Involvement of Bruton's Tyrosine Kinase in FcεRI-dependent Mast Cell Degranulation and Cytokine Production

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Summary

We investigated the role of Bruton's tyrosine kinase (Btk) in FcεRI-dependent activation of mouse mast cells, using *xid* and *btk null* mutant mice. Unlike B cell development, mast cell development is apparently normal in these *btk* mutant mice. However, mast cells derived from these mice exhibited significant abnormalities in FcεRI-dependent function. *xid* mice primed with anti-dinitrophenyl monoclonal IgE antibody exhibited mildly diminished early-phase and severely blunted late-phase anaphylactic reactions in response to antigen challenge in vivo. Consistent with this finding, cultured mast cells derived from the bone marrow cells of *xid* or *btk null* mice exhibited mild impairments in degranulation, and more profound defects in the production of several cytokines, upon FcεRI cross-linking. Moreover, the transcriptional activities of these cytokine genes were severely reduced in FcεRI-stimulated *btk* mutant mast cells. The specificity of these effects of *btk* mutations was confirmed by the improvement in the ability of *btk* mutant mast cells to degranulate and to secrete cytokines after the retroviral transfer of wild-type *btk* cDNA, but not of vector or kinase-dead *btk* cDNA. Retroviral transfer of Emt (= Itk/Tsk), Btk's closest relative, also partially improved the ability of *btk* mutant mast cells to secrete mediators. Taken together, these results demonstrate an important role for Btk in the full expression of FcεRI signal transduction in mast cells.

Mast cells and basophils play pivotal roles in the initiation of allergic reactions. Cross-linking of the high-affinity receptor for IgE (FcεRI) on these cells activates intracellular signaling pathways that lead to degranulation and release of histamine and other preformed mediators, de novo synthesis and release of lipid mediators, and secretion of preformed and de novo synthesized cytokines (1, 2). These bioactive mediators are thought to lead to allergic inflammation.

FcεRI consists of one molecule of an α subunit that is capable of binding to IgE, one molecule of a β subunit with four transmembrane segments, and two molecules of disulfide-bonded γ subunits (3). None of these subunits have discernible enzyme structures, but both the β and γ subunits have the immunoreceptor tyrosine-based activation motif (ITAM; references 4, 5).[1] After FcεRI cross-linking, tyrosine phosphorylation of several intracellular proteins is the earliest recognizable activation event (6). The importance of protein tyrosine kinases (PTKs) in FcεRI-mediated mediator secretion has been demonstrated

[1]*Abbreviations used in this paper:* BMMC, bone marrow–derived cultured mast cells; Btk, Bruton's tyrosine kinase; ITAM, immunoreceptor tyrosine–based activation motif; NFAT, nuclear factor of activated T cells; PCA, passive cutaneous anaphylactic; PH, pleckstrin homology; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase; SCF, stem cell factor; SH, Src homology.

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by showing that treatment with a variety of PTK inhibitors can abrogate FcεRI-dependent activation of mast cells (7, 8). Two specific PTKs, Lyn and Syk, that belong to the Src and Syk/ZAP families, respectively, were shown to be essential for FcεRI-mediated mast cell activation (9–11). According to a generally accepted hypothesis (12), Lyn that is associated with the β subunit in unstimulated cells is activated upon FcεRI cross-linking. Subsequently, activated Lyn phosphorylates tyrosine residues within the ITAM sequences in the β and γ subunits. Phosphorylated ITAM (phospho-ITAM) in the β subunit recruits new molecules of Lyn through the Src homology 2 (SH2) domain–phosphotyrosine interaction while phospho-ITAM in the γ subunit recruits Syk by the same mechanism (13). Lyn and Syk are activated when bound to phospho-ITAMs (14, 15), and such activated Lyn and Syk in turn phosphorylate downstream targets such as phospholipase C (PLC)–γ.

Three Tec family PTKs, Btk, Emt/Itk/Tsk (Emt), and Tec, are also expressed in mast cells (16, 17). Among them, Btk and Emt are activated upon FcεRI cross-linking, suggesting a functional role in mast cell activation (18, 19). However, in contrast with Lyn and Syk (20–22), these PTKs do not appear to be receptor-associated molecules. Moreover, both Btk and Emt have important roles that are apparently unrelated to their involvement in FcεRI-dependent mast cell activation. Thus, Btk plays an essential role in the differentiation and activation of B lymphocytes: defects in the *btk* gene lead to X-linked agammaglobulinemia in humans (23, 24) and X-linked immunodeficiency (*xid*) in mice (25, 26). In addition, subsequent studies have implicated Btk in a number of signal transduction pathways in immune cells, including those for the B cell antigen receptor (27–29), CD38 (30, 31), CD40 (32), IL-5 (33), IL-6 (34), and IL-10 (35). Emt is considered a "T cell equivalent" of Btk, and is involved in T cell development and early activation events triggered through TCR/CD3 and CD28 (36–38).

Both *xid* (a mutation which results in the substitution of Arg with Cys at residue 28 in the Btk protein) and *btk null* mice exhibit essentially the same phenotype: these mutations lead to reduced numbers of mature conventional B cells, a severe deficiency of B1 B cells, a deficiency of serum IgM and IgG3, and defective responses to various B cell activators in vitro and to immunization with thymus-independent type II antigens in vivo (39, 40).

In this study, we analyzed Btk functions in mast cells in vivo and in vitro. Although *btk* mutant mast cells appear normal in many aspects of development in vitro or in vivo, they exhibited multiple abnormalities in FcεRI-mediated functions. *Btk* mutant mast cells exhibited mild to moderate impairment of FcεRI-mediated degranulation and histamine release, and more severe impairment of FcεRI-mediated cytokine production in vitro. *Btk* mutant mice exhibited correspondingly mild versus severe abnormalities in the early versus late phases of FcεRI-mediated cutaneous inflammatory responses in vivo. Furthermore, we found that both *xid* and *null* mutations of the *btk* gene result in defects in the transcriptional regulation of cytokine genes in mast cells stimulated via FcεRI, and such defects in *btk* mutant mast cells could be improved by retroviral gene transfer of wild-type (wt) *btk* cDNA. These results collectively demonstrate the involvement of Btk in the full expression of FcεRI signal transduction.

Materials and Methods

Passive Cutaneous Anaphylactic (PCA) Reactions. In homologous PCA experiments, 10 μl of various amounts of anti-DNP monoclonal IgE was intradermally injected into the ear of mice. 24 h later, 0.25 ml saline solution containing 1 mg/ml DNP conjugates of BSA ($DNP_{8.7}$-BSA) and 0.5% Evans blue dye was intravenously injected. The amounts of extravasated dye were measured after 30 min by extracting ears with potassium hydroxide as previously described (41). In another type of experiment, CBA/J and CBA/CaHN-*xid*/J mice received 1.0 ml anti-DNP monoclonal IgE antibody intravenously. 24 h later, a skin reaction was elicited by applying 10 μl 0.75% dinitrofluorobenzene acetone–olive oil solution to both sides of the ears. The reaction was assessed by measuring the ear thickness using an engineer's micrometer, Upright Dial Gauge (Peacock, Tokyo, Japan), at the indicated times after antigen challenge (42).

