Btk Plays a Crucial Role in the Amplification of FcεRI-mediated Mast Cell Activation by Kit*

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Stem cell factor (SCF) acts in synergy with antigen to enhance the calcium signal, degranulation, activation of transcription factors, and cytokine production in human mast cells. However, the underlying mechanisms for this synergy remain unclear. Here we show, utilizing bone marrow-derived mast cells (BMMCs) from Btk and Lyn knock-out mice, that activation of Btk via Lyn plays a key role in promoting synergy. As in human mast cells, SCF enhanced degranulation and cytokine production in BMMCs. In Btk−/− BMMCs, in which there was a partial reduction in the capacity to degranulate in response to antigen, SCF was unable to enhance the residual antigen-mediated degranulation. Furthermore, as with antigen, the ability of SCF to promote cytokine production was abrogated in the Btk−/− BMMCs. The impairment of responses in Btk−/− cells correlated with an inability of SCF to augment phospholipase Cγ1 activation and calcium mobilization, and to phosphorylate NFκB and NFAT for cytokine gene transcription in these cells. Similar studies with Lyn−/− and Btk−/−/Lyn−/− BMMCs indicated that Lyn was a regulator of Btk for these responses. These data demonstrate, for the first time, that Btk is a key regulator of a Kit-mediated amplification pathway that augments FcεRI-mediated mast cell activation.

Mast cell activation leads to the release of both preformed and de novo synthesized inflammatory mediators. The intracellular signaling cascade regulating these responses is initiated by aggregation of high affinity receptors for IgE (FcεRI) following antigen binding to receptor-bound IgE (1). However, antigen-induced triggering of mast cells in vivo is likely to occur with a background of stem cell factor (SCF)-mediated Kit activation, as SCF is essential for the growth, differentiation, homing, and survival of mast cells (2). By mimicking this situation in vitro, we have demonstrated that SCF dramatically augments both antigen-mediated degranulation and cytokine generation in these cells (3, 4). Kit-mediated signals are thus required for optimal mast cell degranulation and cytokine production induced by FcεRI aggregation.

Antigen-mediated degranulation and cytokine production are thought to be initiated by the activation of the Src family tyrosine kinase, Lyn (5). The resulting tyrosine phosphorylation of the β and γ chains of FcεRI promotes the binding of the tyrosine kinase Syk to FcεRI (6). This permits the trans/auto-phosphorylation and activation of Syk (7, 8), which in turn phosphorylates the transmembrane adaptor molecules LAT (9) and NTAL (3, 10). These adaptor molecules orchestrate the recruitment of downstream signaling molecules to the receptor-signaling molecular complex by providing docking sites for cytosolic adaptor molecules, including SLP-76, Vav, Gads, Grb2, Gab1, and Gab2 (11) and signaling enzymes such as phospholipase (PL)Cγ1, PLCy2, and phosphoinositide (PI) 3-kinase (12, 13). The subsequent elevation of intracellular calcium levels and activation of protein kinase C (PKC) leads to degranulation (14), whereas activation of the Ras-Raf-MAPK pathway induces arachidonic acid metabolite release (15) and downstream phosphorylation and activation of specific cytokine gene-related transcription factors (16). A parallel pathway controlled by the Src kinase, Fyn, also appears to help regulate FcεRI-dependent mast cell activation (17).

Many of these same signaling events are initiated upon binding of SCF to Kit (18) but are insufficient on their own to induce degranulation (4). Our previous studies have suggested that this may be related to the inability of SCF to induce phosphorylation of LAT (3) and downstream activation of PKC (4). Nevertheless, SCF can potentiate FcεRI-mediated degranulation and phosphorylation of NTAL as well as enhance calcium mobilization (3). How SCF augments these responses, however, was unclear. Given that the tyrosine kinase, Btk, is thought to play a role in the regulation of PLCγ-mediated calcium mobilization for both the β cell receptor (19) and the FcεRI (20), we have examined whether Btk played a similar role in Kit-mediated responses. By use of bone marrow-derived mast cells (BMMCs) from gene-deficient mice, Btk was not only found to be essential for the ability of SCF to potentiate antigen-mediated degranulation but was also found to be required for the ability of Kit to regulate cytokine production in antigen-stimulated cells.

