

Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity

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ABSTRACT Mast cells derived from Bruton's tyrosine kinase (Btk)-defective *xid* or *btk null* mice showed greater expansion in culture containing interleukin-3 (IL-3) than those from wild-type (*wt*) mice. Although the proliferative response to IL-3 was not significantly different between the *wt* and *xid* mast cells, *xid* and *btk null* mast cells died by apoptosis more slowly than their *wt* counterparts upon IL-3 deprivation. Consistent with these findings, the apoptosis-linked c-Jun N-terminal kinase/stress-activated protein kinase (JNK) activity was compromised in these *btk*-mutated cells upon FcεRI crosslinking or upon stimulation with IL-3 or with stem cell factor. p38 activity was less severely, but significantly, affected by *btk* mutation, whereas extracellular signal-regulated kinases were not affected by the same mutation. Btk-mediated regulation of apoptosis and JNK activity was confirmed by reconstitution of *btk null* mutant mast cells with the *wt btk* cDNA. Furthermore, growth factor withdrawal induced the activation and sustained activity of JNK in *wt* mast cells, while JNK activity was consistently lower in *btk*-mutated mast cells. These results support the notion that Btk regulates apoptosis through the JNK activation.

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein-tyrosine kinase of the recently recognized Tec family (1–3). These protein-tyrosine kinases consist, from the N to C termini, of pleckstrin homology, Tec homology, Src homology 3 (SH3), SH2, and SH1 (=kinase) domains. Various defects in the *btk* gene cause X-linked agammaglobulinemia in humans (2, 3) and the Arg to Cys mutation at position 28 results in X-linked immunodeficiency (*xid*) in mice (4, 5). These diseases and the phenotype of *btk* gene knockout mice (6, 7) indicate an essential role of Btk in the development and functions of B lymphocytes. Our previous study showed that Btk is tyrosine-phosphorylated and enzymatically activated upon crosslinking of FcεRI, suggesting a role of Btk in mast cell activation (8). Recent studies have made significant progresses in our understanding of how Btk activity is regulated. Thus, Btk was shown to be activated by phosphorylation of Tyr-551 by a Src family protein-tyrosine kinase, Lyn (9, 10). Activated Btk in turn autophosphorylates Tyr-223 in the SH3 domain (11). On the other hand, Btk is negatively regulated by protein kinase C in mast cells (12). Btk is also implicated in the regulation of intracellular Ca²⁺ concentrations through direct or indirect phosphorylation of phospholipase Cγ2 in chicken B lymphoma cells (13). However, the mechanisms by which Btk promotes normal functions in B cells and other cell types are largely

unknown. In the present study, we provide evidence that Btk regulates the activity of c-Jun N terminal kinase or stress-activated protein kinase (JNK) and the apoptotic process upon growth factor deprivation of mast cells.

MATERIALS AND METHODS

Cells and Stimulation. Bone marrow cells from femurs of CBA/J, CBA/CaHN-*xid*/J (*xid*), B6/129 F2, and *btk null* (6) mice were cultured in IL-3-containing RPMI 1640 medium/10% fetal calf serum with weekly changes of medium for 4–5 weeks before use (14). Cell density was adjusted to 3×10^5 cells per ml at the weekly change of medium. Numbers of live cells excluding trypan blue were counted by a hemocytometer. The phenotype of *xid* and *btk null* mast cells [*xid*-bone marrow-derived mouse mast cells (BMMC) and *btk null*-BMMC] was indistinguishable from *wt* mast cells in their morphology, as revealed by staining cells with May-Giemsa or with Alcian Blue (data not shown). Numbers of IgE binding sites on these mast cells at day 28 were also similar: $4.6 \pm 1.2 \times 10^4$ /cell (CBA/J) vs. $3.7 \pm 2.0 \times 10^4$ /cell (CBA/CaHN-*xid*/J). For FcεRI crosslinking, BMMC primed with anti-dinitrophenyl IgE monoclonal antibody were incubated with dinitrophenyl conjugates of human serum albumin for the indicated intervals. For growth factor stimulation, BMMC were incubated in IL-3-free medium overnight before stimulation with recombinant mouse IL-3 or stem cell factor at 100 ng/ml (both from Kirin Brewery).

