IgE-dependent Activation of Sphingosine Kinases 1 and 2 and Secretion of Sphingosine 1-Phosphate Requires Fyn Kinase and Contributes to Mast Cell Responses

Received for publication, August 12, 2005, and in revised form, November 23, 2005. Published, JBC Papers in Press, November 29, 2005. Published, JBC Papers in Press, November 29, 2005, DOI 10.1074/jbc.M508931200

Ana Olvera1†, Nicole Urtz2‡, Kiyomi Mizugishi3, Yumi Yamashita1, Alasdair M. Gilfillan4, Yasuko Furumoto‡, Haihua Gu††, Richard L. Proia3, Thomas Baumruker5, and Juan Rivera1‡

From the 1Molecular Inflammation Section, Molecular Immunology and Inflammation Branch, NIAMS, 2Genetics of Development and Disease Branch, NIDDK, and 3Laboratory of Allergic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892, 4Novartis Institute for BioMedical Research/Vienna, 59 Brunner Strasse, Vienna A-1235, Austria, and the 5Cancer Biology Program, Division of Hematology and Oncology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Receptor stimulation of a variety of cell types induces the early activation of sphingosine kinase (SphK), a unique lipid kinase that generates the potent and versatile lipid mediator sphingosine-1-phosphate (SIP) (1,2). SIP was demonstrated to function intracellularly, regulating cell survival, cell proliferation, and calcium fluxes (1,3). However, many of the effects of SIP can be attributed to its role as a ligand for a family of five G-protein-coupled receptors, named the endothelial differentiation gene or SIP receptors (SIP1–5) (4,5). This receptor family, and predominantly the SIP1 subtype, is known to regulate diverse biological functions, including cytoskeletal changes, chemotaxis, vascular development, regulation of endothelial cell function, and lymphocyte recirculation (1,4). Two distinct mammalian SphKs (SphK1 and SphK2) have been identified, and both kinases efficiently convert sphingosine to SIP (6,7). SphK1 is activated in response to multiple stimuli, whereas SphK2 activation was only recently demonstrated in response to epidermal growth factor (2,8,9).

In mast cells, the high affinity receptor for IgE (FcεRI) mediates activation of SphK1 has been associated with calcium mobilization and degranulation (10–12). However, the intracellular role of SIP in modulating mast cell calcium responses remains uncertain, in part, due to the failure to identify the intracellular SIP receptors and the organelle(s) from which the calcium is mobilized. Interestingly, mast cells are one of the few cell types that can secrete substantial quantities of SIP upon stimulation, suggesting an important role for this metabolite in their function (13). Studies on the mast cell tumor analog, RBL-2H3, showed that cell migration to an IgE/antigen (Ag) stimulus required transactivation of SIP1 receptors by endogenously generated SIP. Additionally, these studies demonstrated that SIP1 contributes to mast cell degranulation through a second receptor, SIP2 (12).

Our more recent studies demonstrated that the Src protein-tyrosine kinase (Src PTK) Lyn is required for the early phase of SphK1 activation in mast cells (14). This is mediated through the interaction of SphK1 with Lyn, thus promoting SphK1 recruitment to FcεRI. Nonetheless, because SphK1 activity is delayed but not ablated by Lyn deficiency, we investigated what additional signals might be required for SphK activation. Herein, we demonstrate that both isoforms, SphK1 and SphK2, which are expressed in murine and human mast cells (HuuMC), are activated upon FcεRI triggering. Lyn, a second Src PTK crucial for FcεRI-mediated mast cell activation (15), interacted with SphK1 and SphK2 and was required for their activation and for production and secretion of SIP induced by IgE/Ag. Consistent with the previously demonstrated role of SIP in mast cell chemotaxis and degranulation (12), Lyn-deficient BMMC failed to migrate toward an Ag gradient and showed defective degranulation. However, both of these responses were partially restored by the addition of exogenous SIP upon IgE/Ag stimulation.
**Fyn Kinase Regulates Sphingosine Kinases in Mast Cells**

