

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 C_{g1} 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 pre-formed 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 and Gab1, Gab2 (11); and signaling enzymes such as phospholipase (PL)C γ_1 , PLC γ_2 , 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 Fc ϵ RI-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 Fc ϵ RI-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 PLC γ -mediated calcium mobilization for both the B cell receptor (19) and the Fc ϵ RI (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 (W/T) mice used in this study have been described (20). The mice were cross-bred on a C57BL/6 x 129/Sv genetic background. The wild type mice were derived from the same parental lines as the knockout mice. Breeding pairs heterozygous for Lyn, Btk (males were either Btk/Y or +/Y), or both were set up to generate both wild type and knockout mice within the same litter. Whenever possible, littermates were compared directly. All animals were housed

within the same room. The genotype of these mice was confirmed by RT-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, St Louis, MO) in IL-3-free RPMI medium, then rinsed with HEPES buffer (21) containing 0.04% BSA (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: Pepro Tech, Rocky Hill, NJ) for 30 min for the degranulation studies or for the indicated periods of time for the signaling studies. For cytokine mRNA and release studies, cells were similarly sensitized but triggered for 4 h or 10 h respectively in RPMI.

Degranulation Assay - Degranulation was monitored by the release of β -hexosaminidase (β -hex) into the supernatants (22). Briefly, BMMCs, sensitized as above, were triggered in 96-well plates (5 x 10⁵ cells per well, 100 μ l final volumes). The reactions were terminated by centrifugation (3000 RPM) at 4 °C and the supernatants were aliquoted to 96-well plates for β -hex assay. The remaining cells were lysed by adding distilled water and freeze-thawing, then aliquots similarly assayed for β -hex content. Degranulation was then calculated as the percentage of total (cells and supernatants) β -hex 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 then triggered as above at a concentration of 10 x 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 μ l) was added to the lysates and the mixtures were centrifuged for 15 min at 14,000 RPM. Isopropanol (500 μ l) was then added to the aqueous phases and the mixture incubated for 10 min to precipitate RNA. Ten μ g

of RNA was used in the mRNA assay using an *in vitro* transcription kit and pre-designed or custom-designed RPA templates (BD Biosciences, San Diego, CA). RPA was conducted according to the manufacturer's instructions, however, the synthesized radioactive probes labeled with UTP- $[\alpha\text{-}^{33}\text{P}]$ were purified with a probequant G-50 micro column (Amersham Pharmacia Biotech, Piscataway, NJ) instead of ethanol precipitation, and the protected mRNA precipitated with ethanol and ammonium acetate containing Glyco-blue (Ambion, Austin, Tx). The gels were prepared with 80 ml of sequage-6 (National Diagnostics, Inc.), 20 ml of sequage-complete (National Diagnostics, Inc., Atlanta, GA) and 10 % ammonium persulfate (Sigma).

Levels of the secreted cytokines IL-4, IL-6, IL-13, and TNF- α were measured in the supernatants of activated BMMCs by ELISA (Biosource, Camarillo, CA).

Cell Extraction, Immunoprecipitation, and Immunoblotting - Cell lysates and/or immunoprecipitates were prepared as described (23) and aliquots loaded onto 4-12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA). The proteins were then separated by electrophoresis in MES buffer (Invitrogen) as described in the manufacturer's protocol. Following transfer onto nitrocellulose membranes, the proteins were probed for immunoreactive proteins utilizing the following antibodies: goat anti-Btk pAb, goat anti-Tec pAb, anti-Itk pAb, anti-Lyn pAb, anti-Fyn pAb, anti-Blk pAb, anti-Yes pAb, anti-c-Fos pAb, anti-c-Jun pAb, anti-Syk pAb (C-20) (Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-Hck pAb, anti-Src pAb (UBI, Lake Placid, NY), anti-Actin mAb (Clone AC-15) (Sigma, St Louis, Mo), anti-phospho-Btk (pY223) pAb, anti-phospho-Src (pY416) pAb, anti-phospho-AKT (pS473) pAb, anti-phospho-ERK (pT202, pY204), anti-phospho-JNK (pT183, pY185) pAb, anti-phospho-p38 (pT180 pY182) pAb, anti-phospho-c-Jun (pS73) pAb, and anti-phospho-STAT5 (pY694) pAb, anti-phospho-Elk-1 (pS383) pAb, anti-phospho NF κ B (pS536) pAb (Cell signaling, Beverly, MA), anti-phospho-NFAT (pS54) pAb, and anti-phospho-PLC γ_1 (pY783) pAb (Biosource). Unless specified, pAbs were rabbit origin. The immunoreactive proteins were visualized by probing with horseradish peroxidase-conjugated anti-mouse,

anti-goat (Jackson Labs, West Grove, PA) or anti-rabbit IgG (Amersham, Piscataway, NJ), then by ECL (Perkin Elmer, Shelton, CT). As previously (20), and as discussed later, there was no apparent differences in the expression of signaling molecules (apart from Btk and Lyn) in the knock out BMMCs. Therefore protein loading of the samples was normalized by stripping then probing for actin; or alternatively probing identically loaded membranes for either actin or Syk. To quantitate changes in protein phosphorylation, the ECL films were scanned using an ImageQuant 5.0 scanner (Molecular Dynamics, Sunnyvale, CA).

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 re-suspended in HEPES buffer containing 0.04% BSA and sulfinapyrazone (0.3 mM) (Sigma), and then placed in a 96-well black culture plate (20,000 cells/well) (CulturPlat-96 F, Perkin Elmer Life Science). 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 which had not been loaded 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. As 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 W/T, Lyn $^{-/-}$ and Btk $^{-/-}$ / Lyn $^{-/-}$ double knock out BMMCs. The Btk $^{-/-}$, Lyn $^{-/-}$, and Btk $^{-/-}$ / Lyn $^{-/-}$ BMMC 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 Blk, 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 W/T mouse BMMCs (Fig. 1 a,b), however, maximum phosphorylation observed with SCF was of 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,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,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 W/T 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. Figure 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 IFN-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 approximately 50% in both the $Btk^{-/-}$ and $Lyn^{-/-}$ BMMCs when compared to W/T 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 SCF-mediated potentiation of degranulation induced by minimally effective concentrations of antigen as shown previously in Figure 2a.

Production of TNF-α and IL-6 and IL-13 (Fig. 3c, d, and e, respectively) in response to SCF and antigen or both in combination was reduced by approximately 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 to W/T 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 approximately 50% of that in W/T 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 PLC γ_1 - and PI 3-kinase-dependent pathways control Fc ϵ RI-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.

PLC γ_1 and PI 3-kinase activation was monitored by the phosphorylation of PLC γ_1 or AKT, respectively (13). Both SCF and antigen stimulated PLC γ_1 phosphorylation in W/T 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,f). We were unable to detect PLC γ_2 phosphorylation in response to SCF and antigen utilizing a commercially available anti-phospho-PLC γ_2 . However, following immunoprecipitation with an anti-PLC γ_2 antibody then probing with an anti-phosphotyrosine antibody, we observed that, although both antigen and SCF induced PLC γ_2 phosphorylation, these responses were not additive (data not shown). In contrast to PLC γ_1 phosphorylation, the effects of SCF and antigen on AKT phosphorylation (Fig. 4 c,d,g,h) were not additive. Rather, antigen induced a decrease in the more predominant SCF-mediated AKT phosphorylation (Fig. 4 c,d). This was likely due to 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,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 W/T cells is absent in the Lyn $^{-/-}$ and Btk $^{-/-}$ / Lyn $^{-/-}$ BMMCs (Fig. 4 g,h).