**EXPERIMENTAL PROCEDURES**

**Mast Cells**—The Btk−/−, Lyn−/−, Btk−/−/Lyn−/− knock-out, and wild type (WT) mice used in this study have been described previously (20). The mice were cross-bred on a C57BL/6 × 129/Sv genetic background. The wild type mice were derived from the same parental lines as Btk−/−/Y or Lyn−/−/Y, or both were set up to generate both wild type and knock-out mice within the same litter. Whenever possible, littersmates were compared directly. All animals were housed within the same room. The genotype of these mice was confirmed by reverse tran-
scription-PCR of tail biopsies and by immunoblot analysis of proteins extracted from the BMMCs derived from these mice. Bone marrow obtained by femur lavage was cultured in RPMI 1640 medium containing IL-3 as described (13). The studies were then conducted on BMMCs after 4–6 weeks in culture.

**Cell Activation**—For degranulation and signaling studies, cultured BMMCs were sensitized overnight with anti-mouse monoclonal dinitrophenyl (DNP) IgE (100 ng/ml) (Sigma) in IL-3-free RPMI medium and then rinsed with HEPES buffer (21) containing 0.04% bovine serum albumin (Sigma). The cells were triggered in the same buffer with DNP-human serum albumin (HSA) (0–100 ng/ml) and/or murine SCF (0–100 ng/ml; PeproTech, Rocky Hill, NJ) for 30 min for the degranulation studies or for the indicated periods for the signaling studies. For cytokine mRNA and release studies, cells were similarly sensitized but triggered for 4 or 10 h, respectively, in RPMI.

**Degranulation Assay**—Degranulation was monitored by the release of β-hexosaminidase into the supernatants (22). Briefly, BMMCs, sensitized as above, were triggered in 96-well plates (5 × 10⁵ cells per well, 100 μl final volumes). The reactions were terminated by centrifugation (3000 rpm) at 4°C, and the supernatants were aliquoted to 96-well plates for β-hexosaminidase assay. The remaining cells were lysed by adding distilled water and freeze-thawing, and then aliquots were similarly assayed for β-hexosaminidase content. Degranulation was then calculated as the percentage of total (cells and supernatants) β-hexosaminidase content found in the supernatants following challenge.

**Cytokine Production**—RNAse protection assays (RPA) were utilized to measure mRNA levels for multiple cytokines and chemokines following cell activation. Cells were sensitized and then triggered as above at a concentration of 10 × 10⁶ cells/ml. Messenger RNA was extracted by lysing the cells with 1 ml of TRIzol (Invitrogen) for 5 min at room temperature. Chloroform (200 μl) was added to the lysates, and the mixtures were centrifuged for 15 min at 14,000 rpm. Isopropyl alcohol (500 μl) was then added to the aqueous phases, and the mixture was incubated for 10 min to precipitate RNA. Ten μg of RNA was used in the mRNA assay by using an in vitro transcription kit and pre-designed or custom-designed RPA templates (BD Biosciences). RPA was conducted according to the manufacturer’s instructions; however, the synthesized radioactive probes labeled with α³²PUTP were purified with a probe-quant G-50 microcolumn (Amersham Biosciences) instead of ethanol precipitation, and the protected mRNA was precipitated with ethanol (3000 rpm) at 4°C, and the supernatants were aliquoted to 96-well plates for subsequent studies by ELISA (BIOSOURCE, Camarillo, CA).

**Identification of Phosphorylated Proteins**—Following the degranulation studies, cells were lysed by PBS containing 0.04% bovine serum albumin and sulfinpyrazone (0.3 mM) (Sigma), and then placed in a 96-well black culture plate (20,000 cells/well) (CulturPlat-96 F, PerkinElmer Life Sciences). Fluorescence was measured at two excitation wavelengths (340 and 380 nm) and an emission wavelength of 510 nm. The ratio of the fluorescence readings was calculated following subtraction of the fluorescence of the cells that had not been loaded with Fura-2 AM.

