

Tyrosine Phosphorylation and Activation of Bruton Tyrosine Kinase upon FcεRI Cross-Linking†

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Tyrosine phosphorylation of several cellular proteins is one of the earliest signaling events induced by cross-linking of the high-affinity receptor for immunoglobulin E (FcεRI) on mast cells or basophils. Tyrosine kinases activated during this process include the Src family kinases, Lyn, c-Yes, and c-Src, and members of another subfamily, Syk and PTK72 (identical or highly related to Syk). Recently, some of us described two novel tyrosine kinases, Emb and Emt, whose expression was limited to subsets of hematopoietic cells, including mast cells. Emb turned out to be identical to Btk, a gene product defective in human X-linked agammaglobulinemia and in X-linked immunodeficient (*xid*) mice. Here we report that FcεRI cross-linking induced rapid phosphorylation on tyrosine, serine, and threonine residues and activation of Btk in mouse bone marrow-derived mast cells. A small fraction of Btk translocated from the cytosol to the membrane compartment following receptor cross-linking. Tyrosine phosphorylation of Btk was not induced by either a Ca²⁺ ionophore (A23187), phorbol 12-myristate 13-acetate, or a combination of the two reagents. Coimmunoprecipitation between Btk and receptor subunit β or γ was not detected. The data collectively suggest that Btk is not associated with FcεRI but that its activation takes place prior to protein kinase C activation and plays a novel role in the FcεRI signaling pathway.

Cross-linking of the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on mast cells and basophils induces a variety of morphological and biochemical changes, culminating in the release of chemical mediators (17). Among early intracellular signaling events, tyrosine phosphorylation of several proteins is thought to be of primary importance (2, 4, 7, 18, 21, 26, 40). Thus, protein-tyrosine kinase (PTK) inhibitors prevent not only tyrosine phosphorylation of cellular proteins but also other early events, including the production of inositol 1,4,5-trisphosphate and histamine release (18). Therefore, PTK activation is thought to be proximal to the activation of phospholipase C (PLC) and protein kinase C (PKC), two enzymes considered essential for mast cell activation.

Two subfamilies of PTKs are implicated in this process. The Src family PTKs p56^{lyn} in RBL-2H3 (a rat basophilic leukemia cell line) and p62^{c-yes} in PT-18 (a mouse mast cell line) were found to be associated with FcεRI and activated upon receptor cross-linking (8). p60^{c-src} in RBL-2H3 cells was also activated subsequent to receptor cross-linking, although its association with the receptor was not detected. Members of another PTK subfamily, p72^{syk} and PTK72 (which is identical or highly related to p72^{syk}), were also shown to be activated by FcεRI cross-linking (3, 16). The association of p72^{syk} with the receptor depends on tyrosine phosphorylation of the γ subunit of the receptor (3).

In a recent report, some of us described two novel PTK genes expressed in murine mast cells (37). One, termed *emb*, turned out to be identical to the *btk* gene, whose mutations in humans and mouse strain CBA/N cause X-linked agammaglobulinemia (34, 36) and X-linked immunodeficiency (i.e., the

xid phenotype) (29, 33), respectively. The product of the *btk* gene together with the *tec* (23) and *emt* (= *itk* or *tsk*) (12, 15, 31, 37) gene products constitutes a novel PTK subfamily. The present study was carried out to elucidate the function of Btk in mast cell activation, and this report describes tyrosine phosphorylation and activation of Btk upon cross-linking of FcεRI on murine mast cells. In an attempt to localize the putative site of Btk action relative to other signaling events in the FcεRI signaling pathway, experiments on subcellular localization, associated molecules, and the effects of pharmacological agents on the Btk kinase have also been performed.