Transfection. pMX-puro vector, which is a puromycin-resistant derivative of pMX-neo (15), was used to construct *btk* expression vectors. K430R and *xid* (R28C) mutant *btk* cdNAs were made by PCR-assisted *in vitro* mutagenesis and verified by a limited sequence determination. Transfection of Bosc-23 packaging cells (16) with these vectors was done with a Lipofectamine reagent (Life Technologies), and recovered retroviruses ($3\text{--}10 \times 10^5$ colony-forming units/ml) were used to infect *btk null*-BMMC. Selection of transfected cells in the presence of 1.5 μg/ml puromycin for 2 to 3 weeks yielded sufficient numbers of cells for experiments.

Mitogen-Activated Protein Kinase (MAPK) Assays. *Extracellular signal-regulated kinases (ERKs)*. Cells were lysed, and fractions of lysates were directly analyzed by SDS/PAGE

Abbreviations: Btk, Bruton's tyrosine kinase; BMMC, bone marrow-derived mouse mast cells; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; FcεRI, high-affinity IgE receptor; IL, interleukin; JNK, c-Jun N terminal kinase or stress-activated protein kinase; wt, wild-type; GST, glutathione S-transferase.

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followed by immunoblotting with anti-phospho-MAPK (New England Biolabs). The same blot was reprobed with anti-p44/42 MAPK antibody (New England Biolabs) to confirm equal loadings. The rest of lysates were immunoprecipitated with anti-ERK1 (Zymed), and *in vitro* immune complex kinase assays were done using myelin basic protein (MBP) as a substrate in the presence of [γ - 32 P]ATP. Reaction products were analyzed by SDS/PAGE and autoradiography.

p38. p38 immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). The same blot was reprobed with anti-p38 (Santa Cruz Biotechnology) to control loadings. In *in vitro* kinase assays, immune complexes precipitated with anti-p38 (a gift from J. Han) were also incubated with MBP as substrate.

JNKs. Kinase activity was measured on immune complexes precipitated with anti-JNK1 or anti-JNK2 using glutathione *S*-transferase (GST)-ATF-2 or GST-c-Jun(1-79) as substrate. Both antibodies were from Santa Cruz Biotechnology.

Analysis of Apoptosis and DNA Synthesis. Mast cells depleted of growth factor for the indicated intervals were stained with propidium iodide and analyzed by FACScan (Becton Dickinson). Genomic DNAs were isolated and analyzed by agarose gel electrophoresis. Mitogenic response of mast cells to IL-3 was measured by incubating cells (5×10^5 /ml, 0.2 ml) in mouse recombinant IL-3 for 18 h in the presence of [3 H]thymidine during the last 6 h. Thymidine incorporation into the acid-insoluble fraction was counted.

RESULTS

Defective Apoptosis of the *btk*-Mutated Mast Cells. Culturing murine bone marrow cells in IL-3-containing medium for 4–5 weeks give rise to an almost pure (>95%) population of immature mast cells (BMMC). Starting from similar numbers of bone marrow cells, i.e., $2.0 \pm 0.2 \times 10^7$ (*xid*) and $1.8 \pm 0.1 \times 10^7$ (*wt*), numbers of BMMC obtained from *xid* mice were significantly greater than those from *wt* mice after 28 days of culture: $3.0 \pm 0.8 \times 10^8$ (*xid*) vs. $1.6 \pm 0.4 \times 10^8$ per femur (*wt*) (Fig. 1A), although the proliferative response of *xid*-BMMC to recombinant mouse IL-3 was not significantly different from that of *wt*-BMMC (Fig. 1B). Since medium was changed once a week during the culture of bone marrow cells, we considered the possibility of insufficient supply of IL-3. As shown previously (17), BMMCs die by apoptosis upon growth factor withdrawal. Cell survival was compared between *wt*- and *btk*-mutated mast cells under such conditions. Numbers of live *xid*-BMMC declined more slowly than those of *wt*-BMMC (Fig. 2A). Cytofluorometric analysis of propidium iodide-stained cells (Fig. 2B) and agarose gel electrophoresis of genomic DNAs (Fig. 2C) revealed that both *wt*- and *xid*-BMMCs die by apoptosis and that the apoptotic death of *xid*-BMMC is delayed by approximately 48 h compared with *wt*-BMMC. Similarly, *btk null*-BMMC died by apoptosis in the absence of IL-3 more slowly than *wt*-BMMC (Fig. 2C and D). Furthermore, *btk null*-BMMC reconstituted with the *wt btk* cDNA underwent apoptosis more rapidly than *btk null*-BMMCs reconstituted with the vector or K430R *btk* cDNA, as revealed by earlier DNA fragmentation (Fig. 2E). Therefore, these results demonstrate that Btk regulates apoptosis induced by growth factor deprivation in mast cells.