**Intracellular Calcium Determination**—Calcium flux was measured in the BMMCs following loading of the cells with Fura-2 AM ester (Molecular Probes, Eugene, OR) as described (13). Cells were loaded with Fura-2 AM for 30 min at 37°C, rinsed, and resuspended in HEPES buffer containing 0.04% bovine serum albumin and sulfinpyrazone (0.3 mM) (Sigma), and then placed in a 96-well black culture plate (20,000 cells/well) (CulturPlat-96 F, PerkinElmer Life Sciences). Fluorescence was measured at two excitation wavelengths (340 and 380 nm) and an emission wavelength of 510 nm. The ratio of the fluorescence readings was calculated following subtraction of the fluorescence of the cells that had not been loaded with Fura-2 AM.

**RESULTS**

**Kit Induces Lyn-dependent Phosphorylation of Btk in BMMCs**

To investigate the role of Btk in Kit-mediated responses, we utilized BMMCs derived from the bone marrow of Btk−/− mice. Because previous studies had suggested that Btk and Lyn had both redundant and opposing functions in antigen-dependent mast cell (20) and B cell activation (24), we compared the responses in the Btk−/− BMMCs to those obtained in WT, Lyn−/−, and Btk−/−/Lyn−/− double knock-out BMMCs. The Btk−/−, Lyn−/−, and Btk−/−/Lyn−/− BMMCs genotypes were confirmed by probing lysates from these cells for Btk and Lyn (data not shown). The levels of expression of the other Tec kinases, including TEC and Itk (as controls for Btk) and other Src kinases, including Fgr, Fyn, Hck, c-Src, and Yes (as controls for Lyn), were unaffected in these cells, apart from a slight reduction in the expression of Fgr in the Lyn−/− and Btk−/−/Lyn−/− BMMCs (data not shown).

Both antigen and SCF induced the phosphorylation of Btk in WT mouse BMMCs (Fig. 1, a and b); however, maximum phosphorylation observed with SCF was of a lesser magnitude than that observed with antigen. Although there was little evidence of synergy in the responses at early time points (0–120 s), when cells were co-stimulated with SCF and antigen, Btk phosphorylation was more sustained than was observed with the individual stimulants. As expected, this phosphorylation was not detected in the Btk−/− BMMCs (Fig. 1, c and d). In addition, the phosphorylation of Btk was substantially reduced in the Lyn−/− BMMCs indicating that the phosphorylation of Btk was largely dependent on Lyn.

Stimulation of BMMCs with antigen, but not SCF, resulted in an increase in the phosphorylation of the Src kinases (Fig. 1, e and f), although Src kinases were constitutively phosphorylated to some degree. In the Lyn−/− BMMCs there was virtually no phosphorylation of the Src kinases in both stimulated and non-stimulated BMMCs. Thus, the major Src kinase phosphorylated both constitutively and inducibly by antigen in the BMMCs was Lyn. However, overexposure of the gels revealed that SCF, but not antigen,
also resulted in a lesser phosphorylation of another Src kinase that was not Lyn (Fig. 1e). There was little change in the phosphorylation of the Src kinases in the Btk−/− BMMCs, thus confirming that the phosphorylation of Btk is downstream of Lyn.

**SCF Augments FcεRI-mediated Degranulation and Cytokine Generation in Mouse BMMCs**

To establish that SCF potentiated FcεRI-dependent responses in WT mouse BMMCs as was the case in human mast cells (3, 4), we examined degranulation and cytokine production in response to SCF, antigen, or both in combination. Fig. 2a shows that SCF, at concentrations up to 100 ng/ml, induced little degranulation. When added concurrently with antigen, however, SCF induced a marked concentration-dependent potentiation of antigen-mediated degranulation. Similarly, SCF and antigen acted in synergy to increase the message of multiple cytokines, including IL-1α, IL-1β, IL-4, IL-6, IL-13, TNF-α, and interferon-γ (Fig. 2b). To confirm that the potentiation of cytokine message levels translated into increases in cytokine protein, the release of TNF-α, IL-6, and IL-13 was examined by ELISA 10 h following challenge with SCF with or without antigen. Again, as in human mast cells (4), cytokine secretion was minimally elevated in response to either SCF or antigen alone, but when added in combination, there was a marked synergistic enhancement of cytokine production (Fig. 2, c–e).