MATERIALS AND METHODS

Reagents. Antidinitrophenyl (anti-DNP) IgE and DNP conjugates of human serum albumin (DNP-HSA) were provided by Kimishige Ishizaka and Teruko Ishizaka, respectively. Antiphosphotyrosine monoclonal antibody (MAb) 4G10 and anti-PLC-γ1 MAbs were purchased from Upstate Biotechnology Inc. Anti-FcεRI β subunit MAb and polyclonal anti-FcεRI γ subunit antibody were provided by Jean-Pierre Kinet (National Institutes of Health). Anti-PKC MAb (MC5) and anti-Lyn antibody were obtained from Santa Cruz Biotechnology. Purified mouse recombinant interleukin-3 (IL-3) and a soluble form of stem cell factor (SCF) expressed in *Escherichia coli* were gifts from Kirin Brewery Co. Phorbol 12-myristate 13-acetate (PMA), A23187, and other chemicals of the highest available purity were obtained from Sigma unless otherwise indicated.

Cells. Bone marrow-derived mouse mast cells (BMMC) and PT-18 mouse mast cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, and culture supernatants of mouse IL-3 gene-transfected cells. BMMC (>95% pure mast cells) were used after 4 weeks of culture. Passive sensitization of mast cells with anti-DNP IgE and stimulation with DNP-HSA

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were performed as described previously (18). In some experiments, BMMC depleted of IL-3 for 16 to 18 h were incubated with 100 ng of either mouse recombinant IL-3 or mouse SCF per ml for various lengths of time before cell lysis. Growth stimulation was monitored by [³H]thymidine uptake as described previously (11). In one experiment (see Fig. 1C), BMMC were incubated with ³²P_i for the last 4 h of the IgE sensitization period. One millimolar Na₃VO₄ was added 30 min prior to antigen stimulation.

Cell lysis and immunoprecipitation. Cells were lysed in 1% Nonidet P-40 on ice for 30 min unless otherwise indicated. Lysates cleared by centrifugation (15,000 × *g*, 10 min, 4°C) were immunoprecipitated with anti-Btk antiserum (34) or other antibodies. Immune complexes were recovered with Pansorbin (Calbiochem) or anti-rabbit-IgG-coated agarose (Sigma). In some experiments, cell lysates were precleared by incubation with normal rabbit serum and Pansorbin before immunoprecipitation with anti-Btk antibody.

Immunoblotting. Proteins in immune complexes or cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Incubation with primary antibody, washings, incubation with horseradish peroxidase-conjugated secondary antibody, and washings were followed by detection with an enhanced chemiluminescence kit (Amersham).

Immune complex kinase reactions. Immune complexes were incubated with [γ -³²P]ATP in the presence or absence of acid-denatured enolase (Sigma). Reaction solutions mixed with Laemmli sample buffer were analyzed by SDS-PAGE, blotted, and subjected to autoradiography (9). In some experiments (see Fig. 5), ³²P-labeled proteins were released from immunoglobulins and Pansorbin by incubation at 95°C for 3 min with 1% SDS and 10 mM dithiothreitol and then diluted 10-fold with Nonidet P-40 lysis buffer before reimmunoprecipitation.

Phosphoamino acid analysis. ³²P-labeled proteins on Immobilon-P pieces were incubated with 6 N HCl at 110°C for 2 h. Hydrolysates were lyophilized, then dissolved in a solution containing cold phosphoamino acids, and separated in two dimensions. First-dimensional separation was done by electrophoresis, and second-dimensional separation was done by ascending chromatography (15a). Radioactive phosphoamino acids were detected by autoradiography. The positions of phosphoamino acids were determined by staining with ninhydrin.

Subcellular fractionation. Cells were fractionated as described previously (5). Briefly, cells were homogenized in a hypotonic buffer. Nuclei were pelleted through a 1 M sucrose cushion. Supernatants were fractionated into pellets (membrane fraction) and supernatants (cytosol) by centrifugation at 100,000 × *g* for 30 min at 4°C. Pellets were solubilized in 1% Nonidet P-40-containing buffer. Btk, PKC, and PLC- γ 1 in each fraction were detected by immunoblotting with specific antibodies. The purity of each fraction was monitored by measuring the cytosol marker enzyme lactate dehydrogenase activity as described previously (31a). The distribution of this enzyme in a typical experiment was 84% cytosol, 12% nuclear material, and 4% particulate.