Increased JNK Activity upon Growth Factor Withdrawal and Its Defect in *btk null* Mast Cells. In a neuronal model system (18), apoptosis induced by growth factor deprivation was linked to the sustained activation of JNK and p38, while survival was controlled by the opposing effect of ERKs. Apoptosis induced by various stresses that activate the sphingomyelin pathway was shown to be dependent on the JNK pathway (19). Therefore, we measured JNK activity in *wt*- and *btk null*-BMMCs upon growth factor withdrawal (Fig. 3). JNK activity in *wt*-BMMC increased upon growth factor with-

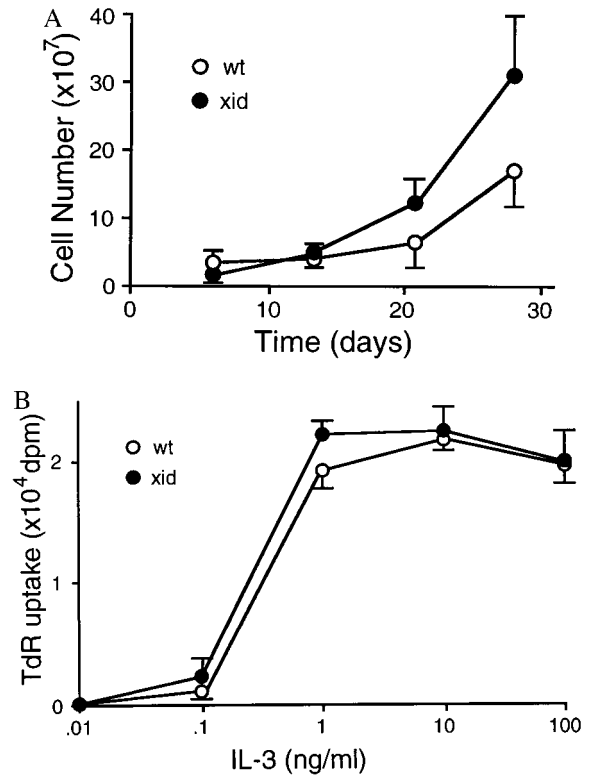


FIG. 1. Growth of mast cells and their proliferative response to IL-3. (A) Growth curve of bone marrow-derived mast cells in IL-3-containing medium. Bone marrow cells obtained from CBA/J (*wt*) or CBA/CaHN-*xid*/J (*xid*) were cultured as described (14). Cell density was adjusted to 3×10^5 per ml weekly. Live cell numbers were counted weekly. Shown is a representative culture from more than 10 experiments. (B) BMMCs (5×10^5 /ml, 0.2 ml/well) derived by 4 weeks of culture of bone marrow cells from CBA/J (*wt*) and CBA/CaHN-*xid*/J (*xid*) were cultured in the indicated concentrations of mouse recombinant IL-3 for 18 h in the presence of [3 H]thymidine during the last 6 h. Thymidine incorporation into the acid-insoluble fraction was counted. Two similar experiments were performed with essentially the same results.

drawal. The peak activity of JNK1 was observed 6–9 h after the removal of growth factor, and JNK1 activity in *wt*-BMMC was higher than that in *btk null*-BMMC at any tested time point throughout the time course (0–24 hr). The activity of another MAPK, p38, in *wt* mast cells was also induced by growth factor deprivation, and it was also higher than that in *btk null* mast cells, whereas ERK1 activity was not induced by starvation, and it was slightly higher in *btk null* cells than that in *wt* cells (Fig. 3). These results are consistent with the notion that Btk regulates apoptosis by regulating JNK and p38.