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Mouse monoclonal or rabbit polyclonal antibodies specific to the following proteins were used in this study: phospho-Akt (Cell Signaling Technologies), Akt (Pharmingen/BD Biosciences), anti-Myc (Upstate Biotechnology, Inc., Lake Placid, NY), anti-FLAG (clone M2; Sigma), extracellular signal-regulated kinase, Src, Lyn, and Fyn (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-V5 (Invitrogen). Antibodies to SphK2 were made by immunizing rabbits (Strategic Biosolutions) with affinity-purified glutathione S-transferase fusion proteins (glutathione S-transferase-SphK2 (Nt) and glutathione S-transferase-SphK2 (Ct)); IRDye<sup>TM</sup>800- or Cy5.5-conjugated anti-rabbit and anti-mouse were from Rockland. S1P and sphingosine were from Biomol. Human myeloma IgE (Calbiochem) was biotinylated as described (16). PP2 and LY294002 were from Calbiochem; human or mouse recombinant interleukin-3 (IL-3) and stem cell factor (SCF), and human recombinant interleukin 6 (IL-6) were from PeproTech. The Ag dinitrophenyl-human serum albumin and Streptavidin were from Sigma. RPMI, StemPro-34 culture media, and fetal bovine serum were from Invitrogen, and σ-glutamyl, penicillin, and streptomycin were from Biofluids. The TNT T7 quick-coupled transcription/translation system was obtained from Promega. Lipoxygenase Plus was from Invitrogen. Disposable chemotaxis chambers were from NeuroProbe, Inc. (Eugene, OR). TLC plates were from EMD Chemicals, Inc.; and [γ-<sup>32</sup>P]ATP and [<sup>35</sup>S]methionine were purchased from MP Biomedicals and Amersham Biosciences, respectively.

**Bone Marrow Isolation and BMMC Culture**—Fny<sup>−/−</sup> (SV129 × C57/BL6 (N4)), Gab2<sup>−/−</sup> (SV129 × C57/BL6 (N4) (17)), and wild type (WT) mice (SV129 × C57/BL6 (N4)) were maintained and used in accordance with National Institutes of Health (NIH) guidelines. SphK1<sup>−/−</sup> and the WT littermates were previously reported (18) and maintained as above. Bone marrow was isolated from 5–6-week-old wild-type or gene-disrupted mice, and BMMC were cultured in RPMI medium supplemented with 20 ng/ml IL-3 and 20 ng/ml SCF as previously described (19). Cells were generally grown for a minimum of 4 weeks and used when greater than 95% of the population expressed FcRI.

**HuMCIsolation and Culture**—HuMCs were developed from CD34<sup>+</sup> cells in StemPro-34 culture medium containing IL-6 (100 ng/ml), and stem cell factor (100 ng/ml), as described (20). IL-3 (30 ng/ml) was included for the first week of culture. Experiments were conducted on these cells 8–10 weeks after the initiation of culture, at which point the population was greater than 99% mast cells.

**Transfection of Cells**—HEK-293 cells were transfected with V5 or c-Myc-tagged mouse SphK1 or FLAG-tagged mouse SphK2 using Lipofectamine Plus as previously described (7). HEK-293 transfectants were harvested and lysed by freeze-thawing in Buffer A (50 mM Tris (pH 7.4), 100 mM KCl, 1% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 5 mM sodium pyrophosphate, 10 μg/ml leupeptin, apro tin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). The cytosolic fraction obtained by centrifugation at 100,000 × g for 60 min at 4 °C was used for the measurement of SphK activity or measurements of S1P and sphingosine levels in BMMC. For co-immunoprecipitation experiments, HEK-293 cells were co-transfected with V5-SphK1 or FLAG-SphK2 and murine Lyn, Fyn, or a catalytically inactive mutant (K296N) of Fyn. Forty-eight h after transfection, cells were lysed in Buffer B (borate-buffered saline containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml apro tin, leupeptin, and pepstatin, 5 mM sodium pyrophosphate, 50 mM NaF, and 1 mM sodium orthovanadate), and the lysates were used for immunoprecipitation of the Src kinases.

**Ag Stimulation, Immunoblots, and Immunoprecipitations**—HuMCs were sensitized overnight in Stem Pro-34 growth medium without IL-6 or SCF, containing biotinylated human IgE (100 ng/ml) and then triggered by the addition of streptavidin at a final concentration 100 ng/ml as described (16). BMMC were washed and incubated in SCF-free medium for 20–24 h and then sensitized with 1 μg/ml anti-dinitrophenyl mouse IgE in IL-3–free medium containing 2% fetal bovine serum for an additional 3 h. BMMC were washed twice and resuspended in Tyrode’s solution/bovine serum albumin buffer (37 °C, 20 mM HEPES buffer (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM glucose, and 0.05% bovine serum albumin). Cells were then stimulated with 100 ng/ml Ag unless otherwise indicated. In some experiments, cells were incubated with LY294002, an inhibitor of PI3K, for 20 min at 37 °C prior to Ag stimulation. Preparation of BMMC lysates, immunoprecipitations, and immunoblotting procedures were as previously described (15), except that the secondary antibodies used for protein detection were labeled with dyes sensitive to the infrared range and detected by an Odyssey infrared imaging system (LI-COR Biosciences). For immunoblots of cell membrane proteins, cells were resuspended in Buffer A and lysed by freeze-thawing. Cell debris and nuclei were removed by centrifugation at 800 × g for 10 min, and the clarified lysates were centrifuged at 100,000 × g for 1 h. Pellets, containing cellular membranes, were washed twice with PBS and solubilized in SDS-PAGE sample buffer.