Btk-deficient BMMCs exhibited a slight reduction in PLC γ_1 phosphorylation in response to antigen and SCF (Fig. 4 e,f). However, the ability of SCF to potentiate antigen-mediated PLC γ_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,f). In Lyn $^{-/-}$, as well as Btk $^{-/-}$ / Lyn $^{-/-}$ BMMCs, the ability of antigen in the absence or presence of SCF to induce PLC γ_1 phosphorylation was completely blocked. As a result, the synergistic increase in PLC γ_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, whereas Lyn was required for phosphorylation of PLC γ_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 W/T 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) was still observed. These responses, however, were substantially lower and less sustained than those observed in the W/T BMMCs. In contrast, in the Lyn $^{-/-}$ BMMCs, the increase in calcium levels was delayed but eventually reached levels that were similar to those in W/T 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 that this was at the level of PLC γ_1 activation but downstream of PI 3-kinase activation.

MAP kinases 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 MAP kinases and specific

transcription factors. In W/T BMMCs, the phosphorylation of the ERK1/2, JNK, and p38 MAP kinases was augmented by co-stimulation with antigen and SCF compared to the effects of the individual stimulants added alone (Fig. 6). There was no reduction, in the synergistic phosphorylation of ERK1/2 in the $Btk^{-/-}$ and $Lyn^{-/-}$ BMMCs (Fig. 6 a,b) and only a slight reduction in the $Btk^{-/-} / Lyn^{-/-}$ BMMCs. In contrast, the synergy between antigen and SCF in the phosphorylation of p38 MAP kinase 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 $Btk^{-/-}$ and $Btk^{-/-} / Lyn^{-/-}$ 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 κ B (27-29). We thus examined these responses in the W/T and kinase-deficient BMMCs. Both SCF and antigen induced the synthesis of Jun. However, these responses were not additive (Fig. 7 a,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,d). We observed no marked differences in levels of Jun in the kinase-deficient BMMCs (Fig. 7 a,b) apart from slightly higher levels in the $Lyn^{-/-}$ and $Btk^{-/-} / Lyn^{-/-}$ BMMCs challenged with SCF and antigen concurrently compared to the levels in W/T BMMCs treated in a similar manner. In addition, no marked defects in the overall levels of phosphorylated Jun (Fig. 7 c,d) were observed in these cells compared to the W/T responses. However, by taking into account the different levels of Jun expression in these cells (Fig. 7 a,b), there was a reduction in the phosphorylation of Jun per unit mass in response to SCF and antigen in combination in the $Btk^{-/-}$, $Lyn^{-/-}$ and $Btk^{-/-} / Lyn^{-/-}$ BMMCs (Fig 7e), which reflected the observed changes in JNK phosphorylation described in Fig. 6 e. The lack of changes in the overall levels of phosphorylated Jun together with the relatively

minor changes in Fos expression (Fig. 7 f,g) in these cells, however, suggests that the reduction in cytokine production in the $Btk^{-/-}$, and $Btk^{-/-} / Lyn^{-/-}$ BMMCs was unlikely to be due to defects in the induction and/or phosphorylation of AP1 components.

Both NF κ B (Fig 7 h,i) and NFAT (at the activating S54) (Fig. 7 j,k) were phosphorylated in response to SCF and antigen in the W/T BMMCs. The phosphorylation of NF κ B, in contrast to the phosphorylation of NFAT was additive when these agents were added concurrently. The phosphorylation of NF κ B 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 NF κ B, 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 Fc ϵ RI-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 which is utilized by Kit for augmenting the activation of mast cells via Fc ϵ RI. As such, Btk may provide the link between receptor-proximal events and integrated downstream signaling by Kit and Fc ϵ RI. 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 previously shown in human mast cells (3,4), SCF was found to markedly potentiate degranulation and augment cytokine production in W/T mouse BMMCs (Fig. 2). The enhancement of degranulation could be attributed to a synergistic increase in PLC γ_1 phosphorylation and resultant enhanced calcium

mobilization. Examination of the kinetics of these and other signaling responses including the phosphorylation of Btk (Fig. 1) and NTAL (data not shown) suggested that this may be due to an SCF-dependent conversion of the normally transient FcεRI-mediated responses to a more sustained response. As previously reported (20), degranulation in response to antigen was reduced by approximately 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 to 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 W/T 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 less 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 $PLC\gamma_1$ phosphorylation in these cells (Fig. 4). Btk is known to activate $PLC\gamma$ by phosphorylating conserved activation tyrosine residues in the SH2-SH3 domain linker region in both $PLC\gamma_1$ (pY771, pY783) and $PLC\gamma_2$ (pY753, pY759) in B cells (37). We noted that, although Lyn was absolutely required for the phosphorylation of pY783 in $PLC\gamma_1$ in response to antigen in BMMCs, the phosphorylation of this residue was only partially reduced in the individual responses to antigen and SCF in the $Btk^{-/-}$ BMMCs. Nevertheless, Btk was

absolutely required for the Kit-mediated enhancement of the phosphorylation of $PLC\gamma_1$ (pY783) in response to antigen. These data suggest that, in activated mast cells, the phosphorylation of the critical pY783 in $PLC\gamma_1$ 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 previously reported 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 MAP kinases, ERK1/2, JNK, and p38 (Fig. 6), and 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 S54 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 due to 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 PI 3-kinase

by antigen which was observed in the W/T BMMCs.

TNF- α , IL-6, and IL-13 genes are regulated by binding of the transcription factors, NFAT, NF κ B (40-42) and the AP1 complex to their promoter regions as regulated by AKT (32) and MAP kinases including JNK and p38 (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 NF κ B. 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 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-PLC γ 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 S54 (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 NF κ B and NFAT phosphorylation 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 NF κ B and, ultimately reduced cytokine production in the Btk^{-/-} and Btk^{-/-} / Lyn^{-/-} BMMCs.

As summarized in Figure 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 and that Btk is an essential player in the amplification pathway which 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 Fc ϵ RI-mediated degranulation.

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FOOTNOTES

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The abbreviations used are: Ag, antigen; Btk, Bruton's tyrosine kinase; Btk^{-/-} / Lyn^{-/-}, Btk, Lyn double knock out; BMMC, bone marrow-derived mast cell; FcεRI, high affinity receptor for IgE; IL, interleukin; mAb, monoclonal antibody; pAb, polyclonal antibody; PI 3kinase, phosphoinositide 3kinase; PLC, phospholipase C; SCF, stem cell factor; TNF, tumor necrosis factor.

FIGURE LEGENDS

Figure 1. Expression and phosphorylation of Btk and Src kinases in W/T, Btk^{-/-}, Lyn^{-/-}, and Btk^{-/-} / Lyn^{-/-} BMMCs. Cells were sensitized overnight with mouse monoclonal anti-DNP-IgE (100 ng/ml) in media without IL-3, then following rinsing, challenged with SCF (100 ng/ml) or Ag (DNP-HSA: 100 ng/ml) or Ag and SCF added concurrently (100 ng/ml) for the indicated times (a,b), 120 s (c,d) or 30 s (e,f). Proteins were then extracted from the cells and, following separation by gel electrophoresis, were probed for the indicated phosphorylated proteins. In (e) the blots from the Lyn^{-/-} and Btk^{-/-} / Lyn^{-/-} BMMCs were

subsequently over-exposed to reveal the Src kinases phosphorylated in response to SCF in the absence or presence of Ag. The blots are representative of $n=3$. In (b), the symbols are (?), Ag; (?), SCF; (?), SCF and Ag concurrently. The data in (b), (d) and (f) were generated by scanning the blots in (a), (c), and (e) respectively, then normalizing to the maximal response obtained with antigen alone. In (d) and (f), the order of bars for each cell type is control, Ag, SCF, and Ag+SCF. The dashed line in (f) represents the constitutive phosphorylation in W/T cells.

