Defects in Bruton’s tyrosine kinase (Btk) result in B cell immunodeficiencies in humans and mice. Recent studies showed that Btk is required for maximal activation of JNK, a family of stress-activated protein kinases, induced by several extracellular stimuli including interleukin (IL)-3. On the other hand, IL-3-induced JNK activation is dependent on Ras. In the present study we have investigated whether Ras is involved in Btk-mediated JNK activation in BaF3 mouse pro-B cells. Overexpression of wild-type Btk protein in these cells enhanced JNK activation upon IL-3 stimulation, whereas expression of kinase-dead Btk partially suppressed JNK activation. Induced expression of the dominant negative Ras(N17) in the cells overexpressing wild-type Btk suppressed JNK activation. Importantly, overexpression of Btk enhanced the level of the GTP-bound, active form of Ras in response to IL-3 stimulation. Btk overexpression also increased the Shc-Grb2 association induced by IL-3 stimulation. Expression of either N17Ras or V12Ras did not impose any effects on Btk kinase activity. These data collectively indicate that Ras plays a role of an intermediate signaling protein in Btk-mediated JNK activation induced by the IL-3 signaling pathway.

Defects in Bruton’s tyrosine kinase (Btk) lead to X-linked agammaglobulinemia in humans (1, 2) and X-linked immunodeficiency (xid) in mice (3, 4). In addition to its crucial role in B lymphocyte development, Btk is implicated in signal transduction triggered by several immune cell receptors and cytokine receptors (for review, see Refs. 5–7). For example, we showed recently that Btk plays an important role in cytokine production induced by cross-linking of the high affinity IgE receptor (FceRI) in murine mast cells (8, 9). This protein-tyrosine kinase (PTK), a representative member of the Tec PTK family, is also required for apoptosis induced by anti-IgM stimulation, γ-irradiation, or growth factor deprivation (10–12).

A biochemical function of Btk is to mobilize a signaling pathway leading to the activation of stress-activated protein kinases, JNK/SAPK (JNK), in response to various stimuli including interleukin (IL)-3 (12). JNK is a subfamily of mitogen-activated protein kinases (MAPKs) involved in cell growth, differentiation, and transformation (for review, see Refs. 13 and 14). MAPK, in general, is activated by phosphorylation of critical threonine and tyrosine residues in the region between subdomains VII and VIII by a dual specificity kinase, generally termed MAPK kinase. MAPK kinases are activated by MAPK kinase kinases. In the case of JNK, two MAPK kinases, MKK4 (=SEK1 and JNKK) and MKK7, are known to activate JNK, and numerous MAPK kinase kinases for JNK have been described recently (15–22). Furthermore, transfection studies demonstrated that Rho family GTPases, Rac1 and Cdc42, regulate JNK activity (23, 24). PAK65, a mammalian homolog of Sacccharomyces cerevisiae Ste20, is activated directly by a GTP-bound form of Cdc42 or Rac1 (25–28) and activates MEKK1, a MAPK kinase kinase, under some conditions (29). In some situations these Rho family proteins are regulated by Ras, a prototypic GTPase (for review, see Refs. 30 and 31). Terada et al. (32, 33) showed recently that Ras regulates the catalytic activities of JNK as well as ERK, a prototypic MAPK, upon IL-3 stimulation.

IL-3 binding to the IL-3 receptor complex induces the activation of several nonreceptor PTKs: Jak1, Jak2, Lyn, Fes, and Tec (for a review, see Ref. 34). Therefore, several proteins are tyrosine phosphorylated in the IL-3-simulated cells (35–37). Because Lyn and other Src family PTKs can phosphorylate Btk at the tyrosine residue (Tyr-551) in the activation loop and activate the enzymatic activity of the latter PTK (38), Tec may also be activated by the same mechanism. Similar to other cytokines, IL-3 stimulation leads to the activation of Ras (39, 40). Tyrosine phosphorylation of Shc, an adaptor molecule with a phosphotyrosine binding domain at its amino terminus and a Src homology 2 (SH2) domain at its carboxyl terminus, and its association with Grb2, another adaptor protein composed of an SH2 domain flanked by two SH3 domains, are also induced by IL-3 (41–44). Although the precise mechanisms for linking these observations have not been elaborated in the IL-3 signaling system, many insights were obtained in growth factor receptor signaling systems. Some growth factor receptors with intrinsic PTK activity activate Ras through the direct or indirect recruitment of Grb2 adaptor protein (for a review, see Ref. 45). For example, ligand-bound and thereby auto-phosphorylated epidermal growth factor receptor binds Grb2 as well as Shc. The SH2 domain of Grb2 binds directly to the tyrosine-phosphorylated receptor or through interactions with Shc. Sos, a guanine nucleotide exchange factor for Ras, constitutively bound to Grb2 through SH3-proline-rich sequence interactions is then mobilized to the plasma membrane where Sos interacts with Ras. GDP-bound inactive Ras is then turned on to the active GTP-bound form (for review, see Ref. 44).

