 cells, Syk-deficient cells show the profound inhibition of the BCR-induced tyrosine phosphorylation of Btk at 10 min after receptor cross-linking, implicating that this Btk phosphorylation is mediated by Syk. Taken together, these data suggest that the initial Btk phosphorylation and sustained phosphorylation are mediated by coordinated actions of Lyn and Syk after BCR cross-linking.

Our conclusion is somewhat inconsistent with the previous reports using COS cell and fibroblast expression systems (6, 7). In these systems, Lyn is able to phosphorylate Btk, whereas Syk is not. One of the possibilities about this disparity between the heterologous systems and DT40 B cell system is that Syk is maximally activated through transphosphorylation at Tyr\textsuperscript{518} and/or Tyr\textsuperscript{519} by Lyn in DT40 cells (16). In contrast, overex-
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