**Kit-mediated Degranulation and Cytokine Production in Btk−/−, Lyn−/−, and Btk−/−/Lyn−/− BMMCs**

As reported (20), antigen-mediated degranulation was reduced by ~50% in both the Btk−/− and Lyn−/− BMMCs when compared with
FIGURE 2. The effects of SCF and Ag on degranulation (a) and cytokine production (b) in WT BMMCs are shown. Cells were sensitized overnight and then challenged with SCF (a, indicated concentrations; b–e, 100 ng/ml) or Ag (DNP-HSA) (a, indicated concentrations; b–e, 100 ng/ml) or Ag and SCF added concurrently (a, indicated concentrations; b–e, 100 ng/ml) for 30 min for β-hexosaminidase (β-hex) release (a), 4 h for RPA (b), or 10 h for ELISA (c–e). The samples were then processed and the assays conducted as described under “Experimental Procedures.” The data in a are presented as means ± S.E. (n = 4–6) of separate experiments conducted in duplicate, in b are representative of n = 3, and in c–e are means ± S.E. (n = 3). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 3. The effects of Ag (a) or Ag and SCF added concurrently (b) on degranulation and on secreted TNF-α (c), IL-6 (d), and IL-13 production (e) in WT, Btk−/−, Lyn−/−, and Btk−/−/Lyn−/− BMMCs. Cells were sensitized overnight, then challenged with Ag (DNP-HSA, 100 ng/ml) for the indicated times (a) or with the indicated concentrations of SCF in the absence or presence of the indicated concentrations of Ag for 30 min (b). Degranulation was then assessed by monitoring the release of β-hexosaminidase (β-hex) as described under “Experimental Procedures.” For cytokine release in c–e, cells were similarly sensitized but challenged for 10 h. The media were then collected and assayed for the indicated cytokines by ELISA. The data in a are presented as means ± S.E. (n = 3); in b as means ± S.E. (n = 5) apart from SCF alone which is n = 2. c–e, the data are presented as means ± S.E. (n = 2–4), and the order of bars for each cell type is control, Ag, SCF, and Ag + SCF.
WT controls and was virtually abolished in the Btk\(^{-/-}\)/Lyn\(^{-/-}\) double knock-out BMMCs (Fig. 3a). SCF was unable to potentiate the residual antigen-mediated degranulation (i.e. 10–15%) in the Btk\(^{-/-}\) and the Lyn\(^{-/-}\) BMMCs and the minimal degranulation in the Btk\(^{-/-}\)/Lyn\(^{-/-}\) double knock-out BMMCs (Fig. 3b). This is in contrast to SFC-mediated potentiation of degranulation induced by minimally effective concentrations of antigen as shown previously in Fig. 2a.

Production of TNF-α and IL-6 and IL-13 (Fig. 3, c–e, respectively) in response to SCF and antigen or both in combination was reduced by ~50% in the Btk\(^{-/-}\) BMMCs, potentiated in the Lyn\(^{-/-}\) BMMCs, and virtually reduced to background levels in the Btk\(^{-/-}\)/Lyn\(^{-/-}\) double knock-out BMMCs, as compared with WT BMMCs. In contrast to degranulation, however, an additive response to the combination of SCF and antigen was still observed in Btk\(^{-/-}\) BMMCs, although the net response was still ~50% that in WT BMMCs. In the Btk\(^{-/-}\)/Lyn\(^{-/-}\) double knock-out BMMCs, however, production of cytokines was virtually ablated. Similar responses were observed at the message level as determined by RPA (data not shown).

**SCF- and Antigen-induced Signaling Studies in Btk\(^{-/-}\) and Lyn\(^{-/-}\) BMMCs**

**Activation of PLCγ1 and PI 3-Kinase—**Our previous studies suggested that the ability of SCF to potentiate antigen-mediated degranulation was associated with an enhancement of calcium mobilization (4). As both PLCγ\(_1\)- and PI 3-kinase-dependent pathways control FceRI-mediated degranulation in human mast cells via regulation of calcium mobilization (13), we next examined whether these signaling events were ablated in the Btk\(^{-/-}\), Lyn\(^{-/-}\), and Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs.