In the present report, transfection studies of IL-3 responsive...
pro-B cells with an inducible Ras expression system demonstrated that Ras plays a role in mediating the activation signal from Btk to JNK activation. Btk also enhanced IL-3-induced association between Shc and Grb2, therefore accounting for the involvement of Ras in this pathway.

EXPERIMENTAL PROCEDURES

Materials—Recombinant mouse IL-3 was a generous gift from Kirin Brewery Co. BaF3-N6 and BaF3-V2 cell lines were described previously (32). Anti-JNK1/p(C17), anti-Btk(M138), and anti-Grb2(C23) antibodies were obtained from Santa Cruz Biotechnology. Anti-phospho-tyrosine antibody was purchased from New England Biolabs, and anti-Shc and anti-phosphotyrosine (4G10) antibodies from Upstate Biotechnology Inc. Retroviral expression vectors, pMXPuro-btk and pMXPuro-btk(K430R), were described previously (12). Pansorbin and hygromycin B were purchased from Calbiochem and enhanced chemiluminescence reagents from NEN Life Science Products. G418 was from Alexis Corporation. Other chemicals of the highest grade were obtained from Sigma Chemical Company unless otherwise mentioned.

Cell Culture and Transfection—BaF3-N6, BaF3-V2 cell lines and their transfectants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mg/ml G418, 1 mg/ml hygromycin B, and mouse IL-3 (culture supernatant of mouse IL-3 gene-transfected cells). For the btk transfectants, 1.2 μg/ml puromycin was included in the culture medium. Bone marrow cells from femurs of B6/129 F2 (wild type) and btk null mice with the same genetic background were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, and IL-3. After 4 weeks of culture, more than 95% of the cells are immature mast cells. These mast cells were incubated overnight with IgE anti-dinitrophenyl monoclonal antibody and stimulated with antigen, dinitrophenyl conjugates of human serum albumin, for 15 min before restimulation with IL-3 for the indicated intervals.

Analyses of Ras-bound Guanine Nucleotide—Analyses of Ras-bound guanine nucleotide in BaF3-N6 transfectants, btk(K430R), were described previously (48). Ras guanine nucleotide exchange activity and ERK phosphorylation were examined upon IL-3 stimulation, we transfected the BaF3-N6 transfectants, btk(K430R), with a LipofectAMINE reagent (Life Technologies, Inc.). Retroviruses (5–6 × 105 colony-forming units/ml) in the culture supernatants were used to infect BaF3-N6 and BaF3-V2 cell lines. Transfected cells were selected in the presence of 1.2 μg/ml puromycin for 2 weeks and cloned by limiting dilutions.

Induction of Ras Expression and IL-3 Stimulation—Expression of a dominant negative ras (N17Ras) gene in BaF3-N6 transfectants and a constitutively active ras (V12Ras) gene in BaF3-V2 transfectants was induced by culturing the cells in the presence of 5 mM IPTG for 18 h at 37 °C during the last 6 h of IPTG treatment, the cells were cultured in RPMI 1640 medium containing 1 mg/ml bovine serum albumin and 25 mM HEPES (pH 7.25) at 37 °C for the indicated periods.

Immunoprecipitation and Immunoblotting—IL-3-stimulated cells were lysed in 1% Nonidet P-40, 0.5 mM PMSF, 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 16.5 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μM p-nitrophenyl p’-guanidinobenzoate, 1 μM pepstatin, and 0.1% NaN3 on ice for 30 min, followed by centrifugation at 15,000 × g for 10 min at 4 °C. For JNK kinase assays, the cells were lysed in WCE lysis buffer (25 mM HEPES (pH 7.5), 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton, 2 μM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 0.5 mM sodium orthovanadate). Cell lysates were incubated with the indicated antibodies for 90 min on ice. Immune complexes were precipitated by a brief centrifugation after incubation with Pansorbin for another 30 min at 4 °C. Precipitated proteins were subjected to immune complex kinase assays or solubilized directly with SDS sample buffer and resolved by SDS-PAGE followed by blotting onto Immobilon-P membranes (Millipore). Blots were blocked and incubated sequentially with a primary antibody, with a secondary antibody conjugated with horseradish peroxidase, and with enhanced chemiluminescence reagents before exposure to Reflection autoradiography films (NEN Life Science Products). Results were analyzed by densitometry.