Defective JNK Activation in *btk*-Mutated Mast Cells upon Fc ϵ RI Crosslinking or Growth Factor Stimulation. Activities of ERKs, JNKs, and p38 were measured in BMMCs prepared from *wt* and *btk null* mice before and after Fc ϵ RI crosslinking. In addition to ERK1 and ERK2, the activation of which was previously shown (20, 21), JNK1, JNK2, and p38 were activated in *wt*-BMMC upon Fc ϵ RI crosslinking (Fig. 4). Both ERK1 and ERK2 were activated equally well in *btk null*-BMMC (Fig. 4A), while p38 activity, as assessed by tyrosine phosphorylation, was slightly affected in *btk null* mast cells (Fig. 4B). In these cells tyrosine phosphorylation of p38 returned to the basal level earlier than that in *wt* mast cells. In contrast, the activities of JNK1 and JNK2 were drastically compromised in *btk null*-BMMC (Fig. 4C). As measured by immune complex kinase assays using GST-ATF-2 as a substrate, kinase activities of both JNK1 and JNK2 in *btk null*-BMMC were less than one tenth of those in *wt*-BMMC at its peak activity (15 min) (Fig.

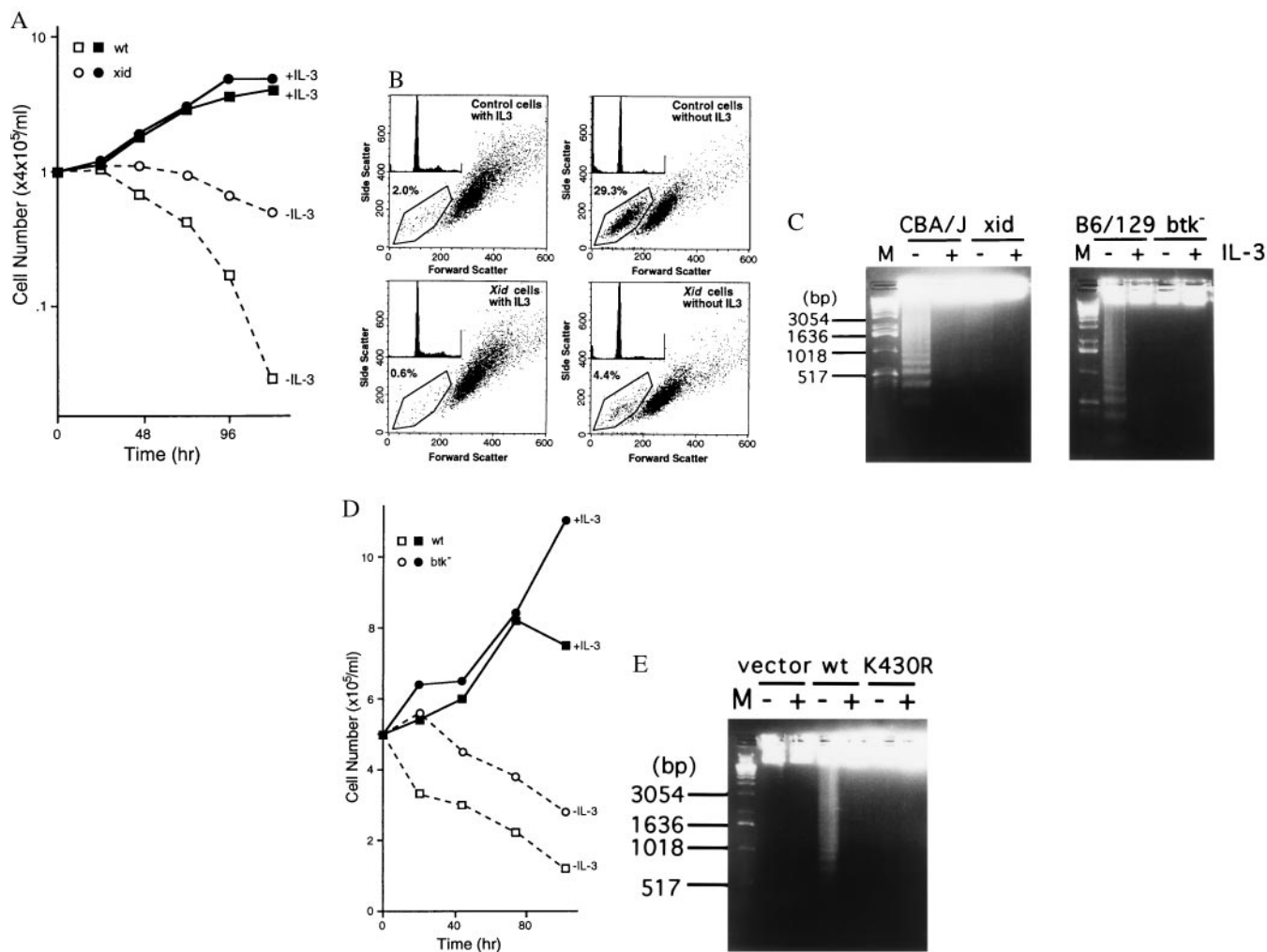


FIG. 2. *xid* and *btk* null mast cells die by apoptosis upon growth factor deprivation more slowly than the *wt* cells. (A) Survival curve of *wt* (CBA/J)- and *xid*-BMBCs in the presence or absence of IL-3 in the culture medium. Numbers of live cells excluding trypan blue were counted. Average values from duplicate samples are shown. Deviations between duplicate samples were less than 15% in all samples. Representative of six similar experiments. (B) Cytofluorometric analysis of propidium iodide-stained BMBCs. Cells were stained 30 h after IL-3 was removed from the medium. Percents of apoptotic cells are indicated. Insets show profiles of propidium iodide-stained cells. (C) DNA fragmentation was analyzed by agarose gel electrophoresis of genomic DNAs from BMBCs (4×10^5 cells) obtained before or 48 h (CBA/J and *xid*) or 10 h (B6/129 F2 and *btk* null) after growth factor deprivation. A 1-kb ladder (Life Technologies) was used as a molecular size marker (M). (D) Survival curve of *wt* (B6/129 F2)- and *btk* null-BMBCs in the presence or absence of IL-3. Experiments were done in duplicate with live cell numbers of less than 15% deviations among duplicate samples. Another experiment with a similar result was done. (E) DNA fragmentation was analyzed by agarose gel electrophoresis of genomic DNAs from *btk* null-BMBCs reconstituted with vector, *wt*, or K430R *btk* cDNAs prepared before (+) or 6 h after (-) growth factor deprivation.