RESULTS

Btk is tyrosine phosphorylated upon FcεRI cross-linking. Although a 72-kDa protein was originally described as a major PTK substrate whose phosphorylation was induced by FcεRI cross-linking (2), subsequent studies (3, 25) have shown that several proteins in this size range are phosphorylated on

tyrosine residues. Since Btk is a 77-kDa PTK, we tested the possibility that Btk itself becomes phosphorylated on tyrosine. BMMC were passively sensitized with anti-DNP IgE and then stimulated by a multivalent antigen, DNP-HSA, for various lengths of time. Precleared detergent lysates were immunoprecipitated with anti-Btk antiserum, and immune complexes were subjected to immunoblot analysis. As shown in Fig. 1A, probing with antiphosphotyrosine MAb (4G10) demonstrated that the level of tyrosine phosphorylation of a 77-kDa protein increased markedly from a low basal level after FcεRI cross-linking. The increase in tyrosine phosphorylation of this protein was detected in less than a minute and reached the maximal level (more than 20-fold over the basal level) around 1 to 3 min after stimulation. Thereafter, the tyrosine phosphorylation level decreased and returned to the basal level within 60 min (Fig. 1A and data not shown). The identity of the 77-kDa protein as p77^{btk} was confirmed by reprobing the same stripped blot with anti-Btk antibody. Other tyrosine-phosphorylated proteins, i.e., 170-, 115- to 120-, 65-, and 62-kDa proteins that were seen after a longer exposure and that were coprecipitated by anti-Btk antibody, remain to be identified. For comparison, tyrosine phosphorylation of Lyn was also examined (Fig. 1A). Rapid and transient induction of the Lyn tyrosine phosphorylation was observed, which is consistent with the previous report on the activation of Lyn in RBL-2H3 cells (8). In order to further confirm the increased tyrosine phosphorylation of p77^{btk}, tyrosine-phosphorylated proteins were affinity purified by incubation of lysates of unstimulated or antigen-stimulated BMMC with antiphosphotyrosine MAb followed by elution with a phosphotyrosine analog, *p*-nitrophenyl phosphate. Tyrosine-phosphorylated proteins were analyzed by immunoblotting with anti-Btk antibody. Essentially the same result as the one described above was obtained (data not shown). Similar experiments were done with a mouse mast cell line, PT-18 (Fig. 1B). Tyrosine phosphorylation of Btk increased upon FcεRI cross-linking by 1.5- to 3-fold. This lower relative increase is apparently due to the high basal level of tyrosine phosphorylation in this cell line.

We next metabolically labeled cellular proteins with ³²P and determined whether Btk is phosphorylated on other residues. The phosphorylation level of Btk in IgE- and antigen-stimulated BMMC was higher than that in unstimulated cells (Fig. 1C, left). Phosphoamino acid analysis revealed an increase not only in tyrosine phosphorylation but also in serine and threonine phosphorylations (Fig. 1C, right). Immunoprecipitation with anti-Btk from ³²P-labeled cell lysates demonstrated that Btk is associated with several phosphorylated proteins (e.g., >200-, 200-, 160-, 110-, and 65-kDa proteins), some of which may correspond to the tyrosine-phosphorylated, Btk-associated proteins shown in Fig. 1A.

Pharmacological agents such as PMA and A23187 partially mimic mast cell activation. PMA activates PKC and p95^{av}, a hematopoietic cell guanine nucleotide exchange factor for Ras (13). On the other hand, A23187 mobilizes Ca²⁺ from intracellular storage sites. A short-term (10-min) treatment of BMMC with 100 nM PMA, 100 nM A23187, or a combination of the two failed to increase tyrosine phosphorylation of Btk (Fig. 2). Furthermore, FcεRI cross-linking induced Btk tyrosine phosphorylation to nearly the full extent even after prolonged (18-h) PMA treatment (Fig. 2, lane 8). Chronic treatment with PMA down-regulated α and β isoforms of PKC, as determined by immunoblotting with anti-PKC (MC5) MAb (data not shown). These data suggest that Btk tyrosine phosphorylation occurs proximal to the increase in the intracellular Ca²⁺ concentration and the activation of PKC, events known to occur subsequent to FcεRI cross-linking.