In Vitro Kinase Assays—Btk activity was measured by autophosphorylation. Btk immune complexes were washed with lysis buffer and then with kinase buffer (minus ATP) before incubation with kinase buffer (50 mM Tris-HCl (pH 7.2), 2 mM MgCl2, 10 mM MnCl2, 0.1% Nonidet P-40, 10 μM ATP, and 10 μCi of [γ-32P]ATP) at 25 °C for 10 min. Reaction products were analyzed by SDS-PAGE and blotted onto Immobilon-P membranes followed by autoradiography.

RESULTS AND DISCUSSION

Btk Is Activated by IL-3 Stimulation—Our previous study demonstrated that JNK activation in response to IL-3 stimulation is up-regulated by Btk in mouse bone marrow-derived mast cells. Thus, IL-3-induced JNK activity at its peak in btk null mast cells was less than half of that in wild-type mast cells (12). These data suggest that Btk is involved in IL-3 signaling pathways, although a previous study reported that Btk is not activated by IL-3 (49). To reevaluate this possibility, we deprived BaF3 pro-B cells of serum and IL-3 for 6 h and then restimulated the cells with IL-3. In vitro kinase assays were performed on Btk immune complexes. As shown previously (49), no apparent changes in Btk autophosphorylation activity were observed during IL-3 stimulation for 30 min (Fig. 1, top panel).

Equal amounts of Btk protein in the immunoprecipitates were detected by reprobing the same blot with anti-Btk antibody (Fig. 1, bottom panel). However, Btk is known to be associated with serine/threonine kinases, one of which was identified to be protein kinase Cβ (50). To eliminate the effects of contaminating serine/threonine kinases, the blot containing the Btk autophosphorylation products was probed with anti-phosphotyrosine antibody. The result clearly showed that tyrosine phosphorylation of Btk is increased by IL-3 stimulation (Fig. 1, middle panel), suggesting that Btk is involved in IL-3 receptor signal transduction. Because Tec, a prototype PTK of this family, was shown previously to be activated by IL-3 stimulation in the same cell line (49), IL-3 receptor occupancy leads to the activation of these Tec family PTKs.

Btk Overexpression Enhances JNK Activity in BaF3 Cells—Terada et al. showed that IL-3-induced JNK activation is dependent on Ras in BaF3 cells (33). They described BaF3-N6 and BaF3-V2 cell lines equipped with the inducible expression vectors for dominant negative Ras(S17N) and constitutively active Ras(G12V) proteins, respectively. To investigate whether Ras is involved in the Btk-mediated JNK activation pathway induced by IL-3 stimulation, we transfected the BaF3-N6 cell line with the wild-type btk cDNA. JNK catalytic activity and ERK phosphorylation were examined upon IL-3.
Role of Ras in Btk-dependent JNK Activation

stabilization in two representative transfected cell clones, BaF3-N-wt8 and BaF3-N-wt9. Expression of 2–3-fold more Btk protein over the endogenous protein was observed in these transfectants by immunoblotting (Fig. 2A). Serum- and IL-3-starved BaF3-N6 transfected cells were stimulated with 100 ng/ml mouse IL-3 for 30 min. JNK1 kinase activity was measured in an in vitro immune complex kinase assay using GST-c-Jun(1–79) as substrate. As shown in Fig. 2B (upper), increases in JNK kinase activity were observed in N-wt8 and N-wt9 cell clones compared with those in vector-transfected cells. Clone N-wt9 exhibited a stronger JNK activity than clone N-wt8. This finding correlates with the higher level of Btk expression in N-wt9. Expression of JNK1 protein detected by immunoblotting cell lysates with anti-JNK1 antibody revealed no significant change among the transfectants (Fig. 2B, lower panel). Kinetic studies showed that overexpression of Btk does not change the time course of IL-3-induced JNK activation, and it peaks around 20–40 min (data not shown) as shown previously (33). Further, these results suggest that overexpressed Btk enhances JNK activation in a mouse pro-B cell line and also confirmed our previous data with mouse bone marrow-derived mast cells (12). ERK activity was also evaluated by immunoblotting with anti-phospho-MAPK antibody. After normalized against ERK loading controls, ERK phosphorylation at the critical tyrosine residue (Tyr-204) induced by IL-3 stimulation was not significantly changed in either Btk-overexpressing cells (Fig. 3C, -IPTG and data not shown). Although BaF3-N-wt9 cells had approximately 50% more ERK amounts for an unknown reason, the other clone, BaF3-N-wt8, had amounts of ERK proteins similar to those in the vector-transfected cells or nontransfected cells (data not shown). These results are consistent with our previous data that Btk did not affect phosphorylation and enzymatic activity of ERKs in FceRI-stimulated mast cells (12).