For co-immunoprecipitations of Fyn and SphK2 from BMMC, cells (6 × 10<sup>6</sup>/sample) were stimulated with Ag and subsequently lysed at 4 °C in Buffer B. In some experiments, BMMC lysates were mixed with lysates of HEK-293 cells transfected with murine FLAG-Sphk2 and incubated for 30 min at 4 °C with gentle agitation prior to immunoprecipitation. Cell lysates were incubated for 3.5 h with rabbit anti-Fyn prebound to protein A-Sepharose. The immunoprecipitated material was washed twice with Buffer B and twice with Buffer A without β-mercaptoethanol or KCl and assayed for the presence of SphK2 activity in Buffer A containing 200 mM KCl as described below. Alternatively, the immunoprecipitated material was resolved by SDS-PAGE and Fyn, and SphK2 was detected by Western blotting. Immunoprecipitations of proteins generated by <i>in vitro</i> transcription/translation were performed as described (14). Briefly, purified human recombinant Fyn, Lyn, or Src proteins and <i>in vitro</i> transcribed/translated human [<sup>35</sup>S]methionine-labeled SphK1 or SphK2, were allowed to form complexes for 1 h at room temperature and then isolated with the corresponding antibodies prebound to protein G-Sepharose for 2 h at 4 °C. Immunoprecipitated proteins were resolved by SDS-PAGE (4–20% Tris-glycine gels), and [<sup>35</sup>S]SphK1 or -2 was detected by autoradiography.

**Expression of Fyn Kinase in Fyn-deficient BMMC and Sphingosine Kinase Activity**—Fyn-deficient BMMC were reconstituted with wild type or a catalytically inactive mutant (K296N) of murine Fyn using a lentiviral expression vector for transduction. Briefly, cells were transduced with virus and incubated for 72 h prior to use. Transient expression (as determined by green fluorescent protein expression) was ∼70–80%. To determine SphK activity, IgE-sensitized BMMC were activated with 100 ng/ml Ag for the indicated time. Reactions were stopped by the addition of 3 ml of cold phosphate-buffered saline containing 100 μM sodium orthovanadate. Cells were pelleted, resuspended in Buffer A, and lysed by freeze-thawing. The resulting cell lysate was centrifuged at 21,000 × g for 30 min, and 20 μg of the soluble fraction (which also

---

*Y. Furumoto and J. Rivera, manuscript in preparation.*
Fyn Kinase Regulates Sphingosine Kinases in Mast Cells

SphK1 and -2 Are Activated upon FcεRI Triggering of BMMC and HuMC—Aggregation of the FcεRI in murine BMMC, human BMMC, and RBL-2H3 has been reported to cause the activation of SphK1 (10–12). Another mammalian sphingosine kinase, SphK2, shows more abundant transcript expression in mast cells (12). Using conditions that differentiate between SphK1 and SphK2 activities (Fig. 1A), we found that SphK2 was a prominent SphK activity in wild type (WT) BMMC (Fig. 1B). To further verify that SphK2 activity can be measured independently of SphK1, we assayed both SphK1 and SphK2 activities in BMMC from SphK1-null mice. As shown in Fig. 1B, SphK1-deficient BMMC had similar levels of SphK2 activity when compared with WT cells. Some residual activity of SphK1 was observed, but this probably resulted from overlapping SphK2 activity, given the noted overlap of activities ranging from 8 to 25 pmol/mg/min and SphK1 ranging from 6 to 24 pmol/mg/min (data not shown). Nonetheless, a weak reactivity was consistently detected at the apparent molecular mass of 70 kDa.