Cell Culture and Stimulation. Bone marrow cells taken from mouse femurs were incubated in the presence of IL-3 as previously described (7). After 4 wk of culture, cells (>95% mast cells, termed BMMCs for bone marrow–derived cultured mast cells) were incubated overnight with anti-DNP IgE antibody. Unless otherwise indicated (e.g., in cells stimulated for release of histamine or leukotrienes, see below), sensitized cells were stimulated for 24 h with 30 ng/ml DNP conjugates of human serum albumin (DNP-HSA) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-ME, 2 mM glutamine, and IL-3. For most retroviral transfection experiments, bone marrow cells cultured in the presence of IL-3 for 2–3 wk were expanded in the presence of both IL-3 and recombinant rat stem cell factor (SCF; gift of Kirin Brewery Co., Tokyo, Japan) for another 1–2 wk. At this point, >95% of the cells were mast cells, termed sBMMCs (for SCF-maintained BMMCs).

Northern Blot Analysis. Total cellular RNAs were isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. RNAs fractionated by formaldehyde/agarose gel electrophoresis were blotted onto nitrocellulose membranes. Mouse TNF-α (obtained from the American Type Culture Collection, Rockville, MD) and c-*myc* (a gift from D.R. Green, La Jolla Institute for Allergy and Immunology, La Jolla, CA) cDNA fragments were gel-purified and ^{32}P-labeled with a Megaprime DNA labeling kit (Pharmacia Biotech, Piscataway, NJ). Membranes were hybridized with ^{32}P-labeled probe purified through Elutip-d (Schleicher & Schuell, Keene, NH). Hybridized bands were detected by autoradiography.

Immunoblot Analysis. Immunologically stimulated cells were lysed in 1% NP-40–containing buffer. Cleared lysates were directly analyzed by SDS-PAGE or immunoprecipitated with polyclonal anti–TNF-α antibodies (Genzyme Corp., Cambridge, MA) before SDS-PAGE. Proteins in gels were electrophoretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were blocked, incubated with anti–TNF-α, anti-Btk (43), anti-Emt (44), or other appropriate primary antibodies, and then with horseradish peroxidase–conjugated secondary antibody. Immunoreactive bands were detected by an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Transfection. Murine *btk* cDNA in pME18S vector (16) was used for in vitro mutagenesis using two-step PCR procedures (45) to generate K430R and other *btk* mutants. The *wt* and mutant *btk* cDNAs confirmed by sequencing were inserted into the Moloney murine leukemia virus–based retroviral vectors, pMX-neo or pMX-puro (46). Retroviruses were generated by transient transfection of BOSC-23 packaging cells (47) with Lipofectamine (GIBCO BRL, Gaithersburg, MD). BMMCs or sBMMCs derived from male *xid* or *btk null* mice were infected with these retroviruses in the presence of 10 μg/ml polybrene. Selection with G418 (for *xid*-BMMCs and *xid*-sBMMCs) or puromycin (for *btk null–BMMCs* and *btk null*–sBMMCs) was started 48 h after infection. Mass populations of G418- or puromycin-resistant cells were grown and then cultured in the absence of selection drug for 48 h before immunological stimulation.

Measurements of Secreted Histamine, Cytokines, and Leukotrienes. Histamine released into the media during a 45-min stimulation was measured by an automatic fluorometric assay (48). Concentrations of antigen (ED_{50}) for half maximal histamine release was estimated using 77% (wt) and 79% (xid) as maximal responses. TNF-α, IL-2, IL-4, IL-6, and GM-CSF secreted into the media for 24 h were measured by ELISA kits (Endogen, Woburn, MA). Leukotrienes secreted into media for 30 min were analyzed by an enzyme immunoassay kit for leukotriene $C_4/D_4/E_4$ (Amersham Corp.).

Transcriptional Activity Assay with Luciferase Reporter Constructs. Luciferase reporter constructs, mouse IL-2 (−321), nuclear factor of activated T cells (NFAT)d–luc, NFκB–luc, and c-*fos*–luc have been previously described (49, 50). To engineer the human TNF-α (−200)–luc, PCR was done to amplify a DNA fragment containing the TNF-α promoter region (−199 to +68). This PCR fragment was inserted into the SmaI/BglII site of pGL3-Basic vector (Promega Corp., Madison, WI). 1.0–1.5 × 10^7 mast cells were transfected with 5–10 μg reporter plasmids by electroporation at 400 V, 950 μF using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were sensitized with anti-DNP monoclonal IgE antibody overnight, and left unstimulated or stimulated with 30 ng/ml DNP-HSA for 8 h before cell harvest. Cells were lysed in 0.2% Triton X-100 in 100 mM potassium phosphate buffer (pH 7.8)/1 mM dithiothreitol. Luminescence of cleared lysates was measured after addition of luciferin solution using a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA).

Quantitation of Tissue Mast Cells. Tissue mast cells in ear skin were quantified by light microscopy at ×400 by an observer who was unaware of the identity (i.e., mouse genotype) of the individual specimens, in 1-μm, Epon-embedded, Giemsa-stained sections, as previously described (51). Results were expressed as mast cells (mean ± SEM) per mm^2 of dermis (51).

Results

Mast Cell Development Is Not Affected by btk Mutations. First, we assessed the effects of *btk null* and *xid* mutations on several aspects of mast cell development and phenotype in vivo or in vitro. Mast cells in *wt* and *btk null* mouse ear skins were similar in their morphology and anatomical distribution (data not shown) and numbers: 125 ± 27.9/mm^2 of dermis (129/C57BL F2) versus 123 ± 32.2/mm^2 of dermis (*btk null*). The phenotypes of BMMCs were indistinguishable between *wt* (CBA/J) and *xid* (CBA/HCaN-*xid*/J) as well as between *wt* (129/C57BL F2) and *btk null* mice in their morphology when the cells were stained with May-Giemsa or with Alcian Blue (data not shown), and in numbers of IgE binding sites: (4.6 ± 1.2) × 10^4/cell (CBA/J) versus (3.7 ± 2.0) × 10^4/cell (CBA/HCaN-*xid*/J); (7.9 ± 2.4) × 10^4/cell (129/C57BL F2) versus (8.2 ± 2.9) × 10^4/cell (*btk null*). The *wt*- and *btk null*–BMMCs were similar in the expression of various signaling proteins, including FcεRI β and γ subunits, Lyn, Syk, Grb2, Shc, Sos, H-Ras, PLC-γ1, SPY75 (= HS1), protein kinase C (PKC; α, βI, βII, δ, ε, η, θ, and ζ isoforms), ERK1/2, JNK1/2, p38, PAK65, SEK1, and c-Jun (data not shown). Therefore, we concluded that either *btk null* or *xid* mutations apparently do not significantly interfere multiple aspects of mast cell development in vivo and in vitro.

Effects of btk Mutations on Anaphylactic Reactions In Vivo. We next tested the effects of the *btk* mutations on mast cell activation events induced by FcεRI cross-linking. Two types of PCA experiments were carried out. Mice primed by intradermal injection of anti-DNP IgE for 24 h were injected intravenously with antigen and Evans blue dye. Extravasation of Evans blue dye, due to increased blood vessel permeability as a result of PCA reactions, was quantified. The extravasation of Evans blue dye during the first 30 min of the PCA reactions, which is dependent mainly on histamine and serotonin released from activated mast cells (52), was slightly but significantly reduced at all the tested IgE doses in *xid* mice compared with *wt* mice (Fig. 1 *A*). To examine another type of PCA reaction (42), mice were sensitized with anti-DNP IgE 24 h before a solution of 0.75% dinitrofluorobenzene (hapten) was applied epicutaneously to the ear skin. *xid* mice exhibited little or no IgE/antigen-specific edema, whereas *wt* mice exhibited a prominent response that was detectable 4 h or later after antigen stimulation (Fig. 1 *B*). This late reaction is known to be at least partly due to TNF-α secreted from activated mast cells (51). Indeed, injection into the PCA-inducing site of *wt* mice with a neutralizing antibody to TNF-α just before antigen application significantly suppressed the development of the edema associated with the late phase of the reaction, as measured 24 h after antigen stimulation (Fig. 1 *C*). Significant defects in both the early and late phases of PCA reactions were also observed in *btk null* mice (data not shown).