We also transfected the BaF3-V2 cell line with a kinase-dead (K430R) mutant of btk cDNA and performed the same experiments with the transfected cells. A representative clone, BaF3-V-K/R1, expressed four times more Btk(K430R) protein over the endogenous Btk in the vector-transfected cells (Fig. 3A). Transfection of the K430R btk cDNA did not change JNK1 protein levels (Fig. 3B). As shown in Fig. 3B (–IPTG), JNK activation upon IL-3 stimulation was slightly lower (20% and 25% lower in two experiments) in BaF3-V-K/R1 than in vector-transfected cells, suggesting that the kinase-dead mutant of Btk has a weak dominant negative effect. However, Btk(K430R) expression exhibited little, if any, effect on ERK phosphorylation (Fig. 3C, –IPTG).

**Effects of N17Ras and V12Ras on JNK Catalytic Activity in Wild-type btk and Kinase-dead btk Transfected BaF3 Cells.** Respectively—To investigate the effect of Ras on Btk-mediated JNK activation pathway, we induced the expression of the dominant negative Ras (N17Ras) in BaF3-N6 transfected and the constitutively active Ras (V12Ras) in BaF3-V2 transfected cells in the presence of 5 mM IPTG. As shown in Fig. 3B, the enhancement in JNK activation induced by Btk overexpression was almost totally abrogated in both N-wt8 and N-wt9 cells (compare the lanes between –IPTG and +IPTG). On the other hand, JNK activation was enhanced slightly (by 20–60% at its peak activity) by expression of V12Ras in both vector- and btk(K430R)-transfected BaF3-V2 cells (Fig. 3B). These results suggest that Ras is involved in the Btk-mediated JNK activation pathway.

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**FIG. 2. JNK and ERK activities in the BaF3-N6 transfectants.** Panel A, expression of Btk in vector- or wild-type btk-transfected BaF3-N6 cells. Total cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-Btk antibody. Panel B, cells in the presence or absence of IPTG were starved and restimulated with IL-3 for 30 min as described under “Experimental Procedures.” Cell lysates were immunoprecipitated with anti-JNK1. Immune complex kinase assays were done using GST-c-Jun(1–79) as substrate. Reaction products were analyzed by SDS-PAGE followed by autoradiography (upper). The GST-c-Jun bands are indicated. Results are representative of three similar experiments. The amounts of JNK1 protein in cell lysates were measured by immunoblotting (lower). Panel C, lysates of IL-3-stimulated BaF3-N-wt9 and vector control cells were analyzed by immunoblotting with anti-phospho-MAPK (upper). Positions of phospho-ERK1 and phospho-ERK2 are indicated. The same blot was reprobed with anti-MAPK (lower). Positions of ERK1 and 2 are indicated. Results are representative of three similar experiments.

**FIG. 3. JNK and ERK activities in the BaF3-V2 transfected cells.** Panel A, Btk expression was analyzed as described in the legend to Fig. 2A. A representative transfected, BaF3-V-K/R1, expresses ~4-fold more Btk(K430R) protein over the endogenous Btk in the vector-transfected cells. Panel B, cells were starved and restimulated with IL-3 for the indicated intervals. JNK1 immune complex kinase assays were carried out as described in the legend to Fig. 2B (upper). The amounts of JNK1 protein in cell lysates were measured by immunoblotting (lower). Panel C, immunoblotting of the total cell lysates with anti-phospho-MAPK was performed as described in the legend to Fig. 2C and followed by reprobing with anti-MAPK. Results shown in panels B and C were repeated in another experiment.