PLCγ\(_1\) and PI 3-kinase activation was monitored by the phosphorylation of PLCγ\(_1\) or AKT, respectively (13). Both SCF and antigen stimulated PLCγ\(_1\) phosphorylation in WT BMMCs, and the combination of both stimuli resulted in an additive and more sustained phosphorylation than that induced by either stimulant alone (Fig. 4, a, b, e, and f). We were unable to detect PLCγ\(_1\) phosphorylation in response to SCF and antigen by utilizing a commercially available anti-phospho-PLCγ\(_1\). However, following immunoprecipitation with an anti-PLCγ\(_1\) antibody and then probing with an anti-phosphotyrosine antibody, we observed that although both antigen and SCF induced PLCγ\(_1\) phosphorylation, these responses were not additive (data not shown). In contrast to PLCγ\(_1\) phosphorylation, the effects of SCF and antigen on AKT phosphorylation (Fig. 4, c, d, g, and h) were not additive. Rather, antigen induced a decrease in the more predominant SCF-mediated AKT phosphorylation (Fig. 4, c and d). This was likely because of Lyn-mediated down-regulation of PI 3-kinase activation, as the inhibitory response was reversed in the Lyn\(^{-/-}\) and Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs but not the Btk\(^{-/-}\) BMMCs (Fig. 4, g and h). The lack of synergistic enhancement of AKT phosphorylation in the Lyn\(^{-/-}\) and Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs likely reflects the fact that both a Lyn-dependent inhibitory pathway, potentially via SHIP (25), and a Lyn-dependent activation pathway, potentially via Syk (26), for antigen-induced PI 3-kinase activation are blocked in these cells. This conclusion is further supported by the fact that the slight increase in AKT phosphorylation observed in response to antigen in the WT cells is absent in the Lyn\(^{-/-}\) and Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs (Fig. 4, g and h).

Btk-deficient BMMCs exhibited a slight reduction in PLCγ\(_1\) phosphorylation in response to antigen and SCF (Fig. 4, e and f). However, the ability of SCF to potentiate antigen-mediated PLCγ\(_1\) phosphorylation was completely blocked in the Btk\(^{-/-}\) BMMCs, and the combination of antigen and SCF appeared to result in phosphorylation levels that were slightly lower than that observed with antigen alone (Fig. 4, e and f).

**FIGURE 4. Phosphorylation of PLCγ\(_1\) (a, b, e, and f) and AKT (c, d, g, and h) in response to Ag, SCF, or SCF in the presence of Ag in WT (a–d), Btk\(^{-/-}\), Lyn\(^{-/-}\), and Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs (e–h).** Cells were sensitized and treated with control buffer (C), Ag (DNP-HSA: 100 ng/ml), SCF (100 ng/ml), or Ag and SCF added concurrently (100 ng/ml) for the indicated times (a–d) or for 120 s (e–h), and proteins were extracted. Following gel electrophoresis, the proteins were probed with antibodies recognizing phosphorylated PLCγ\(_1\) (p-PLCγ\(_1\)), phosphorylated AKT (p-AKT), or actin. The blots are representative of n = 3–4. The data in b, d, f, and h were generated by scanning the blots in a, c, e, and g, respectively, and then normalizing to the maximal response obtained with antigen (b and f) or SCF (d and h) alone. The symbols used are as follows: ●, Ag; ▲, SCF; ▼, SCF and Ag concurrently in b and d; f and h, the order of bars for each cell type is control, Ag, SCF, and Ag + SCF.