Figure 2. The effects of SCF and Ag on degranulation (a) and cytokine production (b) in W/T BMMCs. Cells were sensitized overnight, 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 -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 in “Experimental Procedures”. The data in (a) are presented as means $\pm$ S.E.M. ($n=4-6$) of separate experiments conducted in duplicate, in (b) are representative of $n=3$, and in (c-e) are means $\pm$ S.E.M. ($n=3$).

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 W/T, Btk $^{-/-}$, Lyn $^{-/-}$, and Btk $^{-/-}$ / Lyn $^{-/-}$ BMMCs. Cells were sensitized overnight, then challenged in (a) with Ag (DNP-HSA: 100 ng/ml) for the indicated times or in (b) with the indicated concentrations of SCF in the absence or presence of the indicated concentrations of Ag for 30 min. Degranulation was then assessed by monitoring the release of β -hex as described in “Experimental Procedures”. For cytokine release in (c-e), cells were similarly sensitized, but challenged for 10 h. The media was then collected and assayed for the indicated cytokines by ELISA. The data in (a) are presented as means $\pm$ S.E.M. ($n=3$), in (b) means $\pm$ S.E.M. ($n=5$) apart from SCF alone which is $n=2$. In (c-e), the data are presented as means $\pm$ S.E.M. ($n=2-4$); and the order of bars for each cell type is control, Ag, SCF, and Ag+SCF.

Figure 4. Phosphorylation of PLC γ_1 (a,b,e,f) and AKT (c,d,g,h) in response to Ag, SCF, or SCF in the presence of Ag in W/T (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 extracted. Following gel electrophoresis, the proteins were probed with antibodies recognizing phosphorylated PLC γ_1 (p-PLC γ_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, then normalizing to the maximal response obtained with antigen (b) and (f) or SCF (g) and (h) alone. The symbols are (?), Ag; (?), SCF; (?), SCF and Ag concurrently in (b) and (d). In (f) and (g), the order of bars for each cell type is control, Ag, SCF, and Ag+SCF.

Figure 5. Calcium mobilization in response to SCF (100 ng/ml), Ag (100 ng/ml), and Ag and SCF added concurrently (100 ng/ml) (Ag+SCF) in W/T, Btk $^{-/-}$, Lyn $^{-/-}$ and Btk $^{-/-}$ / Lyn $^{-/-}$ BMMCs. (a) Synergistic elevation of calcium levels in W/T BMMCs. The data are representative of $n=5$ conducted in duplicate. (b) Antigen-mediated calcium mobilization, (c) SCF-mediated calcium mobilization and (d) calcium mobilization in response to Ag and SCF added concurrently in W/T, Btk $^{-/-}$, Lyn $^{-/-}$ and Btk $^{-/-}$ / Lyn $^{-/-}$ BMMCs (as indicated). The data are presented as means $\pm$ S.E.M. ($n=5$). In (e) and (f), the data from the W/T BMMCs challenged with SCF and Ag from (d), the Btk $^{-/-}$ or Lyn $^{-/-}$ BMMCs challenged with Ag and SCF concurrently from (d) and the Btk $^{-/-}$ or Lyn $^{-/-}$ BMMCs challenged with Ag alone from (b) during the first 300 s have been re-plotted to demonstrate that the potentiation of antigen-mediated calcium flux by SCF is absent in the Btk $^{-/-}$ and Lyn $^{-/-}$ BMMCs. Standard errors in (b), (c) and (d) have been omitted for clarity, but are similar to those in (e) and (f).

Figure 6. Phosphorylation of the MAP kinases ERK1/2 (a,b), p38 (c,d) and JNK (e,f) in response to Ag, SCF, or Ag and SCF added concurrently in W/T, Btk^{-/-}, Lyn^{-/-}, and Btk^{-/-} / Lyn^{-/-} BMMCs. Cells were sensitized and treated with control buffer (C), Ag (DNP-HSA: 100 ng/ml), SCF (100 ng/ml) or Ag and SCF (100 ng/ml) for 30 min, and proteins extracted. Following gel electrophoresis, the proteins were probed with antibodies recognizing phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38), or phosphorylated JNK (p-JNK), or actin. The blots are representative of n=3. The data in (b), (d) and (f) were generated by scanning the blots in (a), (c), and (e) respectively, then normalizing to the maximal response obtained with antigen alone. In these panels (f), the order of bars for each cell type is control, Ag, SCF, and Ag+SCF.

Figure 7. Synthesis and/or phosphorylation of transcription factors in response to Ag, SCF, or Ag and SCF added concurrently in W/T, Btk^{-/-}, Lyn^{-/-}, and Btk^{-/-} / Lyn^{-/-} BMMCs. Cells were sensitized and treated with control buffer (C), Ag (DNP-HSA: 100 ng/ml), SCF (100 ng/ml) or Ag and SCF (100 ng/ml) for 30 min, and proteins extracted. Following gel electrophoresis, the proteins were probed with antibodies recognizing total (a,b,e) and phosphorylated Jun (p-Jun) (c,d,e), total Fos (f,g), phosphorylated NFκB (p-NFκB) (h,i) and phosphorylated NFAT (p-NFAT) (j,k). The blots are representative of n=3. The data in (b), (d), (g), (i), and (k) were generated by scanning the blots in (a), (c), (f), (h), and (j), respectively, then normalizing to the maximal response obtained with antigen alone. The data in (e) were generated by normalizing the data in (d) with the data in (b). In these panels, the order of bars is control, Ag, SCF, and Ag+SCF.

Figure 8. The potential role of Btk in the amplification pathway utilized by Kit for the potentiation of FcεRI-mediated mast cell degranulation and cytokine production. We have restricted this scheme to signaling molecules actually examined in this study and excluded others including, cytosolic adaptor molecules and members of the Ras-Raf-MAP kinase-AP1 cascade for clarity. The readers are referred to references 21, 27 and 28 for specific details about these molecules. In the diagram, the blue line represents the “principle” pathway for the activation of phospholipase Cγ₁ leading to degranulation, and for the activation of the transcription factors NFκB and NFAT leading to cytokine production. The green line represents the “amplification” pathway utilized by Kit for the potentiation of the principle pathway. The dashed lines with question marks represent unresolved stages of this cascade. In this scheme, FcεRI aggregation results in the phosphorylation of the transmembrane adaptor molecules LAT and NTAL, however, Kit activation appears to result in the phosphorylation of NTAL in the absence of detectable LAT phosphorylation (4). The phosphorylation of LAT results in the recruitment and activation of phospholipase (PLC)γ₁ with resulting hydrolysis of membrane-bound phosphoinositide 4,5, bis-phosphate (PIP₂) to yield inositol tris-phosphate (IP₃) and diacylglycerol (DAG). These molecules respectively induce mobilization of intracellular calcium and activation of protein kinase C (PKC) resulting in degranulation. The elevation of intracellular calcium levels also induces NFAT activation via the calcium-regulated phosphatase, calcineurin. Concurrently with these events, though slightly delayed in onset, is the activation of the amplification pathway which results in enhancement of PLCγ₁ activity and calcium mobilization and it is by this mechanism that we hypothesize that Kit potentiates FcεRI-mediated degranulation and cytokine production. As previously described, this pathway appears to be regulated by NTAL (4) and, although not synergistically enhanced by SCF and antigen, by phosphoinositide 3-kinase (PI3K) (50). As both NTAL phosphorylation (4) and AKT phosphorylation (current study) are not abrogated in Btk^{-/-} BMMCs, activation of Btk is likely downstream of these events, although the exact processes by which Btk is activated by this pathway is currently unclear. The activation of Btk by Kit, however, appears to be crucial for the subsequent potentiation of PLCγ₁-mediated calcium mobilization leading to degranulation, and activation of NFAT and NFκB leading to enhanced cytokine production.