Effects of btk Mutations on FcεRI-mediated Degranulation and Cytokine Secretion. To investigate the cellular basis for the diminished PCA reactions in *btk* mutant mice, the capacities to degranulate and release histamine and to produce and secrete cytokines were compared between BMMCs derived from the *wt* mice and the *xid* (or *btk null*) mice. Consistent with the modest defect in the early phase of PCA reactions and the more striking defect in the later phase, *xid*-BMMCs showed relatively mild defects in FcεRI-elicited histamine release but more severe impairments in cytokine secretion compared with *wt*-BMMCs. Maximal histamine responses (70–80% of the cellular content) were similar between *wt*- and *xid*-BMMCs. However, *xid*-BMMCs exhibited a marked reduction in histamine release at suboptimal doses of antigen, and the sensitivity of

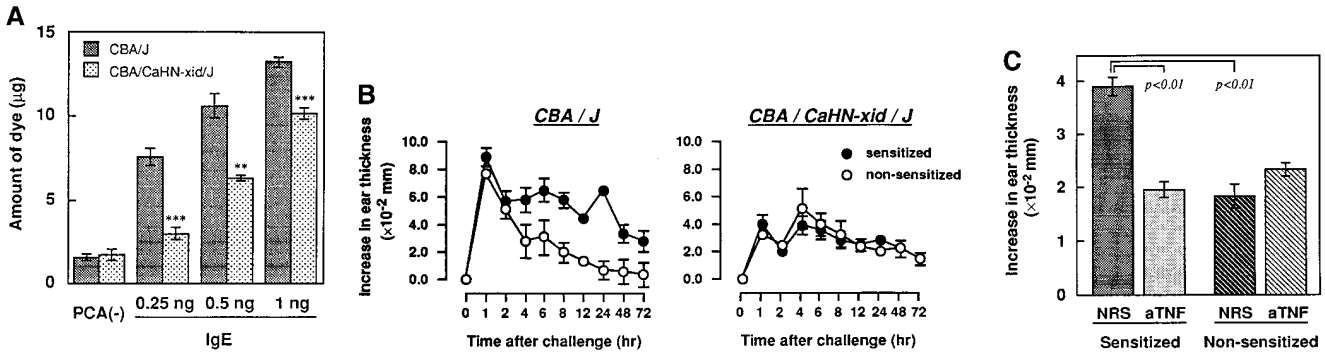


Figure 1. PCA reactions in CBA/J (*wt*) and CBA/CaHN-*xid*/J (*xid*) mice. (*A*) Mice were left unsensitized [*PCA(-)*] or sensitized by intradermal injection of the indicated amounts of anti-DNP IgE in 10 μl solution at the ear 24 h before antigen challenge. Mice were stimulated by intravenous injection of $DNP_{8.7}$-BSA and Evans blue dye for 30 min, and then the amount of extravasated Evans blue dye in the IgE-injected and control ears was measured as previously described (41). Statistical significance is indicated by ** ($P < 0.01$) and *** ($P < 0.001$). (*B*) Mice were left unsensitized or sensitized by intravenous injection of anti-DNP monoclonal IgE 24 h before antigen challenge. 25 μl of 0.75% dinitrofluorobenzene was applied on both sides of the ear, and the ear thickness was measured (42) at the indicated intervals. In *C*, anti–TNF-α antibody (*aTNF*, 80,000 U/0.2 ml/mouse) or normal rabbit serum (*NRS*, 0.2 ml/mouse) was intravenously injected just before antigen challenge in *wt* mice which had been sensitized with IgE and challenged with antigen (hapten) epicutaneously as in B; ear thickness was measured 24 h after antigen challenge.

xid-BMMCs to antigen stimulation was reduced by 3.8-fold compared with *wt*-BMMCs (ED_{50} = 4.4 ng/ml [*wt*] versus 17 ng/ml [*xid*], see Fig. 2 *A*). *Btk null*–BMMCs exhibited a somewhat more severe defect with a reduction in maximal histamine release in addition to reduced antigen sensitivity (Fig. 2 *B*).

In contrast to the relatively mild defect in histamine release, the differences in TNF-α secretion between the *xid*- and *wt*-BMMCs stimulated with an optimal concentration (30 ng/ml) of antigen ranged between 1:3.2 and 1:12 (a mean of 1:6.7, $n = 5$, see Fig. 2 *C*). FcεRI-stimulated secretion of IL-2, IL-6, and GM-CSF was also impaired to a similar extent in *xid*-BMMCs (Fig. 2 *C* and data not shown). Similarly reduced cytokine responses were observed in *btk null* cells (data not shown). *Wt*-, *xid*-, and *btk null*–BMMCs secreted barely detectable amounts (<40 pg/ml) of IL-4 in response to an immunologic stimulation through FcεRI (data not shown). These results suggest that the abnormalities in the expression of PCA reactions in *xid* and *btk null* mice reflect the mildly reduced degranulation and markedly defective cytokine secretion exhibited by *btk* mutant mast cells upon FcεRI cross-linking.

Defects in the Transcription of Cytokine Genes in btk Mutant Mast Cells. To further characterize the defects in TNF-α production and secretion in *xid*-BMMCs, we analyzed levels of TNF-α mRNA and protein in *xid*- and corresponding *wt*-BMMCs. As revealed by Northern blotting (Fig. 3 *A*), in *wt*-BMMCs, TNF-α mRNA was almost undetectable before stimulation but increased dramatically within 1 h after FcεRI cross-linking and decreased within the next few hours. However, under the same conditions of stimulation, *xid*-BMMCs produced less than one fifth the amount of TNF-α mRNA at its peak.

The membrane-bound TNF-α precursor (53), which was barely detectable before stimulation, increased after FcεRI cross-linking and reached a plateau level by 2–3 h after stimulation in *wt*-BMMCs (Fig. 3 *B* and data not shown). However, stimulation of *xid*-BMMCs led to only a slight increase in membrane-bound TNF-α content. Cellular pulse and chase experiments with [^{35}S]methionine showed that there was no significant difference in the intra-