4C). Essentially the same results were obtained when GST-c-Jun(1-79) was used as a substrate (data not shown). Similarly, BMBC derived from *xid* mice showed a reduced activation of JNK compared with CBA/J (*wt*)-BMBC upon Fc ϵ RI crosslinking (data not shown).

To confirm the specific effect of the *btk* mutations on the activation of JNK, *btk* null-BMBC was reconstituted with the expression vectors encoding *wt*, kinase-dead (K430R), or *xid* (R28C) Btk proteins. Upon Fc ϵ RI crosslinking, *btk* null-BMBC reconstituted with the *wt* *btk* cDNA exhibited the JNK activation similar to that in *wt*-BMBC while JNK activation in *btk* null-BMBC reconstituted with the kinase-dead (K430R) *btk* was as low as that in *btk* null-BMBC transfected with the vector control (Fig. 4D). As expected from the *xid* phenotype, *btk* null-BMBC reconstituted with the *btk* cDNA with *xid* mutation showed defective JNK activation. p38 activation upon Fc ϵ RI crosslinking was also restored in the *wt* *btk*, but not kinase-dead *btk*, transfectants (data not shown). Transfection of *btk* null-BMBC with *syk* or *lyn* cDNAs did not restore JNK

activation, indicating that Btk is specifically involved in JNK activation induced by Fc ϵ RI crosslinking (data not shown).

Furthermore, the ability of Btk to regulate JNK was shown when BMBCs deprived of growth factors overnight were stimulated with IL-3 or stem cell factor. Both growth factors induced stronger, transient activation of JNK1 in *wt*-BMBC compared with that in *btk* null-BMBC (Fig. 4E). Collectively, these data show that Btk regulates JNK activity under various conditions.

DISCUSSION

The present study demonstrates that Btk regulates apoptosis as well as kinase activities of JNK and p38. The intact kinase activity of Btk is required for both of the activities. The intactness of the pleckstrin homology domain of Btk was also required for growth factor deprivation-induced apoptosis, since expression of the Btk protein with *xid* mutation (R28C) in the pleckstrin homology domain failed to respond by rapid