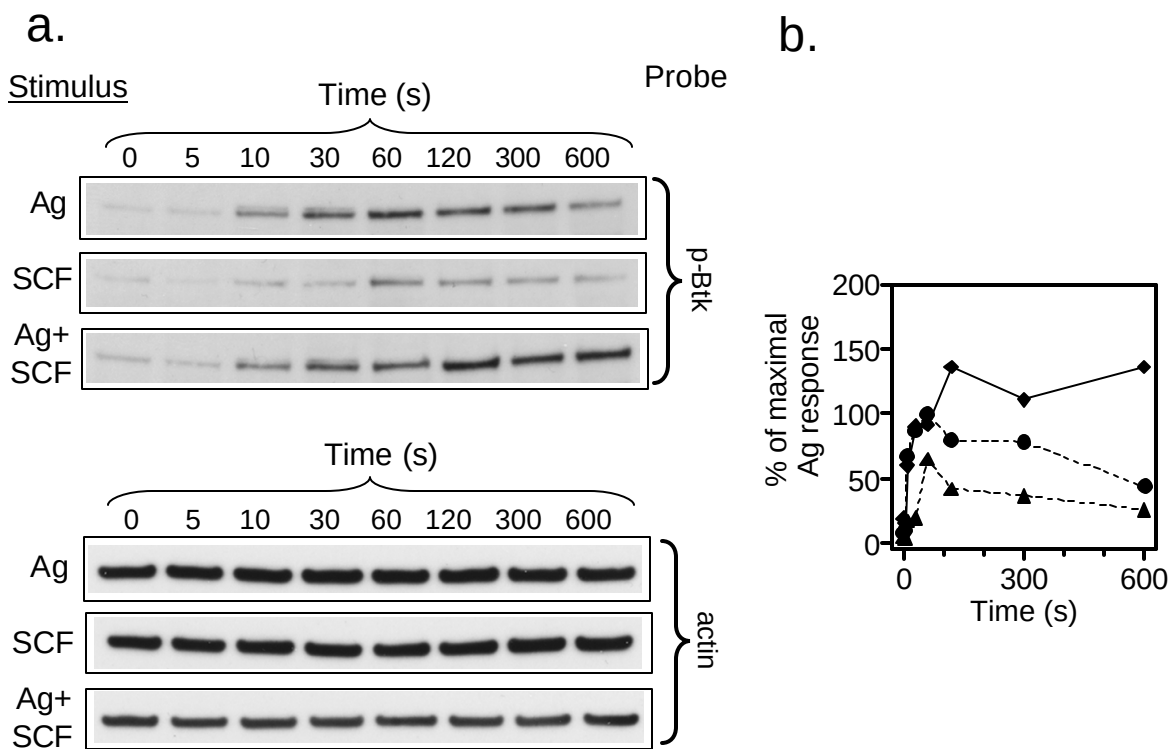
Figure 1.