In Lyn\(^{-/-}\), as well as Btk\(^{-/-}\)/Lyn\(^{-/-}\) BMMCs, the ability of antigen in the absence or presence of SCF to induce PLCγ\(_1\) phosphorylation was completely blocked. As a result, the synergistic increase in PLCγ\(_1\) phosphorylation in response to SCF and antigen added concurrently was reduced to close to baseline in the Btk\(^{-/-}\)/Lyn\(^{-/-}\) double knock-out cells. Thus, although Lyn was required for phosphorylation of PLCγ\(_1\) in response to antigen, Btk was central to the ability of SCF to potentiate this response.
Calcium Mobilization—As with human mast cells (4), SCF and antigen acted in synergy to enhance calcium mobilization in WT BMMCs (Fig. 5a). In the Btk−/− BMMCs, the initial increases in calcium mobilization in response to antigen (Fig. 5b) or SCF (Fig. 5c) when added separately or concurrently (Fig. 5d) were still observed. These responses, however, were substantially lower and less sustained than those observed in the WT BMMCs. In contrast, in the Lyn−/− BMMCs, the increase in calcium levels was delayed but eventually reached levels that were similar to those in WT BMMCs. As was the case with degranulation, the residual calcium flux in the antigen-challenged Btk−/− (Fig. 5e) or Lyn−/− BMMCs (Fig. 5f) could not be further potentiated by SCF. In the Btk−/−/Lyn−/− double knock-out BMMCs, the calcium response to both stimuli was virtually ablated (Fig. 5, b–d).

Taken together, the above data support the concept that the ability of SCF to potentiate antigen-mediated calcium mobilization, hence degranulation, was entirely dependent on Btk, and this was at the level of PLCγ activation but downstream of PI 3-kinase activation.

MAPK and Transcription Factor Phosphorylation—We next examined if the observed deficiencies in cytokine production in the Btk−/−, Lyn−/−, and Btk−/−/Lyn−/− BMMCs correlated to reduced activation of MAPKs and specific transcription factors. In WT BMMCs, the phosphorylation of the ERK1/2, JNK, and p38 MAPKs was augmented by
co-stimulation with antigen and SCF compared with the effects of the individual stimulants added alone (Fig. 6). There was no reduction in the synergistic phosphorylation of ERK1/2 in the Btk<sup>−/−</sup> and Lyn<sup>−/−</sup> BMMCs (Fig. 6, a and b) and only a slight reduction in the Btk<sup>−/−</sup>/Lyn<sup>−/−</sup> BMMCs. In contrast, the synergy between antigen and SCF in the phosphorylation of p38 MAPK and JNK was markedly impaired in all kinase-deficient BMMCs. Paradoxically, Lyn deficiency resulted in enhanced phosphorylation of both ERK1/2 and JNK in antigen-stimulated cells, and as a result, the additive effects of SCF on these responses were less apparent in these cells. Taken together, the above data indicate that the reduction in cytokine production in the Btk<sup>−/−</sup> and Btk<sup>−/−</sup>/Lyn<sup>−/−</sup> BMMCs was associated with similar deficiencies in the p38 and JNK signaling pathway(s) but not the ERK1/2 pathway.

JNK and p38 regulate gene transcription by mediating the phosphorylation of transcription factors, including those of the AP1 complex (Fos and Jun), NFAT, and NF<sub>KB</sub> (27–29). We thus examined these responses in the WT and kinase-deficient BMMCs. Both SCF and antigen induced the synthesis of Jun. However, these responses were not additive (Fig. 7, a and b). The subsequent phosphorylation of Jun in response to SCF and antigen added concurrently was greater than the responses of these agents when added separately (Fig. 7, c and d). We observed no marked differences in levels of Jun in the kinase-deficient
WT BMMCs. The phosphorylation of NFκB and NFAT, in response to SCF and antigen, added separately or concurrently, was partially reduced in the Lyn−/− BMMCs but substantially reduced in the Btk−/− and Btk−/−/Lyn−/− BMMCs (Fig. 7, h−k). Taken together, these studies indicate that the defective phosphorylation of NFAT and NFκB, rather than defective regulation of components of the AP1 complex, could be responsible for the reduced cytokine production in the Btk−/− and Btk−/−/Lyn−/− BMMCs.