Btk(K430R) expression exhibited little, if any, effect on ERK phosphorylation (Fig. 3C, –IPTG).
Role of Ras in Btk-dependent JNK Activation

As shown previously (32), ERK activity, as revealed by immunoblotting with anti-phospho-MAPK antibody, was enhanced by induction of V12Ras (Fig. 3C) and partially suppressed by induction of N17Ras (Fig. 2C), irrespective of overexpression of wild-type or K430R Btk protein in the BaF3-N6 or BaF3-V2 cells, respectively.

Btk Overexpression Enhances Ras Activation upon IL-3 Stimulation—Because Btk overexpression induced a remarkable enhancement in JNK activation in IL-3-stimulated BaF3-N-wt9 cells, we measured the GTP/GDP ratio of Ras in these cells before and after IL-3 stimulation. As shown in Fig. 4, the active GTP-bound form of Ras in a control cell line, BaF3-N-vec, increased upon IL-3 stimulation as described previously (32). The GTP/GDP ratios in BaF3-N-wt9 cells were twice as high as those in BaF3-N-vec. Therefore, this experiment clearly demonstrates that Btk regulates the Ras activity.

Neither N17Ras nor V12Ras Affects Btk Activity in BaF3 Cells—We next examined the effects of the dominant negative and constitutively active Ras mutants on Btk kinase activity. Btk autophosphorylating activity was not affected by the IPTG-induced expression of either N17Ras or V12Ras (data not shown). Because Btk kinase activity is correlated with its tyrosine phosphorylation (51), the blot containing the Btk autophosphorylation products was probed with anti-phosphotyrosine antibody. This latter experiment also confirmed the lack of effects on Btk kinase activity by mutant Ras expression (data not shown). Further, IPTG treatment of the parental BaF3 cells did not change Btk expression or Btk kinase activity. These results are consistent with the notion that Btk works upstream of Ras, and active Ras in turn leads to the activation of JNK.

Btk Overexpression Enhances the Shc-Grb2 Association in BaF3 Cells—To gain insight into how Btk activates Ras, we analyzed the effects of IL-3 stimulation on two Ras-regulatory adaptor proteins, Shc and Grb2, in vector- or wild-type btk-transfected BaF3-N6 cells. Anti-Shc immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody as well as anti-Grb2 antibody. As shown in Fig. 5A, Shc-Grb2 association was increased markedly in the Btk-overexpressing cells (BaF3-N-wt9), although tyrosine phosphorylation levels of Shc were indistinguishable between vector- and btk-transfected BaF-N6 cells. After being normalized for the amount of Shc as revealed by reprobing the blot, the coimmunoprecipitated Grb2 in BaF3-N-wt9 cells at 5 min after IL-3 stimulation was severalfold (5.9- and 8.7-fold in two experiments) more than those in BaF3-N-vec cells. Similar observations were made on FcεRI-stimulated cultured mast cells derived from bone marrow cells of wild-type and btk null mice. Thus, the amount of Grb2 associated with Shc upon FcεRI stimulation was slightly higher (22% and 28% in two experiments) in wild-type mast cells than in btk null mast cells (Fig. 5B). Therefore, it seems that the amount of Grb2 associated with Shc is controlled by expression levels of Btk. Because the Shc-Grb2 association is an early signaling event preceding Ras activation, Btk might up-regulate Ras activation through its effects on the Shc-Grb2 association. In contrast, neither Shc tyrosine phosphorylation nor Shc-Grb2 association changed significantly in the BaF3-V-K/R1 transfectants (data not shown). This might reflect the weak effect by K430R Btk. This also suggests that Btk-enhanced Shc-Grb2 association might require the kinase activity of Btk. Although the precise mechanisms remain to be explored, these results demonstrate collectively that Btk operates upstream of Ras in the IL-3 receptor signaling pathway.

Concluding Remarks—Both Btk and JNK are activated by a variety of extracellular stimuli. Some stimuli, e.g. FcεRI and B cell receptor stimulation, induce the activation of both Btk and JNK (12, 51–55). Although the current study deals mostly with IL-3 receptor-activated signaling pathways, Ras may mediate the Btk activation signal to JNK activation not only in the IL-3 system but also in other signal transduction systems such as the FcεRI system. Indeed, Ras activation was shown by this and the aforementioned stimuli (56–59). Increased association of Shc and Grb2 seems to be part of the Ras activation mechanisms in these signaling processes.

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Involvement of Ras in Bruton's Tyrosine Kinase-mediated JNK Activation
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