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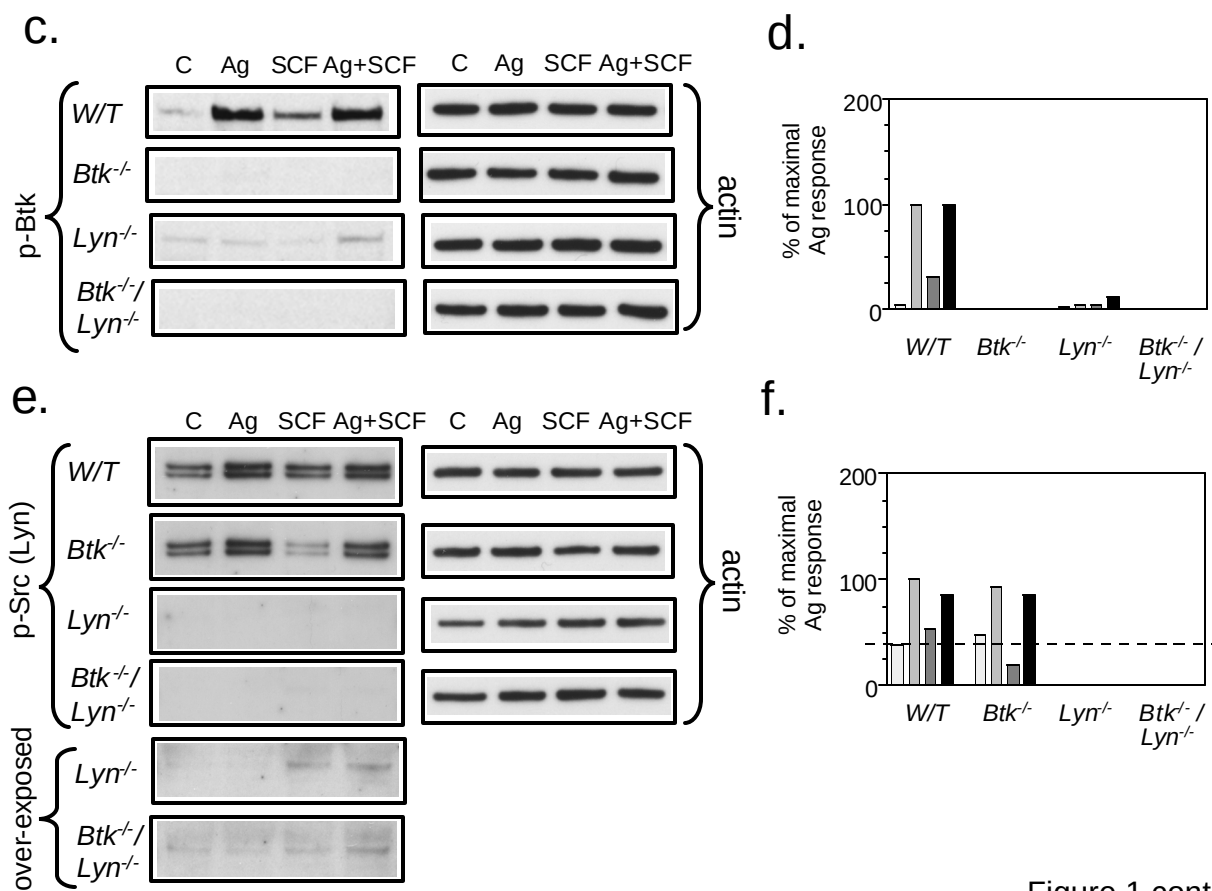
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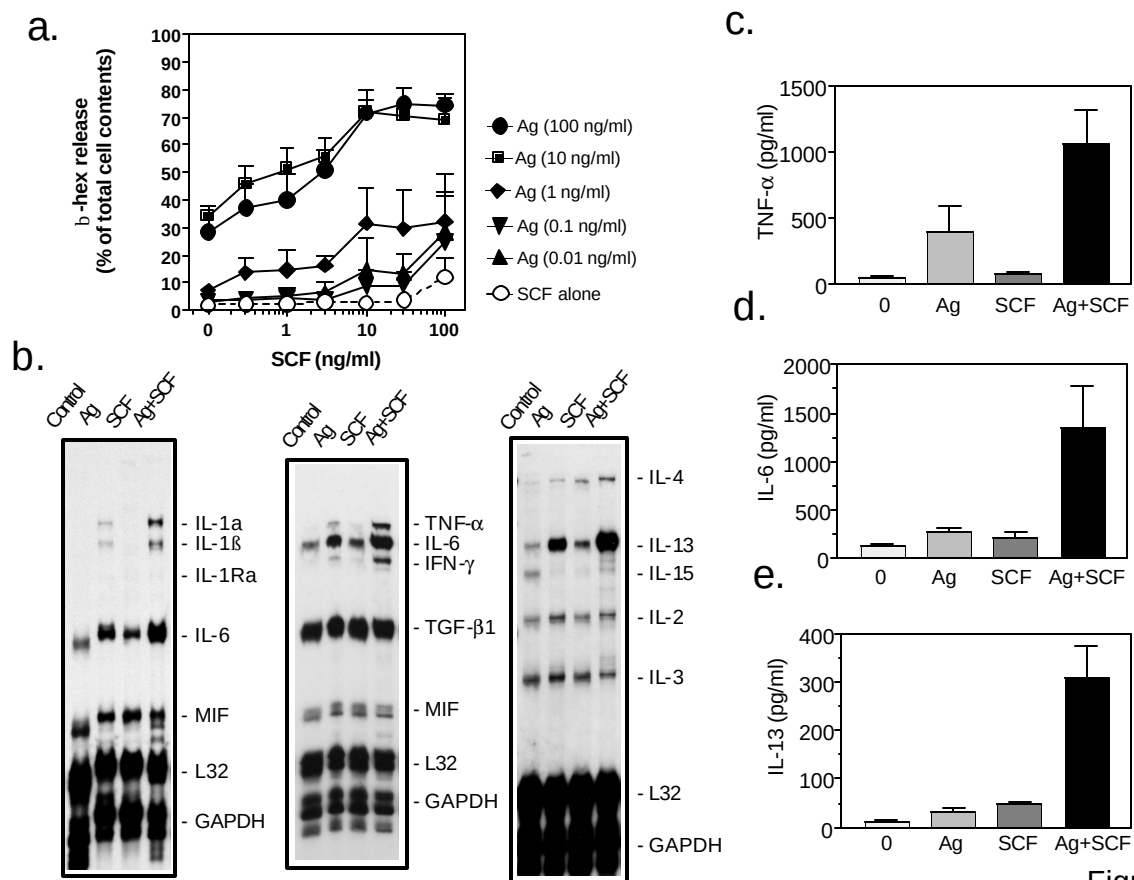
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