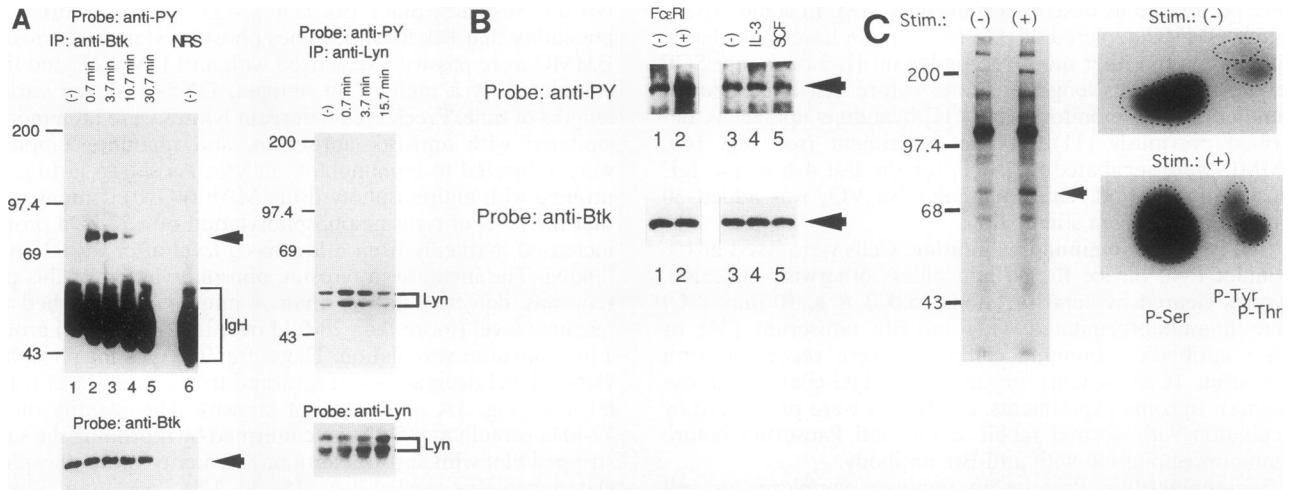


FIG. 1. Phosphorylation of Btk induced by Fc ϵ RI cross-linking. (A) BMMC, sensitized with anti-DNP IgE, were left unstimulated (lanes 1 and 6) or were stimulated with DNP-HSA for the indicated lengths of time (lanes 2 to 5). Cell lysates were immunoprecipitated with anti-Btk serum, normal rabbit serum (NRS), or anti-Lyn. Immune complexes were analyzed by SDS-PAGE and blotted. Membranes were probed with antiphosphotyrosine (PY) MAb (upper left and right) and reprobed with anti-Btk (lower left) or anti-Lyn (lower right) after previously attached immunoglobulins were stripped. The positions of molecular mass standards in kilodaltons are shown at the left of the gels. Btk is indicated by arrowheads. IP, immunoprecipitating antibody. (B) IgE-sensitized PT-18 cells were left unstimulated (lane 1) or were stimulated with antigen for 1 min (lane 2). A separate set of PT-18 cells were depleted of IL-3 for 18 h and then incubated with medium (lane 3), 100 ng of recombinant mouse IL-3 per ml (lane 4), or 100 ng of recombinant mouse SCF per ml (lane 5) for 10 min. Cell lysis, immunoprecipitation with anti-Btk, and immunoblotting first with antiphosphotyrosine MAb (upper) and then with anti-Btk (lower) were done as for panel A. (C) BMMC were metabolically labeled with ^{32}P and left unstimulated (-) or were stimulated with IgE and antigen (Stim.) (+). Cell lysis, immunoprecipitation with anti-Btk, SDS-PAGE, and blotting were done as for panel A. ^{32}P -labeled proteins were visualized by autoradiography (left). Radiolabeled Btk proteins on the excised membranes were subjected to phosphoamino acid analysis. The relative positions of phosphorylated amino acids are indicated at the bottom (right).