Ag stimulation of IgE-sensitized BMMC and HuMC resulted in rapid activation (with similar kinetics) of both SphK1 and SphK2 (Fig. 2, A–C). The activity of both enzymes in HuMC varied, with SphK2 activity ranging from 8 to 25 pmol/mg/min and SphK1 ranging from 6 to 24 pmol/mg/min (data not shown). As we previously reported for SphK1 (14), a biphasic induction of both SphK1 and SphK2 activity was found in BMMC, with the first peaking at 30 s to 3 min and the second more prolonged phase occurring between 15 and 30 min.
Fyn Kinase Regulates Sphingosine Kinases in Mast Cells

The Src Kinase Fyn Is Crucial for IgE-dependent Activation of SphKs and S1P Formation—Our previous study showed that Lyn-deficient BMMC were delayed in activating SphK1 and failed to efficiently translocate SphK1 to Ag-stimulated FcεRI. This suggested that Lyn played an important regulatory role but also that other molecular events are involved in activation of SphKs. Pretreatment of IgE-sensitized BMMC with the Src PTK-selective inhibitor, PP2, prevented activation of both SphK1 and 2 by Ag (Fig. 2, A and B). In fact, pretreatment of Lyn-deficient BMMC with PP2 also ablated the delayed activation of SphK1 and SphK2 seen in these cells (data not shown). This suggested that a second Src PTK was involved in SphK activation. Thus, we explored whether another receptor-proximal Src PTK, Fyn (15), was necessary for activation of SphK1 and SphK2. BMMC from Fyn-deficient mice showed a severe defect in SphK1 activation and demonstrated complete ablation of SphK2 activation (Fig. 2, A and B). It is noteworthy that the basal activity of SphK1 was unchanged in Fyn-deficient BMMC (WT, 4.76 ± 0.59, n = 9; Fyn<sup>−/−</sup>, 4.95 ± 1.19, n = 9), but the basal SphK2 activity was significantly diminished (WT, 8.19 ± 1.19, n = 9; Fyn<sup>−/−</sup>, 3.80 ± 0.86, n = 8, p = 0.0085). This decline in activity was not due to reduced expression of SphK2, as demonstrated in Fig. 2D. Concurrent with IgE-dependent activation of SphKs, WT BMMC showed increased intracellular expression of the SphK product, S1P (Fig. 2E), with a rapid (1–3 min) and a more prolonged phase (20–90 min poststimulation). In agreement with a previous report (24), the intracellular levels of S1P progressively decreased between 30 and 120 min, whereas the amount of S1P secreted to the extracellular medium increased (Fig. 2F). Coinciding with early increases in S1P, there was a concomitant decrease in the levels of sphingosine, the substrate of SphKs (Fig. 2G). Sphingosine levels were generally lower in Fyn-deficient
BMMC as compared with WT. However, the reduced amount of sphingosine was not the cause for the defective SphK activation, since preincubation of Fyn-deficient BMMC with 100 nM to 1 μM sphingosine (which is rapidly incorporated into cellular membranes) did not modify the response to Ag in these cells (data not shown). This was not unexpected, since the basal amount of sphingosine per nmol of total phospholipid (6–12 pmol/nmol phospholipids) in either WT or Fyn-deficient BMMC was much higher than that reported for other cell types (about 0.2 pmol/nmol phospholipids for HL-60, U937, Jurkat T cells, or Swiss 3T3 fibroblasts) (22, 23), suggesting that sphingosine was not limiting in Fyn-deficient BMMC.

To exclude the possibility of impaired SphK induction as a consequence of a developmental defect in Fyn-deficient BMMC, we transduced these cells with a virus encoding wild type or catalytically inactive Fyn kinase. Expression of wild type or mutant Fyn was achieved at levels close to those in WT BMMC (Fig. 3A). Cells transduced with wild type Fyn showed a fully restored activation of both SphK1 and SphK2 upon FcεRI triggering, whereas the catalytically inactive mutant seemingly (statistical significance was not achieved) partially restored their activation (Fig. 3, B and C). That the noncatalytic function of Fyn might contribute to SphK activation was also supported by transduction with a virus encoding lacZ, which did not cause SphK induction. Collectively, the findings demonstrate that Fyn induction of SphK activities can be restored.