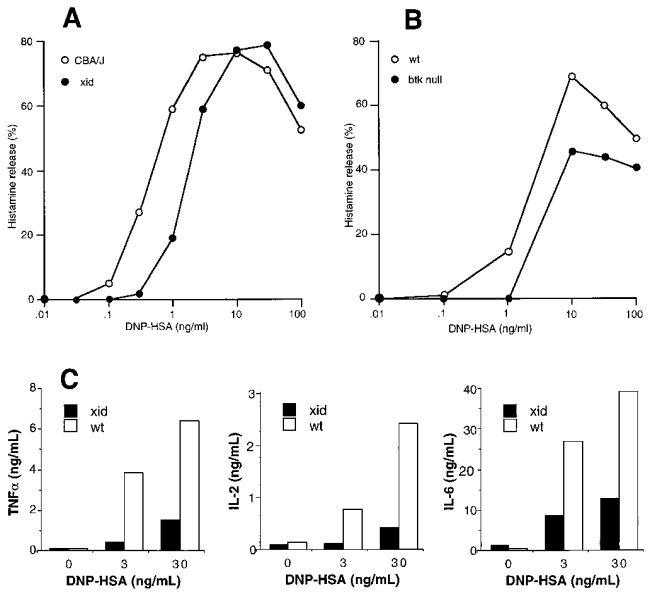


Figure 2. FcεRI-induced histamine release and cytokine secretion from *wt* and *xid*-BMMCs. BMMCs were sensitized by overnight incubation with 1 μg/ml anti-DNP monoclonal IgE antibody. Cells resuspended in Tyrode solution (*A* and *B*) or culture media (*C*) were stimulated by the indicated concentrations of DNP-HSA for 45 min (*A* and *B*) or 24 h (*C*). Histamine released into the buffer was measured by an automatic fluorometric assay (*A* and *B*). Cytokines secreted into the culture media during 24 h were measured by ELISA (*C*). The results of histamine release are mean values of duplicates per point, with SEM <5% of the mean. The results shown in *A* for *xid*-BMMCs and their corresponding *wt*-BMMCs are representative of those obtained in more than five experiments. The results shown in *B* are representative of those from two experiments. Cytokine measurements shown in *C* were performed in triplicate and mean values are shown, with SEM <10% of the mean. The results in *C* are representative of those from at least seven experiments.

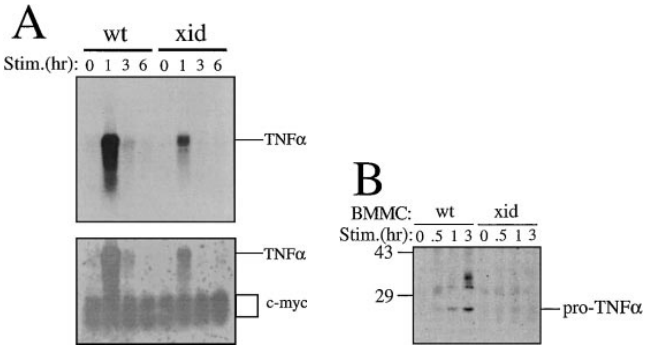


Figure 3. Analysis of TNF-α mRNA and protein in *wt* and *xid* mast cells. (*A*) Northern blot analysis for TNF-α mRNA of total cellular RNAs from immunologically stimulated BMMCs (*top*). The same blot was reprobed with c-*myc* cDNA (*bottom*). FcεRI cross-linking did not change c-*myc* mRNA levels. Because the blot was not stripped before reprobing, the TNF-α mRNA signals were also detected by the second probing. (*B*) Immunoblotting analysis of membrane-bound TNF-α (*pro-TNFα*) in *wt*- and *xid*-BMMCs. Cells were immunologically stimulated via FcεRI for the indicated times. Detergent lysates were analyzed by Western blotting with polyclonal anti–TNF-α antibodies. Positions of molecular mass standards (in kilodaltons) and pro–TNF-α are indicated on the left and right, respectively.

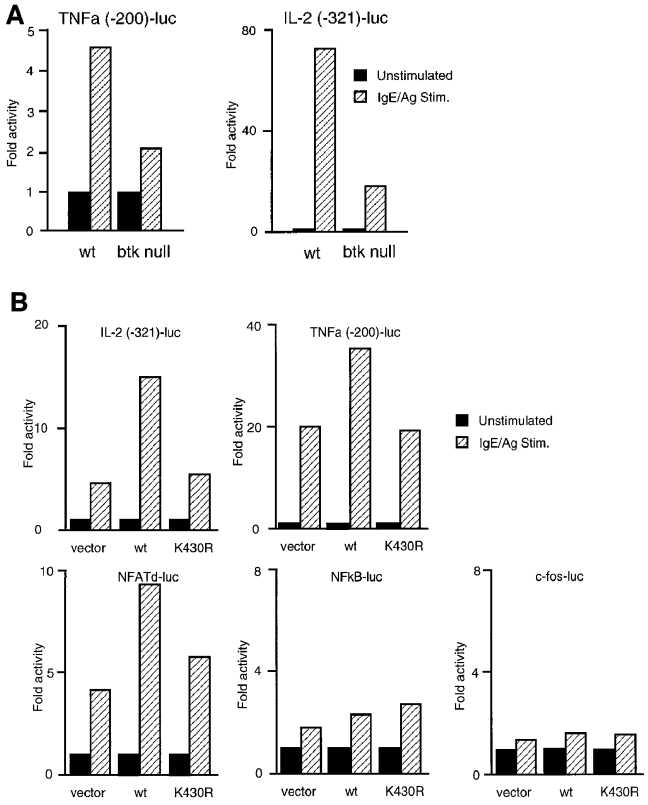


Figure 4. Transcriptional activity of luciferase reporter constructs in *wt* mast cells or *btk null* mast cells reconstituted with *wt* or K430R *btk* cDNAs. Promoter reporter constructs, IL-2 (−321)–luc, TNF-α (−200)–luc, NFAT–luc, NFκB–luc, or c-*fos*–luc, were transfected into *wt* or *btk null* mast cells (*A*) or *btk null* mast cells reconstituted with vector, *wt btk*, or K430R *btk* retroviruses (*B*). Luciferase activity was measured in cell lysates that were prepared 8 h after antigen stimulation or mock stimulation. Fold activities in FcεRI-stimulated cells versus unstimulated cells (= 1) are shown. The results shown are representative of those obtained in at least three experiments of each type.

cellular stability of TNF-α protein between *wt*- and *xid*-BMMCs (data not shown).

Taken together, these data suggest that Btk regulates TNF-α production at the transcriptional level. To test this possibility directly, we transfected BMMCs with luciferase reporter constructs under the control of TNF-α or IL-2 promoters. Transcription of both the TNF-α and IL-2 reporter constructs was strongly induced when *wt*-BMMCs were stimulated by FcεRI cross-linking. In *btk null–BMMCs*, the induced transcriptional activity of the TNF-α (−200)–luc construct was ~50% of that in *wt*-BMMCs (Fig. 4 *A*). Induction of transcriptional activities of the IL-2 (−321)–luc construct was 4–5-fold less in *btk null*–BMMCs compared with its activity in *wt* cells (Fig. 4 *A*). Similar results were obtained when the transcriptional activity of the TNF-α and IL-2 constructs was assessed in *xid*- versus *wt*-BMMCs (data not shown). We then performed additional experiments to assess the specificity of the transcriptional regulation of cytokine genes by Btk. We found that *btk null*–sBMMCs that had been stably transfected with *wt btk* exhibited higher transcriptional activities of the IL-2 (−321)–luc, TNF-α (−200)–luc, and NFAT–luc constructs than the cells transfected with vector or kinase-dead (K430R) *btk* (Fig. 4 *B*). By contrast, all three cell populations exhibited similar low levels of activity for the NFκB and c-*fos* constructs (Fig. 4 *B*).

The NFAT family of transcription factors and AP-1 proteins play essential roles in the expression of the IL-2 (54, 55) and TNF-α genes (56–58). These results indicate that defects in the production/secretion of cytokines upon FcεRI cross-linking in *btk* mutant mast cells are due, at least in part, to the inefficient transcription of these genes and may involve the signal transduction pathways leading to the activation of NFAT and/or AP-1 (Jun–Fos or Jun–Jun dimers). This notion is consistent with our recent data that Btk regulates JNK, an activator of c-Jun (59).