Since Fc ϵ RI cross-linking induces cell proliferation (32) in addition to many other changes in mast cell physiology, we determined whether increased tyrosine phosphorylation of Btk is induced by other mitogenic stimuli. Incubation of growth factor-deprived BMMC or PT-18 cells with IL-3 or SCF for 10 to 30 min did not induce detectable tyrosine phosphorylation of Btk (Fig. 1B and unpublished data). However, both cytokines induced tyrosine phosphorylation of numerous other proteins and stimulated [^3H]thymidine uptake (data not shown). Therefore, these data suggest that tyrosine phosphor-

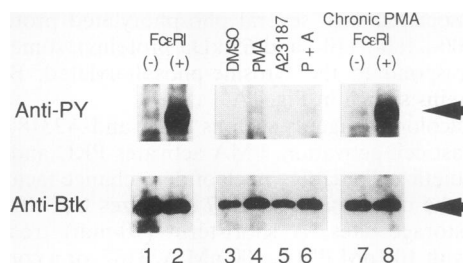


FIG. 2. Effects of PMA and A23187 on Btk tyrosine phosphorylation in BMMC. BMMC were treated with 100 nM PMA (lane 4), 100 nM A23187 (lane 5), both of these reagents (lane 6), or a vehicle, dimethyl sulfoxide (DMSO) (lane 3), for 10 min. Another set of the cells were sensitized overnight with anti-DNP IgE in the presence (lanes 7 and 8) or absence (lanes 1 and 2) of 100 nM PMA and then left unstimulated (lanes 1 and 7) or were stimulated with DNP-HSA for 5 min (lanes 2 and 8). Btk immune complexes prepared from these cells were analyzed by immunoblotting with antiphosphotyrosine (PY) MAb (upper) and subsequently with anti-Btk (lower). The positions of Btk are indicated by arrowheads.

ylation of Btk may be involved in the Fc ϵ RI signaling pathway but not in cell growth in general.

Tyrosine phosphorylation of Btk is accompanied by an increase in its *in vitro* kinase activity. As shown with many PTKs involved in signal transduction pathways for Fc ϵ RI as well as for T- and B-cell antigen receptors (35, 38), tyrosine phosphorylation of PTKs is associated with their activation. We examined, therefore, the kinase activity of Btk in an *in vitro* immune complex kinase assay. Lysates prepared from unstimulated or antigen-stimulated BMMC were immunoprecipitated with anti-Btk antibody, and immune complexes were incubated with [γ - ^{32}P]ATP in the presence or absence of an exogenous substrate, enolase. Reaction products were analyzed by SDS-PAGE and blotted. Autoradiography of the blot (Fig. 3, left) shows a significant increase (1.5- to 3-fold at its peak) in Btk-phosphorylating activity upon Fc ϵ RI cross-linking. The increase in Btk-phosphorylating activity was as rapid as the increase in tyrosine phosphorylation, but Btk phosphorylating activity lasted longer than tyrosine phosphorylation. Although the increases in the Btk-phosphorylating and enolase-phosphorylating activities were relatively small, they were consistently observed in five different Btk preparations. As was shown previously with RBL-2H3 cells (8), we observed a modest increase (\sim twofold) in the Lyn kinase activity to autophosphorylate and phosphorylate enolase (Fig. 3, right). Since phosphoamino acid analysis indicated the presence of a coprecipitating serine/threonine kinase activity in Btk immune complexes (data not shown), the extent of tyrosine phosphorylation of enolase by Btk was examined by probing the same blot with antiphosphotyrosine MAb. The result (Fig. 3, middle) shows that the enolase tyrosine-phosphorylating activity in Btk immune complexes was remarkably increased by Fc ϵ RI cross-