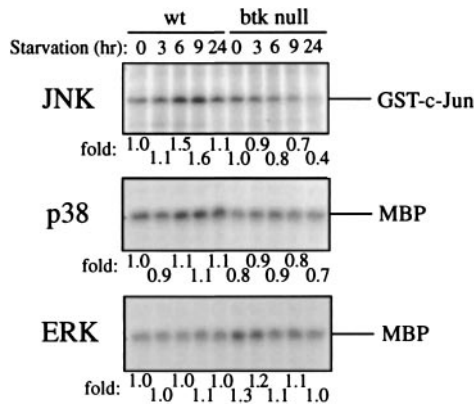


FIG. 3. Activities of MAPK upon growth factor withdrawal. IL-3 was removed from cultures (2×10^6 cells) of *wt*- and *btk null*-BMMCs for the indicated intervals. Cells were lysed and immunoprecipitated with anti-JNK1, anti-p38, or anti-ERK1. Immune complex kinase assays were done, and reaction products were analyzed by SDS/PAGE and autoradiography. -Fold activities measured by densitometry of the substrate bands are shown after normalized to that of *wt* cells before starvation. Positions of substrates, GST-c-Jun(1-79) and MBP, are indicated.

apoptotic cell death. This is in a sharp contrast with radiation-induced apoptosis in DT-40 chicken lymphoma B cells, where *xid* mutated Btk induced apoptosis as well as *wt* Btk did (22).

Increased ceramide levels generated by various stresses induced apoptosis in a JNK-dependent manner (19), and apoptosis of sympathetic neurons after nerve growth factor deprivation is inhibited by a c-Jun dominant negative mutant (23). These previous studies as well as our present study indicate that apoptotic cell death induced by growth factor deprivation is dependent on JNK activity. However, it is noteworthy that the transient activation of JNK does not seem to lead to apoptosis. Thus, either growth factor stimulation or Fc ϵ R1 crosslinking did not induce apoptosis but rather induce DNA synthesis (data not shown). Activation of ERKs induced by these stimuli may oppose JNK activation to suppress apoptosis, as proposed for apoptosis of PC12 cells deprived of nerve growth factor (18). JNK was also implicated in cell growth (24) and transformation (25). However, the mechanism by which Btk regulates JNK is currently unknown. Since Btk is activated rapidly by stimulation of various cell surface receptors, including B cell antigen receptor (26, 27) and Fc ϵ R1 (8), and since it can be activated by Src family kinases (9, 10), it probably works upstream of Ras or Rho family GTPases.