**DISCUSSION**

Previous studies have focused on the role of Btk in regulating BCR-mediated responses in B cells (24) and FcεRI-mediated responses in mast cells (30–32). However, the present studies point to a broader role of Btk as a core component of an amplification pathway that is utilized by Kit for augmenting the activation of mast cells via FcεRI. As such, Btk may provide the link between receptor-proximal events and integrated downstream signaling by Kit and FcεRI. These synergistic interactions likely mirror the situation that would be expected in vivo where SCF is essential for mast cell growth (33), differentiation (34), homing (35), and survival (36).

In the present studies, we have utilized Btk−/− and Lyn−/− mice to demonstrate the essential role for both kinases in the synergistic response to antigen and SCF in BMMCs. As shown previously in human mast cells (3, 4), SCF was found to markedly potentiate degranulation and augment cytokine production in WT mouse BMMCs (Fig. 2). The enhancement of degranulation could be attributed to a synergistic increase in PLCγ1 phosphorylation and the resultant enhanced calcium mobilization. Examination of the kinetics of these and other signaling responses, including the phosphorylation of Btk (Fig. 1) and NTAL, suggested that this may be because of an SCF-dependent conversion of the normally transient FcεRI-mediated responses to a more sustained response. As reported previously (20), degranulation in response to antigen was reduced by ∼50% in both Btk−/− and Lyn−/− BMMCs and was essentially absent in the Btk−/−/Lyn−/− double knock-out BMMCs (Fig. 3). The additive defect in the Btk−/−/Lyn−/− BMMCs, compared with the single knock-out BMMCs, suggested that, although there is some overlap in the regulation and function of these enzymes, Btk and Lyn may also act independently to regulate degranulation (20).

The failure of SCF to enhance the residual antigen-induced degranulation in Btk−/− and Lyn−/− BMMCs indicated that both of these enzymes were essential for the ability of Kit to enhance mast cell degranulation. These observations correlated with deficient calcium signaling in the Btk−/− and Lyn−/− BMMCs (Fig. 5). Although the phosphorylation of Btk was partially regulated by Lyn, these enzymes appear also to have independent roles in mediating the calcium response. For example, the initial increase in the calcium signal observed in WT BMMCs in response to antigen was absent in the Lyn−/− BMMCs, whereas the initial increase in calcium flux in response to antigen and SCF was still evident in the Btk−/− BMMCs. However, this signal was lost sustained resulting in substantially lower maximal calcium levels in these cells.

The inability of SCF to enhance the residual antigen-induced increase in the calcium signal in Btk−/− and Lyn−/− BMMCs was associated with an inability of SCF to augment the residual PLCγ1 phosphorylation in these cells (Fig. 4). Btk is known to activate PLCγ by phosphorylating conserved activation tyrosine residues in the Src homology 2–Src homology 3 domain linker region in both PLCγ1 (Tyr(P)-771 and Tyr(P)-783) and PLCγ2 (Tyr(P)-753 and Tyr(P)-759) in B cells (37). We noted that although Lyn was absolutely required for the phosphorylation of Tyr(P)-783 in PLCγ1, in response to antigen in BMMCs, the phosphorylation of this residue was only partially reduced in the indi-
individual responses to antigen and SCF in the Btk−/− BMMCs. Nevertheless, Btk was absolutely required for the Kit-mediated enhancement of the phosphorylation of PLCγ1 (Tyr(P)-783) in response to antigen. These data suggest that in activated mast cells the phosphorylation of the critical Tyr(P)-783 in PLCγ1 is regulated both by a Lyn-dependent/Btk-dependent pathway and a Lyn-dependent but Btk-independent pathway. It is the Lyn-dependent/Btk-dependent pathway, however, that is central to the amplification signaling cascade that is utilized by Kit to regulate FcεRI-mediated mast cell degranulation. Our results, however, show some discrepancies with past observations in that it had been reported previously that antigen-dependent degranulation is either unchanged (38) or even enhanced (17, 25) in Lyn−/− BMMCs. Nevertheless, our observations agree with reports of reduced antigen-dependent degranulation in these cells (20). The reasons for these apparent discrepancies remain unclear but may reflect different conditions for cell culture as, for example, the presence or absence of SCF.