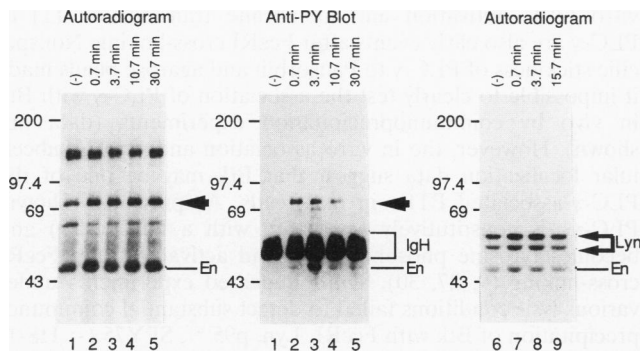


FIG. 3. Activation of Btk enzymatic activity upon FcεRI cross-linking. BMDC stimulation, cell lysis, and immunoprecipitation with anti-Btk or anti-Lyn were done as described for Fig. 1A. Immune complexes were subjected to *in vitro* kinase assays with acid-denatured enolase as an exogenous substrate. Reaction products were analyzed by SDS-PAGE and blotted. The Btk blot was exposed to an X-ray film (left) and then probed with antiphosphotyrosine (PY) MAb (middle). The Lyn blot was exposed to an X-ray film (right). Btk is indicated by arrowheads. The positions of Lyn (arrows), enolase, and immunoglobulin heavy chains are also shown.

linking. The maximal activity (5- to 10-fold over the basal level) was attained around 3 min after antigen stimulation.

As expected from the finding that IL-3 or SCF did not induce tyrosine phosphorylation of Btk, growth stimulation provided by IL-3 or SCF did not change the Btk-phosphorylating and enolase-phosphorylating activities of Btk (data not shown), suggesting that the enhanced activity of Btk following FcεRI cross-linking is specifically coupled to its increased tyrosine phosphorylation.

Btk localizations and FcεRI-stimulated translocation. The rapid activation of Btk suggests that it plays a role in membrane-proximal events upon FcεRI cross-linking. In order to obtain further clues on the function of Btk in mast cells, its subcellular localization was determined. Unstimulated or antigen-stimulated BMDC were fractionated into nuclear, particulate (membrane), and soluble (cytosol) compartments, and Btk proteins in each fraction were detected by immunoblotting (Fig. 4). Btk was found mostly (>90%) in the cytosol in unstimulated cells, while a small portion ($\leq 5\%$) was also found in the membrane fraction. A faint signal of p77^{Btk} was consistently observed in nuclear fractions after a long exposure.

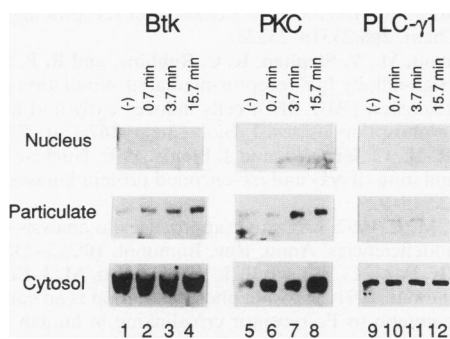


FIG. 4. Subcellular localization of Btk during mast cell activation. IgE-sensitized BMDC were stimulated with antigen for the indicated time intervals prior to subcellular fractionation as described in Materials and Methods. Equivalent amounts of each fraction were analyzed by immunoblotting with anti-Btk, anti-PKC (MC5), or anti-PLC-γ1.

However, we cannot exclude the possibility that this is due to contamination from other compartments. Interestingly, the level of Btk in membrane fractions increased after antigen stimulation. The increase in membrane-associated Btk, detected in less than 1 min, persisted for at least 15 min following receptor cross-linking (Fig. 4). When the same blot was reprobbed with anti-PLC-γ1, we observed a transient increase of PLC-γ1 in the membrane compartment after FcεRI cross-linking, an observation consistent with the previous report on the translocation of PLC-γ1 in RBL-2H3 cells (1). We also reprobbed the same blot with anti-PKC (MC5), which reacts with α, β, and γ isoforms. Consistent with the previous report (25a), the amounts of membrane-associated PKC increased following FcεRI cross-linking. Interestingly, the kinetics of FcεRI-mediated increases in membrane-associated Btk and PKC were similar. This is the first observation that Btk can be present in the membrane compartment despite the lack of apparent membrane-anchoring sequences or known posttranslational modifications for membrane attachment, such as myristylation (as seen in Src family PTKs).