Gene Transfer–mediated Enhancement of the Ability of btk Mutant Mast Cells to Secrete Cytokines and Degranulate. To further investigate the relationship between *btk* mutations and impairment of mast cell functions, we measured cytokine production in *btk* mutant mast cells that had been reconstituted with Btk by stable or transient transfection. For most of these experiments, we used mast cells (sBMMCs) that had been expanded in the presence of both IL-3 and SCF. When *xid*-sBMMCs that had been transfected with *wt btk* cDNA were stimulated by FcεRI cross-linking, we observed a substantial enhancement of TNF-α–producing/secretory ability as compared to that seen in *xid*-sBMMCs that had been transfected with *neo* vector alone, with *xid* or kinase-dead (K430R) mutant *btk* cDNAs, or with *wt syk* or *wt lyn* cDNAs (Fig. 5 *A*). Expression of the transfected genes at comparable levels was confirmed by increased immunoreactive Btk proteins in *wt*, *xid*, and K430R mutant *btk*–transfected cells (Fig. 5 *D*, *left*). Transfectants expressing the constitutively active Btk* protein with the E41K substi-

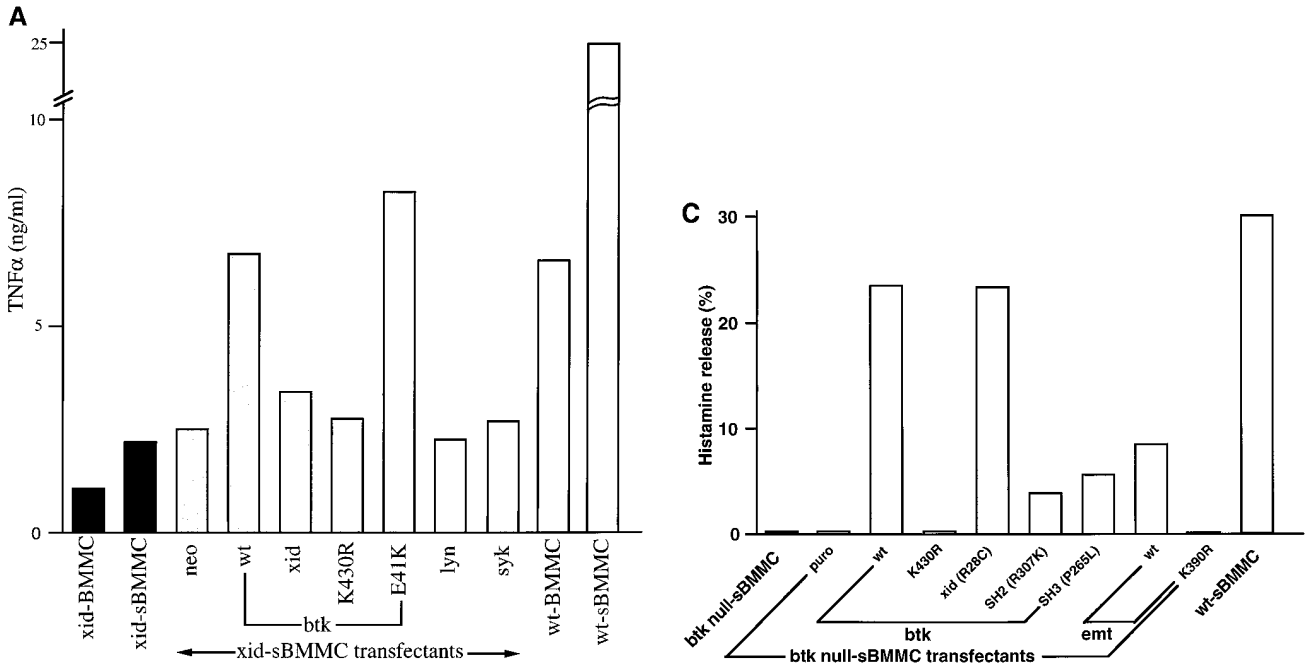


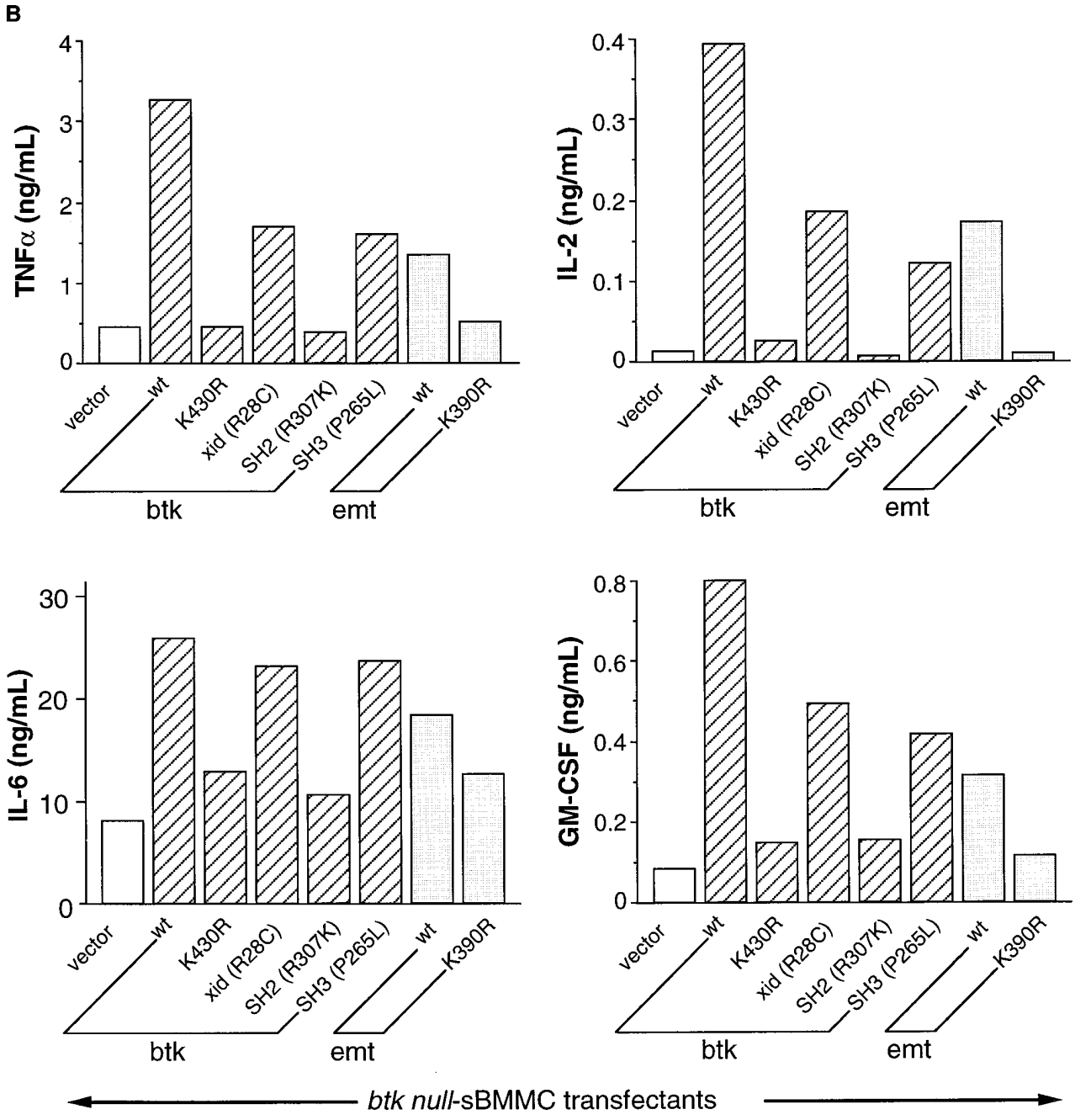
Figure 5. Effects of retroviral transfection of *btk* cDNAs on the ability of *xid* or *btk null* mast cells to produce cytokines or degranulate. (*A*) *xid*-sBMMCs were infected with the retroviruses encoding *wt*, kinase-dead (K430R), *xid*, or E41K mutant *btk* cDNAs. As controls, *xid*-sBMMCs were also transfected with *lyn* or *syk* cDNAs. G418-resistant cells were immunologically stimulated via the FcεRI and the TNF-α secreted during the next 24 h was measured. Levels of TNF-α secreted over 24 h by FcεRI-activated, but nontransfected *xid*-BMMCs, *xid*-sBMMCs, *wt*-BMMCs, and *wt*-sBMMCs, are also shown. The results presented are representative of those obtained in three separate experiments. The TNF-α values of duplicate measurements varied <10%. (*B*) FcεRI-induced secretion of TNF-α, IL-2, IL-6, and GM-CSF from *btk null*–sBMMCs transfected with *wt*, K430R, *xid* (R28C), SH2 (R307K), or SH3 (P265L) mutant *btk* cDNAs; also shown is cytokine secretion from vector control, *wt emt*, or kinase-dead (K390R) mutant *emt*-transfected *btk null*–sBMMCs. (*C*) Histamine release from IgE/antigen (30 ng/ml DNP-HSA)–stimulated *btk null*–sBMMCs transfected with *wt*, K430R, *xid* (R28C), SH2 (R307K), or SH3 (P265L) mutant *btk* cDNAs; results with *wt emt*– or K390R *emt*–transfected cells are also shown. Note that the percentage of histamine release from *wt*-sBMMCs, which had been cultured for a total of 7 wk from the start of bone marrow cell culture, is less than that observed in younger cultures of BMMCs (e.g., see Fig. 2 *B*). In several experiments, 7-wk-old cultures of *wt*-sBMMCs released 10–30% of their cellular content upon FcεRI cross-linking. (*D*) Expression of Btk and Emt proteins in transfected *xid*-sBMMCs (*left*) and *btk null*–sBMMCs (*center* and *right*). Transfected cells were lysed and analyzed by SDS-PAGE and immunoblotting with anti-BtkC (43) or anti-Emt (16) antibodies.