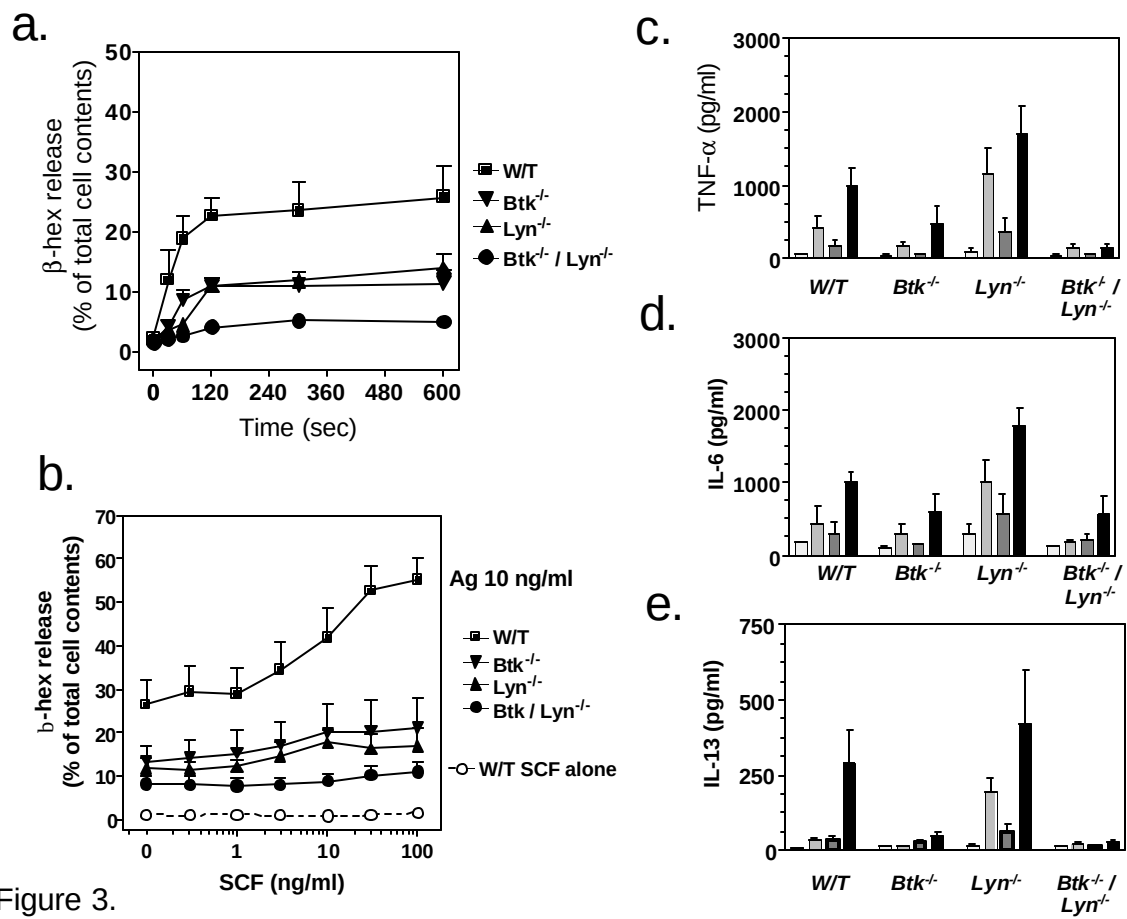
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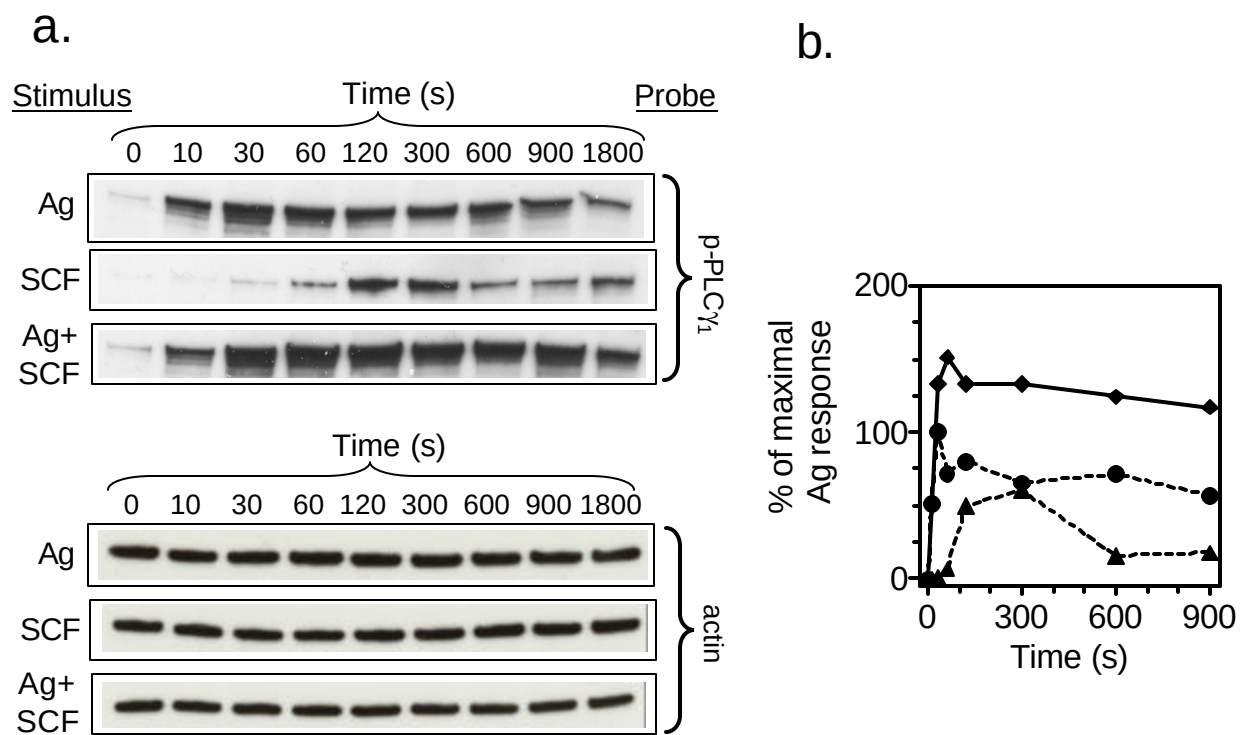
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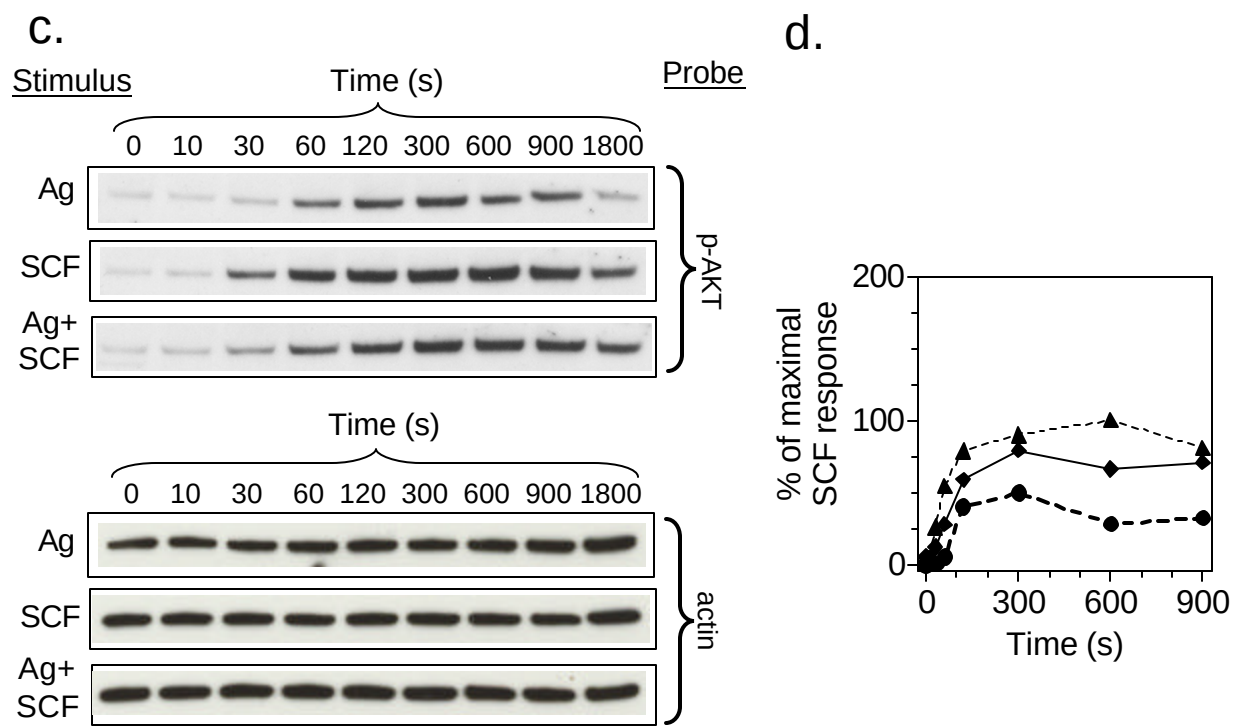