Btk is not associated with FcεRI. Both Src family PTKs, Lyn and c-Yes, and Syk PTK were shown to be capable of associating with FcεRI, suggesting their roles in the receptor-proximal events. We investigated the possibility that Btk might be associated with FcεRI. To this end, unstimulated or IgE- and antigen-stimulated BMDC or PT-18 cells were solubilized with mild detergents (10 mM 3-[(3-cholamidopropyl)-dimethylammonia-1-propanesulfonate [CHAPS], 1% digitonin, or 0.5 to 1% Nonidet P-40) and immunoprecipitated with anti-Btk. Btk immunoprecipitates were analyzed by immunoblotting with anti-FcεRI β MAb or anti-FcεRI γ antibodies. We failed to detect any specific signals for these proteins (data not shown). In reverse immunoprecipitations, anti-FcεRI β or anti-FcεRI γ immunoprecipitates were analyzed by immunoblotting with anti-Btk. Again the results were negative (data not shown). Under the same conditions, anti-Lyn immunoprecipitates contained the proteins immunoreactive with anti-FcεRI β and anti-FcεRI γ (data not shown).

We next adopted a more sensitive method to detect possible association. Immune complexes precipitated with anti-FcεRI γ from CHAPS lysates of unstimulated or IgE- and antigen-stimulated BMDC were subjected to an *in vitro* kinase assay with [γ -³²P]ATP. ³²P-labeled proteins were released from antibodies and Pansorbin and reimmunoprecipitated with anti-Btk or anti-Lyn. As shown in Fig. 5, there were no Btk proteins in the anti-FcεRI γ immunoprecipitates, while strong signals specific for Lyn (p56^{lyn} and p53^{lyn}) were clearly detected. This experiment confirmed the previous observation that more Lyn kinase activity was recruited to FcεRI upon receptor cross-linking (8). Essentially identical results were obtained with *in vitro* kinase assays with anti-FcεRI β immunoprecipitates (data not shown). Therefore, we conclude that Btk is not physically associated with FcεRI.

DISCUSSION

Btk is a member of a recently recognized PTK subfamily displaying several distinct structural features. Members of this subfamily have an amino-terminal pleckstrin homology domain (14, 24) that is followed by SH3 and SH2 domains and have an autophosphorylated tyrosine residue corresponding to Tyr-416 in p60^{c-src}. They lack, however, a carboxy-terminal negative regulatory tyrosine residue corresponding to Tyr-527 in p60^{c-src}. Therefore, it is expected that Btk is regulated in a manner different from that of Src family PTKs, which have both autophosphorylated and negative regulatory tyrosine

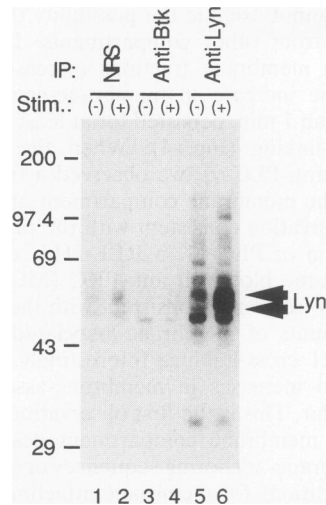


FIG. 5. Fc ϵ RI association with Lyn but not with Btk. Unstimulated (-) or IgE- and antigen-stimulated (Stim.) (+, 3 min with antigen) BMMC were solubilized in 10 mM CHAPS. Immune complexes precipitated with anti-Fc ϵ RI γ were subjected to in vitro kinase assays with [γ - 32 P]ATP. 32 P-labeled proteins released from immunoglobulins and Pansorbin were reimmunoprecipitated with anti-Btk, anti-Lyn, or normal rabbit serum (NRS) and analyzed by SDS-PAGE and autoradiography. IP, immunoprecipitating antibody.