The Requirement of Fyn for Induction of SphK Activity Is Shared by c-KIT but Not by the IL-3 Receptor—We were interested in determining whether the requirement for Fyn kinase in the activation of SphK1 and SphK2 was exclusive to FcεRI. We focused on c-KIT and IL-3 receptors because of previous reports linking these receptors to Fyn kinase (25, 26). c-KIT belongs to the same family of tyrosine kinase receptors as the platelet-derived growth factor receptor, which is known to stimulate SphK in other cell types (27, 28). Similarly, some cytokine receptors have been implicated in the activation of SphKs (29, 30). As shown in Fig. 4, SCF and IL-3 induced the activation of both SphK1 and SphK2. Fyn kinase was absolutely required for c-KIT-dependent activation of SphK1 and SphK2 (Fig. 4, A and B). In contrast, Fyn kinase was dispensable for IL-3 receptor-induced activation of SphK2, although it was more important for the activation of SphK1 (Fig. 4, C and D). Whereas the basal activity of SphK2 was lower in Fyn-deficient BMMC, the
Fyn Kinase Regulates Sphingosine Kinases in Mast Cells

Our findings demonstrate that c-KIT utilizes Fyn kinase for SphK activation, whereas IL-3 receptors use pathways that are considerably or minimally Fyn-dependent for SphK1 activation and SphK2 activation, respectively. Importantly, the results also demonstrate that SphK2 activation is not solely restricted to FcεRI engagement.

**Fyn Kinase Interacts with Both SphK1 and SphK2**—Using an antibody array, we previously found the interaction of SphK1 with Lyn kinase as well as with Lyn kinase (14). The interaction was specific, because antibodies to other Src kinase family members, such as c-Src and c-Yes, were found on this array but did not demonstrate SphK1 interaction (14). We further confirmed the interaction of SphK1 with Fyn by co-immunoprecipitation analysis using purified human recombinant Fyn and [35S]methionine-labeled SphK1, transcribed/translated in an in vitro TNT T7 reticulocyte system (Fig. 5A, upper panel). Immunoprecipitation of Fyn (lanes 1–3) resulted in co-immunoprecipitation of [35S]methionine-labeled SphK1 only when both Fyn and SphK1 were present. Similar experiments were performed for Fyn-SphK2 interaction. As shown in Fig. 5A (lower panel), Lyn immunoprecipitation also caused the co-immunoprecipitation of [35S]methionine-labeled SphK2. These results indicate that Fyn is able to interact with SphKs, and relative quantitation of these in vitro conditions suggested that ~1–2% of the total input of SphK protein was interacting with Fyn. Furthermore, using highly purified SphK1 and Lyn, we were able to show that this interaction, at least in the case of SphK1, is direct (data not shown). We also tested whether Lyn could associate with SphK2, given that our previous studies had focused on SphK1. As shown in Fig. 5A (lower panel), an antibody to Lyn (lanes 1–3) co-immunoprecipitated [35S]methionine-labeled SphK2 only when both purified human recombinant Lyn and [35S]methionine-labeled SphK2, which was in vitro transcribed/translated, were present. In contrast, an antibody to Src failed to co-immunoprecipitate SphK2 from a mixture of purified human...
c-Src kinases as indicated. Shown in the upper panel is the generated product $[^{32}P]S1P$ resolved by TLC at three different exposures (20, 90, and 120 min). In the lower panel, a single exposure is shown, since no differences were observed. B, translocation of SphK2 to cellular membranes after activation in WT, Fyn-deficient (left panels), and Lyn-deficient (right panels) BMMC. The proportion of SphK2 present in membranes (m) as compared with cytosol (c) is also shown in WT, Fyn-deficient, and Lyn-deficient BMMC. Bar graphs represent the fold increase in band intensity calculated by using the Odyssey infrared detection system, and data are normalized to WT at zero time and for protein loading as determined from anti-Lyn or anti-LAT immunoblots. Values represent the average ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.01; statistical significance with respect to unstimulated WT BMMC using a paired t test. C, translocation of SphK2 is restored in Fyn-deficient BMMC transduced with WT Fyn. The Western blot shows membrane SphK2 in WT or Fyn-deficient BMMC transduced with a single vector (WT/h11011, Fyn+/−/h11002), Fyn-deficient BMMC transduced with WT Fyn protein (Fyn+/−/h11007), or Fyn-deficient BMMC transduced with kinase-inactive mutant Fyn protein (Fyn+−/−/h11002) after challenge with Ag for the indicated times. Lyn was used as loading control. The bar graph shows the average ± S.E. of the fold increase in band intensity in four independent experiments calculated as in B. Statistical significance (**, p < 0.01; ***, p < 0.001) with respect to unstimulated cells using a paired t test.