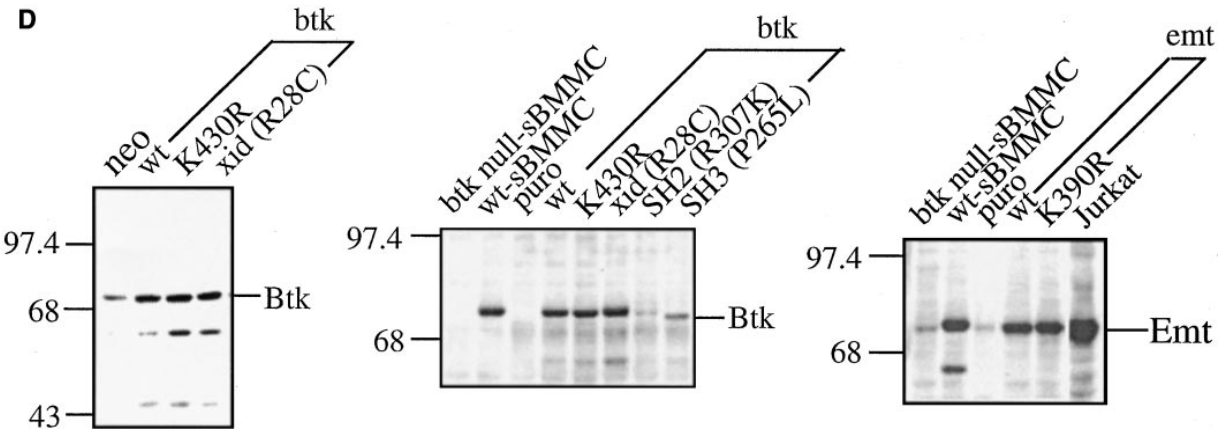
tution (60) exhibited somewhat higher levels of TNF-α secretion than did *wt btk* transfectants (Fig. 5 *A*). Moreover, none of the nontransfected or transfected sBMMCs secreted TNF-α without FcεRI stimulation (data not shown). Results similar to those shown for TNF-α secretion were also obtained when we tested the effects of transfection with *wt* versus various mutant *btk* cDNAs on the ability of FcεRI-stimulated *xid*-sBMMCs to secrete IL-2, IL-6, and GM-CSF (data not shown).

We next investigated *btk null*–sBMMCs transfectants, and in particular analyzed the domain requirements for Btk function in FcεRI-mediated cytokine production as opposed to degranulation. As shown in Fig. 5 *B*, transfection with *wt btk* cDNA greatly enhanced the ability of the cells to produce TNF-α, IL-2, IL-6, or GM-CSF in response to FcεRI-dependent activation, whereas compared with transfection with vector, transfection with kinase-dead (K430R) or SH2 mutant (R307K) *btk* cDNA had little or no effect. On the other hand, relatively low levels of protein expression for the product of the SH2 mutant (R307K) were detected in the stably transfected cells (Fig. 5 *D*, *middle*), indicating that the potential effects of this mutant *btk* in this system have not yet been adequately tested. Notably, transient transfection of *btk null*–sBMMCs with *wt*, but not kinase-dead, *btk* also restored cytokine gene transcriptional activities (data not shown). This finding provides further support for the conclusion that the results reflect the role of Btk in FcεRI signaling, not any role it might have in mast cell differentiation.

Notably, two mutant Btk proteins appeared to be able to partially (for TNF-α, IL-2 and GM-CSF) or fully (for IL-6) normalize 24-h cytokine production with respect to that seen in cells that had been transfected with *wt btk* cDNA (Fig. 5 *B*). One of these, the P265L mutation in the SH3 domain, is equivalent to the function-negative mutation in the SH3 domain of *sem*-5 (61). Therefore, this result suggests that at least partial FcεRI-dependent cytokine secretory function can be expressed in the absence of normal Btk SH3 function.

The other mutant *btk* cDNA that partially restored cytokine secretory ability in *btk null*–sBMMCs was *xid* (Fig. 5 *B*). This result may be related to the fact that levels of *xid* Btk protein in *btk null*–sBMMCs that had been transfected with *xid btk* cDNA were ~20–30% greater than levels of *wt* Btk protein in the corresponding *wt btk* transfectants (by contrast, *xid*-BMMCs express only 1/5 to 1/3 the amount





of Btk protein as do *wt*-BMMCs, data not shown). Thus, in comparison to *xid*-BMMCs, *btk null*–sBMMCs that had been transfected with *xid btk* cDNA greatly overexpress the *xid* Btk.

We previously noted (in Fig. 2) that the defect in FcεRI-dependent degranulation and histamine release exhibited by *xid*-BMMCs was less severe than that observed with *btk null–BMMCs*. Indeed, at optimal levels of FcεRI-dependent stimulation, *xid*-BMMCs gave a histamine release response that was indistinguishable from that of the corresponding *wt*-BMMCs (Fig. 2 *A*). We therefore examined the effect of transfection of *btk null*–sBMMCs with *xid btk* and other mutant *btk*s, as opposed to *wt btk*, on degranulation (as assessed by histamine release) at optimal conditions of IgE sensitization and antigen challenge (Fig. 5 *C*). We found that the profound defect in the histamine release response of *btk null*–sBMMCs under these conditions was nearly fully restored by *wt* or *xid btk* cDNAs, was slightly enhanced by SH2 (R307K) or SH3 (P265L) mutant *btk* cDNAs, but was unaffected (relative to results obtained with vector alone) by the kinase-dead (K430R) mutant *btk* cDNA (Fig. 5 *C*).