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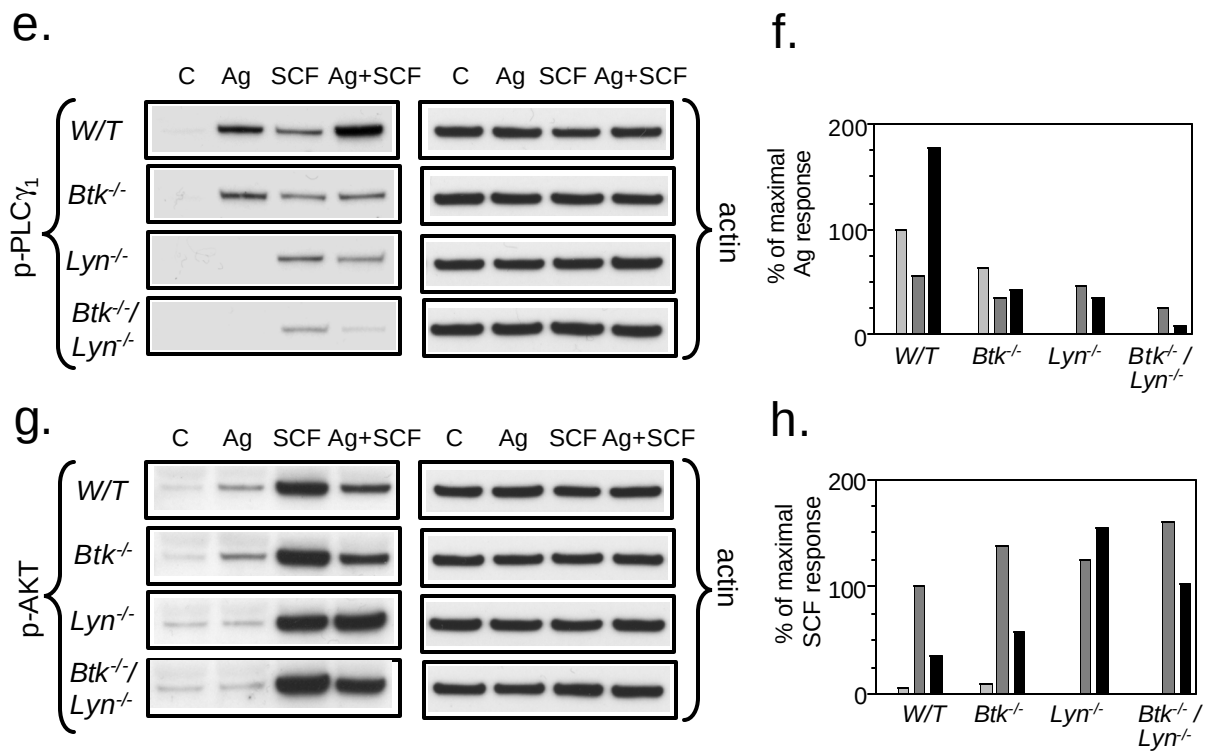
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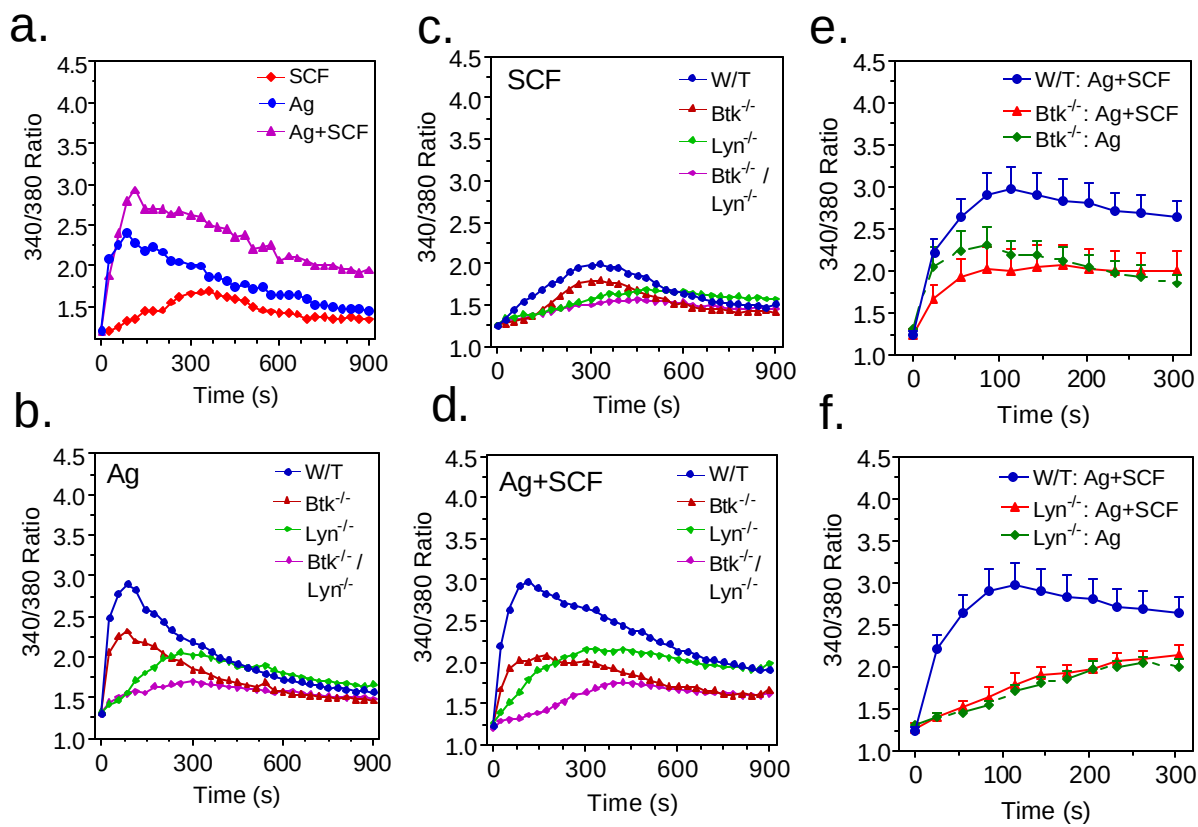
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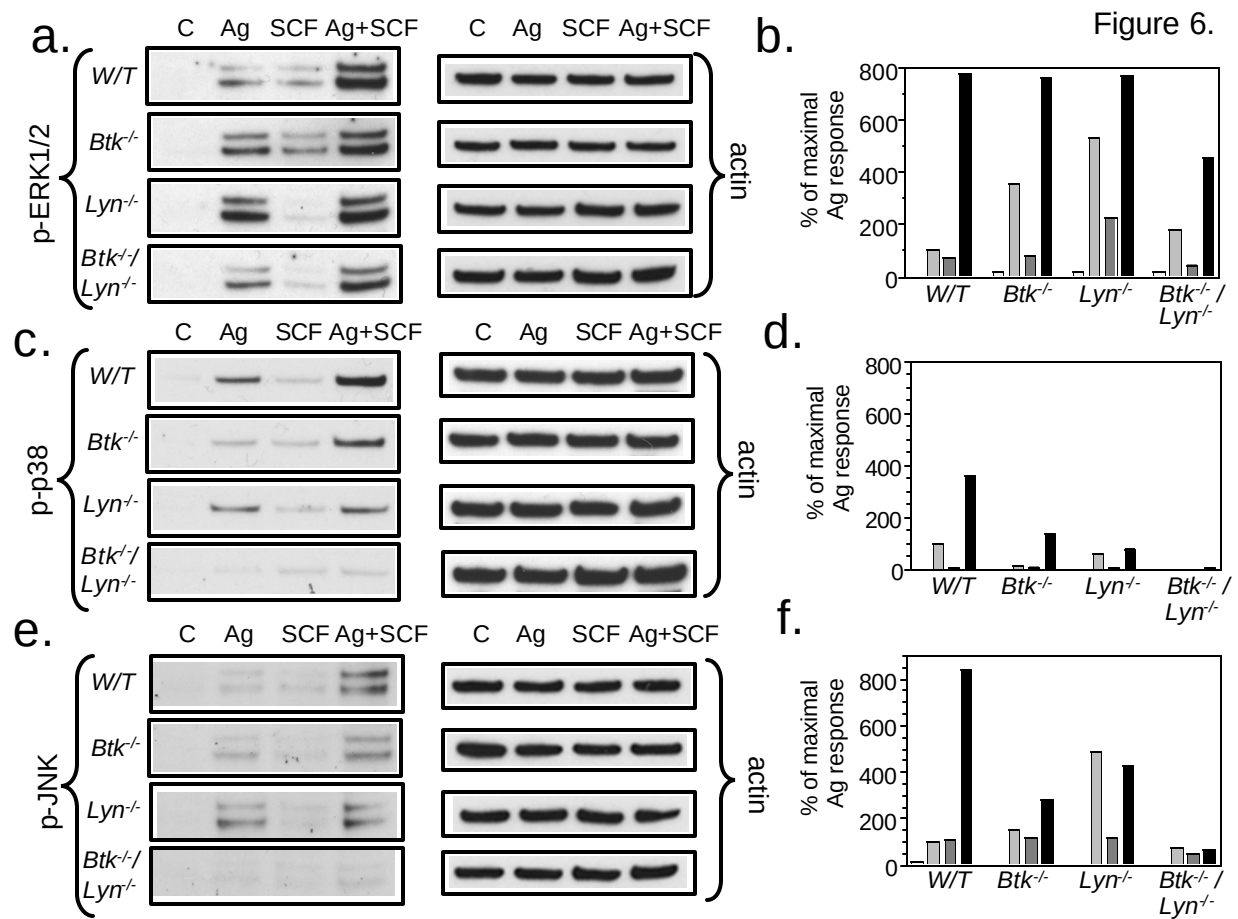