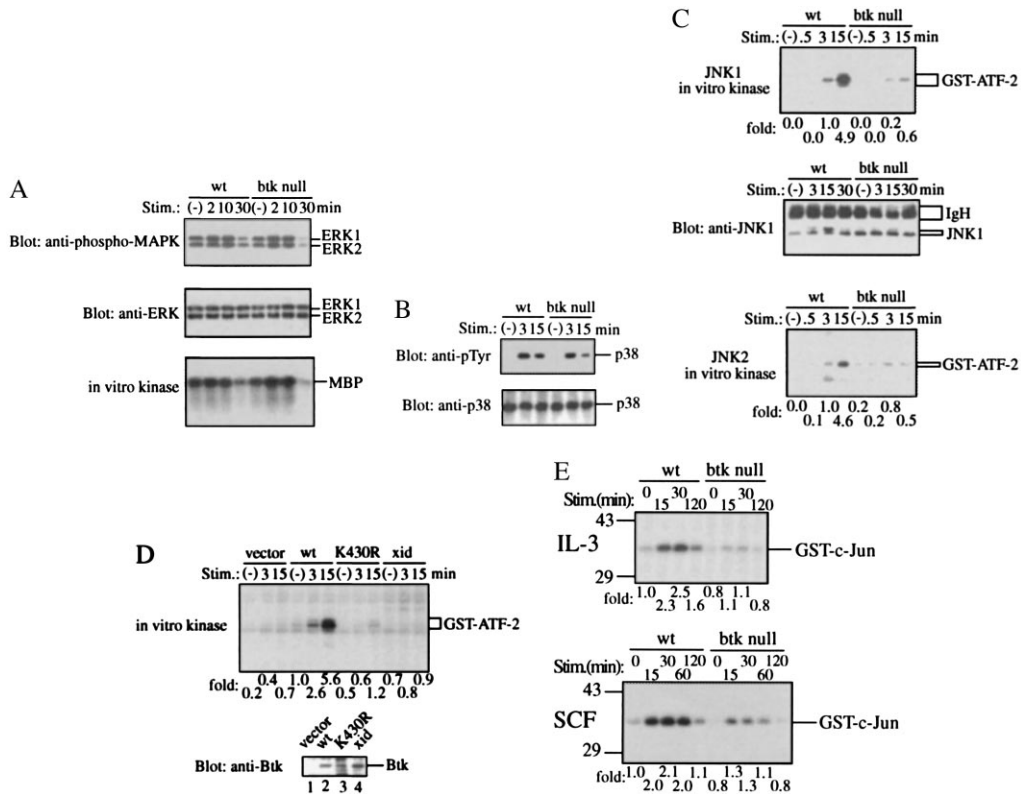


FIG. 4. Defective JNK activation in *btk null*-BMMC upon Fc ϵ R1 crosslinking and stimulation with growth factor. (A) ERK phosphorylation and catalytic activity. BMMCs from B6/129 F2 (*wt*) and *btk null* mice were stimulated through Fc ϵ R1 for the indicated periods. ERK phosphorylation was assayed by immunoblotting with anti-phospho-MAPK (Top). The same blot was reprobbed with anti-p44/42 MAPK antibody to confirm a comparable expression of ERK1/2 (Middle). ERK1 activity was measured by *in vitro* immune complex kinase assays using MBP as a substrate. Representative of three similar experiments. (B) Tyrosine phosphorylation of p38. BMMCs were stimulated as described above. p38 immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody. The same blot was reprobbed with anti-p38. Representative of three similar experiments. (C) JNK activity. Kinase activity was measured on immune complexes precipitated with anti-JNK1 (Top) or anti-JNK2 (Bottom) using GST-ATF-2 as a substrate. -Fold activities measured by densitometry of the autoradiograms were normalized to that of *wt* cells at 3 min poststimulation. The blot onto which the JNK1 kinase reaction products were transferred was probed with anti-JNK1 (Middle). Of note is that the JNK1 band with a slower mobility due to phosphorylation was conspicuous with *wt*-BMMC stimulated for 3-15 min in contrast with the weaker intensity of the corresponding band in *btk null*-BMMC. Representative of three similar experiments. Detection of JNK2 bands by reprobbed of the JNK2 kinase blots was hampered by the closeness of the molecular mass of JNK2 to that of immunoglobulin heavy chains. (D) *Wt*, K430R, and *xid* (R28C) *btk* retroviruses with the puromycin resistance gene were prepared using BOSC-23 packaging cells. Infected, puromycin-selected BMMCs were immunologically stimulated as described in *Materials and Methods*. JNK1 kinase activity was measured. -Fold activities were normalized to that of *wt* cells before stimulation. Lower shows the expression level of Btk in reconstituted cells as revealed by immunoblotting of total cell lysates by anti-Btk antibody. (E) *Wt*- and *btk null*-BMMCs were stimulated with recombinant mouse IL-3 or stem cell factor for the indicated times after an overnight starvation of growth factors. Then, JNK1 activity was measured. -Fold activities were normalized to that of *wt* cells before stimulation.

Activated Cdc42 and Rac eventually lead to activation of JNK through the phosphorylation cascade, PAK65 → MEKK → SEK1 → JNK. Indeed, PAK65 activity in *wt* mast cells upon FcεRI crosslinking was higher than in *btk*-mutated mast cells, and FcεRI-induced JNK activation was at least partially inhibited by a dominant negative SEK1(Lys → Arg) (data not shown). Therefore, Btk appears to regulate JNK through this cascade. The known JNK phosphorylation targets include c-Jun and ATF-2 (24, 28–30). These transcription factors regulate the expression of multiple cytokine genes, including tumor necrosis factor α gene (31). Consistent with the defective JNK activation, *xid-* or *btk null*-BMMCs showed a severe defect in FcεRI-induced secretion of this and other cytokines (D.H., Y.K., Naoki Inagaki, T. Kitamura, W.N.K., M.M.-Y., T.M., W. Han, S.E.H., L.Y., H. Nagai, A. E. Goldfeld, F.W.A., O. N. Witte, and T. Kawakami, unpublished data). Although more subtly affected in *btk* mutated cells, the activity of p38 also seems to be regulated by Btk. To our knowledge, the regulation of apoptosis is the first known biological function of Btk in mast cells. This function appears to be controlled through the regulation of JNK/p38 activity. Studies on mechanisms for JNK/p38 activation by Btk are under way.

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