residues. This study was undertaken in order to determine the properties of Btk in mast cells. In this report we present evidence that Btk is phosphorylated on tyrosine as well as serine and threonine residues and is enzymatically activated upon Fc ϵ RI cross-linking in murine mast cells. Furthermore, a small fraction of Btk, localized in the cytosol in unstimulated cells, translocated to the membrane upon Fc ϵ RI cross-linking. To our knowledge, this is the first study to demonstrate activation of Btk by extracellular stimulation. Similar to Btk in mast cells, Emt, another member of the same subfamily, was phosphorylated on tyrosine in T cells following stimulation through the T-cell-receptor-CD3 complex (19), suggesting similar roles of this PTK subfamily in signal transduction pathways initiated by immune cell antigen receptors. B-cell differentiation is impaired at the pre-B- or pro-B-cell stage by various mutations in the *btk* gene in X-linked agammaglobulinemia patients and CBA/N (*xid*) mice (reviewed in references 6 and 20). However, given that Btk is expressed at similarly high levels in B-lineage cells, myeloid cells, and mast cells, this kinase may have another function, independent of its function in B-cell maturation. Our present study supports the notion that Btk functions in the Fc ϵ RI signaling pathway.

The rapidity of Btk tyrosine phosphorylation and activation, the membrane translocation, and the lack of effect of PMA or A23187 on Btk phosphorylation collectively suggest that Btk may play a role in a very early phase of mast cell activation, prior to the increase in the intracellular [Ca^{2+}] and PKC activation. In this regard, it is interesting first that Btk was purified from a rat basophilic leukemia cell line (RBL-2H3) as a major enzyme that phosphorylates a peptide corresponding to the cytoplasmic sequence of the Fc ϵ RI γ subunit (28). Studies to examine the enzyme-substrate relationship between Btk and Fc ϵ RI γ are under way. Second, the notion of a putative role of Btk in an early phase of mast cell activation is consistent with our preliminary observation that PLC- γ 1 and PLC- γ 2 in lysates of both unstimulated and antigen-stimulated mast cells can interact with bacterial Btk fusion proteins in

vitro (39). Activation and membrane translocation (1) of PLC- γ are also early events after Fc ϵ RI cross-linking. Nonspecific stickiness of PLC- γ to Pansorbin and agarose beads made it impossible to clearly test the association of PLC- γ with Btk in vivo by coimmunoprecipitation experiments (data not shown). However, the in vitro association and similar subcellular localization data suggest that Btk may be one of the PLC- γ -associated PTKs in mast cells. As previously shown, PLC- γ 1 is constitutively associated with a PTK(s) (9) and becomes tyrosine phosphorylated and activated upon Fc ϵ RI cross-linking (9, 27, 30). Third, repeated experiments under various lysis conditions failed to detect substantial coimmunoprecipitation of Btk with Fc ϵ RI, Lyn, p95^{vav}, SPY75 (= HS-1) (10), or Nck (22) as measured by immunoblotting with the corresponding antibodies (data not shown) or by in vitro kinase assays (Fig. 5 and data not shown). In contrast, other Fc ϵ RI-regulated PTKs such as Lyn (8) and p72^{src} (3, 16) associate with Fc ϵ RI. Receptor cross-linking is required for p72^{src}-Fc ϵ RI interactions (3). This difference suggests that Btk may play a role different from that of these Fc ϵ RI-regulated PTKs. Spatiotemporal relations among the Fc ϵ RI-regulated PTKs remain to be established.

In summary, Btk is rapidly phosphorylated and activated upon Fc ϵ RI cross-linking. Btk phosphorylation and activation are not induced by cytokines, PMA, or a Ca^{2+} ionophore. In addition, membrane translocation of Btk and in vitro association with PLC- γ suggest that Btk functions in membrane-proximal events following Fc ϵ RI cross-linking.

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