The synergistic cytokine production in response to SCF and antigen (Fig. 2) in the BMMCs was accompanied by a marked synergistic phosphorylation of the MAPKs, ERK1/2, JNK, and p38 (Fig. 6) and a downstream synergistic phosphorylation of the transcription factors Jun and NFκB (Fig. 7). Although the expression of Fos and Jun was increased by both antigen and SCF, additive or synergistic responses were not observed with the combination of stimuli. Likewise, synergy was not observed in the phosphorylation of the activating Ser-54 residue of NFAT. As reported for antigen-mediated production of IL-2 and TNF-α in BMMCs (20, 39), elevated cytokine message and protein levels for multiple cytokines, including TNF-α, IL-6, and IL-13, in response to SCF were reduced by about 50% in the Btk−/− BMMCs whether antigen was present or not (Fig. 3). The residual synergy observed in the Btk−/− BMMCs was essentially abolished in the Btk−/−/Lyn−/− double knock-out BMMCs, at least for TNF-α and IL-13, despite an apparent enhancement in the Lyn−/− single knock-out BMMCs. The enhanced cytokine production in the Lyn−/− BMMCs may be because of a similar enhancement of ERK1/2 and JNK phosphorylation in response to antigen in these cells. Alternatively, this may reflect the reversal in the Lyn−/− BMMCs of the tonic inhibition of Kit-mediated PI3-kinase by antigen that was observed in the WT BMMCs.

TNF-α, IL-6, and IL-13 genes are regulated by binding of the transcription factors, NFAT and NFκB (40–42), and the AP1 complex to their promoter regions as regulated by AKT (32) and MAPKs, including NFκB (43). The lack of defective AKT and ERK1/2 phosphorylation in Btk−/− and Btk−/−/Lyn−/− BMMCs indicates that the reduced cytokine production observed in these cells was not linked to these signaling molecules. However, the defects in cytokine production in the Btk−/− and Btk−/−/Lyn−/− BMMCs are accompanied by defective p38 and/or JNK signaling.

Of the downstream transcription factors examined, the only defects that correlated with the decreased cytokine production in the Btk−/− and Btk−/−/Lyn−/− BMMCs was the substantial reduction in the phosphorylation of NFAT and NFκB. Despite significant reduction in JNK and p38 phosphorylation in these cells, we observed no apparent decreases in the synthesis and/or the overall total phosphorylation of AP1 components, Jun and Fos, although, on a per unit mass basis, a reduction in the phosphorylation of Jun in response to the combination of SCF and antigen was observed to reflect the changes in JNK phosphorylation. In T cells, NFAT is a target for p38 MAPK (43), and in B cells, NFAT activity is likely regulated by a Lyn-Syk-Btk-PLCγ pathway through activation of the calcium-binding phosphatase, calcineurin (44–46). It should be noted that although NFAT is regulated by dephosphorylation of multiple residues, its activity is also dependent on the calcium- and PKC-dependent phosphorylation of Ser-54 (47, 48) examined in this study. Thus the deficiencies in NFAT phosphorylation in the Btk−/− and Btk−/−/Lyn−/− BMMCs may be explained by the reduced calcium signal and defective p38 phosphorylation in these cells. Similarly, both NFκB and NFAT phosphorylations appear to be regulated by the converging activities of JNK and p38 in B cells (49). Again this might explain how defective phosphorylation of p38 and JNK would lead to similar defects in the phosphorylation of NFAT and NFκB and ultimately reduced cytokine production in the Btk−/− and Btk−/−/Lyn−/− BMMCs.

As summarized in Fig. 8, in this paper we have presented data to support the conclusion that mast cell activation is regulated by both a primary and amplification signaling pathway that Btk is an essential player in the amplification pathway that is utilized by Kit for the potentiation of mast cell mediator release. Moreover, these studies further reinforce the concept that allergic responses to antigen in a physiological setting must be viewed in the context of a background of Kit activation. Finally, these observations may set a novel paradigm for the way in which other stimuli such as adenosine, C3a, IL-3, IL-4, substance P, and chemokines, either induce or potentiate FcεRI-mediated degranulation.

Acknowledgment—We thank Clifford A. Lowell (Department of Laboratory Medicine, University of California, San Francisco) for providing us with knock-out mice.

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