Since Emt is not only closely related to Btk but is activated upon FcεRI cross-linking (19), we also examined whether Emt might influence defects in cytokine production or degranulation in *btk null*–sBMMCs. We found that *btk null*–sBMMCs express endogenous Emt protein (Fig. 5 *D*). Moreover, the overexpression of *wt* Emt protein, but not kinase-dead (K390R) Emt protein, enhanced both FcεRI-dependent cytokine production (Fig. 5 *B*) and histamine release (Fig. 5 *C*) in *btk null*–sBMMCs. However, transfection of *btk null*–sBMMCs with *wt emt* did not restore either cytokine production or histamine release to levels observed in cells which had been transfected with *wt btk* (Fig. 5, *B* and *C*).

Discussion

Btk Functions in Mast Cells and Other Hematopoietic Cells. Btk has been shown to have essential roles in B cell differentiation and activation. Although our in vivo and in vitro studies have thus far revealed no significant effects of the *btk* mutations on mast cell development, we have identified multiple defects in FcεRI-induced activation events in *btk* mutant mast cells. Both degranulation, leading to release of histamine, and production/secretion of several cytokines were mildly or severely impaired, respectively. These defects at the cellular levels probably account for the defective expression of anaphylactic reactions in response to IgE and antigen in *btk* mutant mice. Together with our previous data demonstrating the tyrosine phosphorylation and enzymatic activation of Btk upon FcεRI cross-linking (18), these in vivo and in vitro effects of *btk* mutations have established that Btk has a role in the expression of FcεRI-dependent mast cell function.

Notably, some of the effects of *btk* mutations are milder in mice than in humans. For example, X-linked agammaglobulinemia patients have few mature B cells with no or little immunoglobulin production, whereas *xid* or *btk null* mice have about half the number of B cells as in normal mice (39, 40, 62, 63). Such species differences in the consequences of *btk* mutations raise the possibility that the effects of *btk* mutations on mast cell development or function might be milder in mice than in humans. This possibility is currently being investigated.

The *btk* gene is also expressed in myeloid cells in addition to mast and B cells (16). Hence, we examined the ability of activated macrophages from *btk* mutant mice to secrete TNF-α. We found that lipopolysaccharide stimulation induced the secretion of indistinguishably high levels of TNF-α from *wt* versus *xid* mouse bone marrow–derived macrophages (98% Mac-1^+) cultured in GM-CSF (data not shown). Therefore, the production/secretion of TNF-α seems to be differentially regulated in the two types of cells; it is more dependent on Btk in mast cells than in macrophages. This is not surprising, given the recent data demonstrating that the transcription of the TNF-α gene is regulated in a cell type–specific manner in activated T and B cells (58).

Structural Requirements of Btk for FcεRI-dependent Degranulation and Cytokine Production/Secretion. Btk and Emt have, in order from their NH_2 to COOH termini, pleckstrin homology (PH), Tec homology (TH), SH3, SH2, and SH1 (= kinase) domains. The catalytic activity of Btk was critical for mast cells to exhibit fully normal degranulation and production/secretion in response to FcεRI stimulation. In accord with this finding, transfection of *xid*-sBMMCs with a constitutively active form of Btk, Btk* with E41K mutation (60), resulted in the secretion of even higher levels of TNF-α than did transfection of the cells with *wt* Btk (Fig. 5 *A*). Interestingly, an SH3 mutant (P265L) could induce at least partial restoration of cytokine producing/secretory capacity. Therefore, an intact SH3 domain does not appear to be required for the expression of at least some Btk function in this system.

Remarkably, we also found that *xid* (R28C mutation in the PH domain) Btk, when overexpressed, could, depending on the cytokine, partially or apparently fully restore the cytokine producing/secretory capacity in both *xid* and *btk null* mast cells. The requirement of Btk domains for degranulation is similar to that for cytokine producing/secretory capacity (Fig. 5, *A–C*). Interestingly, *xid* Btk overexpressors released histamine as efficiently as *wt* Btk transfectants upon FcεRI cross-linking with an optimal concentration of antigen (Fig. 5 *C*). At first glance, these results appear inconsistent with the finding that *xid* mast cells express defects in FcεRI-dependent function. However, *xid*-BMMCs express only 1/5 to 1/3 as much Btk protein as do *wt*-BMMCs. By contrast, *btk null*–sBMMCs that had been transfected with *xid btk* cDNA expressed 20–30% more Btk protein than did cells transfected with *wt btk* cDNA, and both types of transfectant greatly overexpressed Btk protein relative to levels in *xid*-BMMCs. Finally, the defects in FcεRI-dependent mast cell function in *xid*-BMMCs are

less severe than those in the *btk null–BMMCs*; in fact, at optimal concentrations of antigen challenge, *xid*-BMMCs released histamine at levels that were indistinguishable from those of *wt*-BMMCs (Fig. 2 *A*). Taken together with the results obtained with the kinase-dead and SH3 mutants, these results suggest that the kinase activity of Btk is strictly required for Btk function in degranulation and cytokine production and secretion, but that other domains, such as the PH and SH3 domains, are not as essential for these functions of Btk in mast cell activation.

Several Btk-associated molecules have been described. PH domain–binding molecules include phosphatidylinositol 4,5-bisphosphate (and related phosphoinositides; references 64–66), the β subunits of GTP-binding proteins (67, 68), PKC (43), and BAP-135 (69). A proline-rich sequence in the Tec homology domain binds SH3 domains of Src family PTKs (70, 71), whereas the SH3 domain of Btk interacts with a protooncogene product, $p120^{c\text{-}cbl}$ (72). Differential binding requirements of some of PH domain–associated signaling molecules may account for the observed differences in the biochemical capacities or biological functions of *xid* versus *wt* Btk.

We found that *wt*, but not kinase-dead (K390R), Emt could partially compensate for the absence of Btk in the expression of FcεRI-dependent mast cell degranulation and cytokine production/secretion. Emt protein levels in *wt* and *btk null* mast cells, as revealed by immunoblotting with an anti-Emt polyclonal antibody that cross-reacts with Btk, were estimated to be ~10% of the Btk level in *wt* mast cells (Fig. 5 *D*), assuming that the antibody binds to Emt and Btk with the same efficiency. Given this finding and the results of our *emt* transfection experiments, it is possible that the retention of limited degranulation and cytokine production capacity in *xid* or *btk null* mutant mast cells may reflect low-level expression of Emt (and/or other Tec family PTKs) in these cells. This possibility remains to be investigated using mast cells devoid of both Btk and Emt (and/or other Tec family) proteins.