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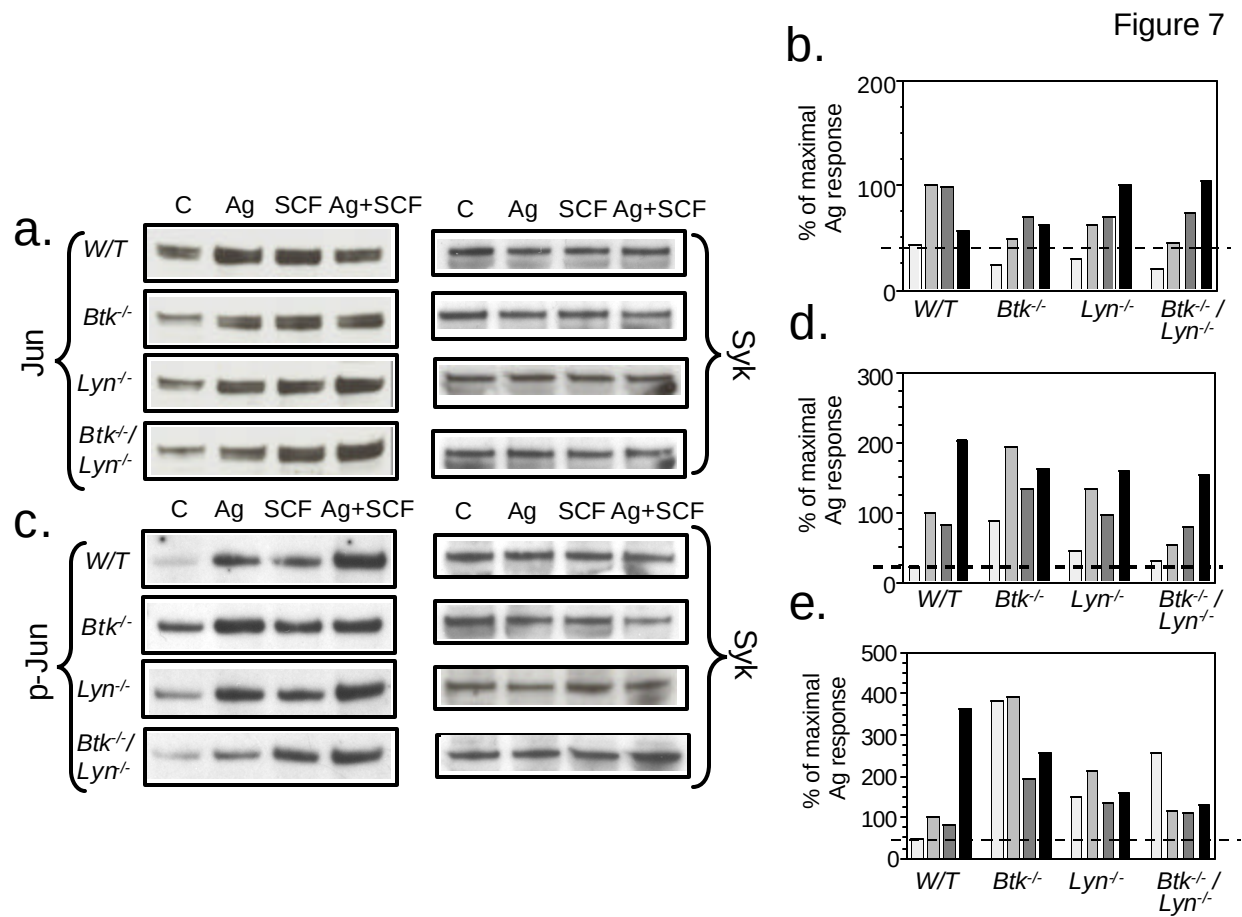
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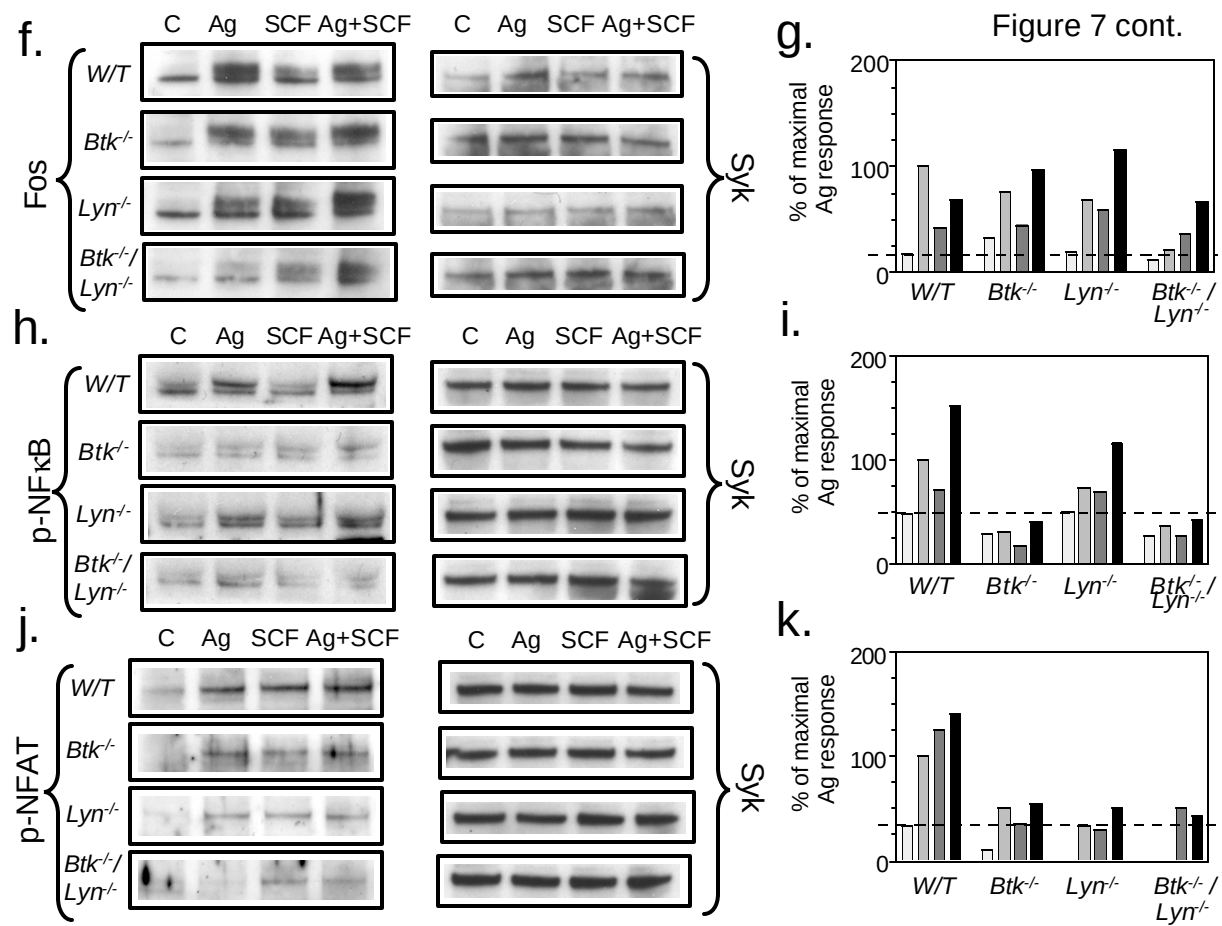
Figure 7. (cont.)

Figure 8.