Btk Regulates the Transcription of Several Cytokine Genes. The abnormalities in the production/secretion of cytokines in *btk* mutant mast cells seem to be at the transcriptional level. Thus, levels of TNF-α mRNA induced by FcεRI cross-linking in *wt* mast cells exceeded that in *xid* mast cells by at least fivefold. This difference in mRNA levels could be due to differences in the transcription rate and/or in the mRNA stability, both phenomena known for IL-2 and other cytokines (73). In addition, the TNF-α mRNA has AU-rich sequences at the 3′ untranslated region that predispose for mRNA degradation and repress its translation (74–76). Although the possibilities of differential cytokine mRNA stabilities and differential derepression of mRNA translation between *wt* and *btk* mutant mast cells are not ruled out by this study, the notion of Btk-mediated regulation of cytokine gene transcriptions can account largely for our data. Thus, promoter reporter assays using the constructs without the 3′ AU-rich sequences demonstrated significant differences between *wt* and *btk* mutant mast cells in the transcriptional activity of the TNF-α and IL-2 promoters and individual *cis*-elements (see below) upon FcεRI cross-linking. By contrast, we found that the *xid* mutation had little or no detectable effect on the posttranslational regulation of TNF-α expression. All of these data are consistent with the fact that the expression of this cytokine is regulated at the transcriptional level by the activation of critical transcription factors in activated T and B cells (56–58, 77).

Similar observations were made with promoter reporter constructs to examine individual *cis*-acting elements. Thus, NFAT activity was activated by FcεRI stimulation, as previously shown in a mast cell line, CPII (78). However, FcεRI-induced transcriptional activation of an NFAT–luciferase construct was lower in *btk null* cells than in *wt* cells. In contrast, NFκB–luciferase and c-*fos*–luciferase activities were induced (at relatively low levels) by FcεRI stimulation, but the extent of transcriptional activation of these constructs was similar in *wt* versus *btk null* mast cells (data not shown). These observations are consistent with the results obtained with *btk null* mast cells that had been transfected with vector, *wt btk*, or kinase-dead *btk* cDNAs (Fig. 4 *B*). NFATp binds to four sites in the TNF-α promoter (58). Together with NFAT, the CRE site just upstream of κ3, an NFATp-binding site, binds c-Jun and ATF-2 transcription factors to activate this gene in response to TCR/CD3 stimulation (57, 58). NFATp binds to five sites in the IL-2 gene promoter (55) and cooperatively binds with c-Fos–c-Jun heterodimers or c-Jun–c-Jun homodimers at these sites (55, 79). Thus, it is likely that the regulation of the TNF-α and IL-2 genes in mast cells also involves NFAT and AP-1 proteins.

Btk-dependent Signaling Pathways. Btk is activated by the phosphorylation of tyrosine-551 by Lyn (80). Activated Btk in turn autophosphorylates Tyr-223 in the SH3 domain (81). Chicken DT-40 B lymphoma cells in which the *btk* gene was knocked out exhibited reduced tyrosine phosphorylation of PLC-γ2 and little $[Ca^{2+}]_i$ rise in response to anti-IgM stimulation, suggesting a role of Btk in the regulation of intracellular Ca^{2+} concentrations through direct or indirect phosphorylation of PLC-γ2 (82). Reaction products of PLC, inositol 1,4,5-trisphosphate, and diacylglycerol, mobilize Ca^{2+} from intracellular storage sites and activate PKC, respectively (for review see reference 83). Increased Ca^{2+} concentrations also lead to the activation and nuclear translocation of NFAT by dephosphorylation of cytoplasmic NFAT by the calcium/calmodulin–dependent phosphatase, calcineurin (for review see reference 84).

Our recent data indicates that Btk regulates JNKs and, to a lesser extent, p38, representing two of the three major MAP kinases (i.e., ERKs, JNKs, and p38) that are activated upon FcεRI cross-linking (59). Thus, upon FcεRI cross-linking, *xid* and *btk null* mast cells exhibited much reduced JNK activation compared with *wt* mast cells. Notably, the activities of ERKs upon FcεRI cross-linking were not significantly different between *wt* and *btk* mutant mast cells. The activity of phospholipase A_2, a key enzyme of arachi-

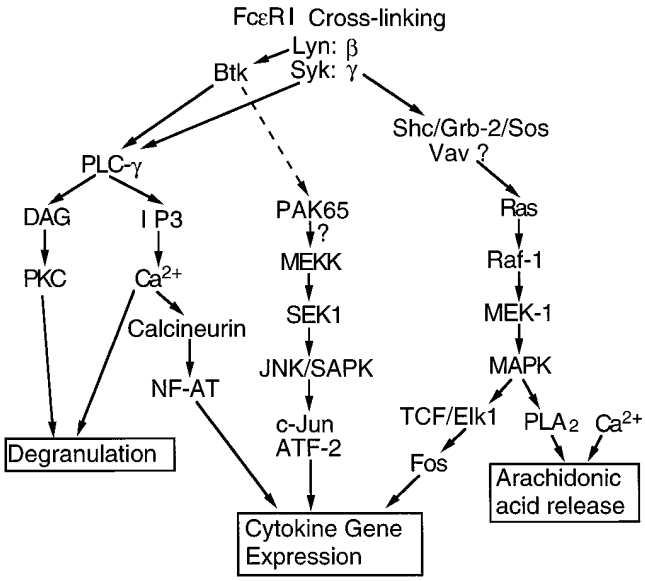


Figure 6. Hypothetical scheme of the roles of Btk in FcεRI-mediated signal transduction. Phosphorylation at Tyr-551 by activated Lyn leads to activation of Btk. Activated Btk in turn may phosphorylate PLC-γ, leading to Ca^{2+} mobilization. This pathway will activate NFAT as a result of dephosphorylation by calcineurin. Btk also can activate the JNK/SAPK pathway, resulting in the activation of c-Jun and ATF-2 by the phosphorylation of critical residues. Active NFAT, c-Jun and ATF-2 then lead to the transcriptional activation of cytokine genes.

donic acid cascade, was shown to be regulated by ERK, which in turn is regulated by Syk in rat basophilic leukemia RBL-2H3 cells (85). Accordingly, the lack of effect of *btk* mutations on ERK activity after FcεRI cross-linking probably explains our finding that leukotriene levels released from *btk null* mast cells were similar to those from *wt* mast cells (data not shown). Similarly, we found that the transcriptional activity of a c-*fos*–luciferase construct was not affected by *btk* mutations in mast cells (c-*fos* is also downstream of ERK).

btk mutations would be expected to impair signaling through JNKs (59). Targets of JNK include the transcription factors c-Jun and ATF-2. JNK phosphorylates the critical residues of the activation domains of these proteins to activate them (86–88). c-Jun and ATF-2, in cooperation with NFAT, were shown to bind to the CRE and κ3 sites, respectively, which are required for the induction of the TNF-α promoter (57, 58). In the case of the IL-2 gene, Fos–Jun heterodimers cooperatively bind with NFAT proteins at four of the five NFAT-binding sites in the IL-2 promoter (55). Taken together with these previous findings, our current data are consistent with the hypothesis that Btk can regulate two arms of the FcεRI signaling process, i.e., the PLC/Ca^{2+}/PKC and JNK signaling pathways (Fig. 6).

On the other hand, the regulation of FcεRI signaling is potentially very complex, and not all of these complexities are illustrated in Fig. 6. For example, we found that the secretion of TNF-α and other cytokines upon FcεRI cross-linking was greater in mast cells that had been cultured in the presence of both IL-3 and SCF as compared with that in cells that had been cultured in IL-3 alone. This might reflect, at least in part, the phosphorylation and enzymatic activation of PLC-γ by c-Kit (89, 90), the receptor for SCF. JNK is also activated transiently by SCF stimulation of BMMCs (59). Therefore, SCF/c-Kit–dependent activation of both PLC/Ca^{2+}/PKC and JNK pathways probably contributed to cytokine gene induction in mast cells that were maintained in IL-3 and SCF.

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