recombinant Src and $[^{35}S]$methionine-labeled SphK2 (lanes 5–7), demonstrating the specificity of this interaction. These in vitro results were confirmed in HEK-293 cells co-transfected with V5-SphK1 or FLAG SphK2 and the Src kinases, Lyn and Fyn. Fig. 5B shows that antibodies to Lyn co-immunoprecipitated SphK1 (about 0.19% of the total) and SphK2 (0.15% of the total) only when Lyn was also overexpressed (upper and lower panels, respectively). Similarly, Lyn antibodies immunoprecipitated SphK1 (about 0.08% of the total) and SphK2 (0.06% of the total) only when Lyn was also overexpressed (Fig. 5B, upper and lower panels, respectively). The reduced interaction in vivo (~0.1%) relative to the in vitro experiments (~1.0%) may simply reflect the amount of Src PTKs present in these assays or specific compartmentalization in vitro that is not reflected in vitro. Catalytically inactive Lyn (KN-Fyn) also interacted with both SphK1 (Fig. 5B, upper panel) and SphK2 (Fig. 5B, lower panel) to almost the same extent as WT Lyn (0.12% of the total SphK1 and 0.05% of the total SphK2), demonstrating that Lyn activity was not required for its interaction with SphKs.

To verify that the interaction between SphK and Fyn was not an artifact of the in vitro system or the overexpression of these proteins in cells, we tested the association of endogenous SphK2 (Fig. 5C), which was the weaker interacting SphK, with Fyn in BMMC from WT and Fyn-deficient mice. This was done by measuring Fyn-associated SphK activity, because the available antibodies to murine SphK1 and SphK2 (including our own) failed to detect small quantities of these enzymes. SphK2 activity was detected in Fyn immune complexes after activation of BMMC, whereas no activity was observed when complexes were derived from Fyn-deficient BMMC. Although the amount of SphK2 activity co-immunoprecipitated with Fyn was a small fraction of the total activity (0.044% at 2 min after activation), the results were highly reproducible, showed an FceRI-induced increase in Fyn-associated activity, and mirrored the amount of total SphK2 found associated with Fyn (0.05–0.06%). To determine whether the increased SphK2 activity in the precipitates was due to increased SphK2 protein associated with Fyn or whether it reflected increased enzymatic activity of SphK2, we preincubated lysates from nonactivated or Ag-activated BMMC with SphK2 from HEK293 cells overexpressing FLAG-SphK2. Antibodies against Fyn, but not LAT, co-precipitated SphK2 (about 0.084% of the total SphK2). After FceRI stimulation, the amount of SphK2 associated
with Fyn increased by 1.8-fold when normalized to the amount of Fyn immunoprecipitated (Fig. 5D). Densitometric quantitation revealed this increased interaction to be consistent in all experiments. This finding suggests that the increased Fyn-associated SphK2 activity, observed after FcεRI stimulation, may result from increased association of Fyn and SphK2. However, because conditions of SphK2 overexpression were used, further experimentation is necessary to validate this viewpoint.

**Fyn Kinase Increases SphK1 and Not SphK2 Activity in Vitro but, Along with Lyn Kinase, Contributes to the Membrane Translocation of SphK2**—To assess the functional consequence of SphK interaction with Src PTKS, we explored the effects of Fyn on SphK1 and SphK2 activities in vitro. As shown in Fig. 6A, the presence of Fyn induced an increase in SphK1 activity (about 8-fold) comparable with that induced by Lyn (about 10-fold) (14), whereas c-Src, which does not interact with SphK1, only slightly affected SphK1 activity (1.5-fold) (data not shown). In contrast, SphK2 activity was unaffected by Fyn, Lyn, or Src (Fig. 6A and data not shown).

SphK1 was shown to translocate to membranes when activated by various stimuli; however, the translocation of SphK2 had not been studied. We sought to determine whether engagement of the FcεRI results in the movement of SphK2 to cell membranes and whether Fyn or Lyn deficiencies affected such movement. We found that SphK2 is translocated rapidly to membrane fractions (8–19% of total SphK2) after activation (Fig. 6B), although the translocation was not quantitatively as pronounced as the previously reported translocation of SphK1 (11, 12). The amount of enzyme present in the membrane fraction decreased after 3 min (not shown), and by 20 min, the amount of SphK2 detected in membranes was comparable with that found in resting conditions (ranging from 5 to 13% of the total SphK2) (Fig. 6B). Consistent with the effects of Fyn and Lyn on SphK activation, no significant translocation of SphK2 was observed in either Fyn- or Lyn-deficient cells (Fig. 6B). Interestingly, the amount of SphK2 in the membrane of resting Fyn-deficient BMMC was reduced relative to wild type cells. Reconstitution of the expression of wild type Fyn kinase in Fyn-deficient BMMC resulted in significant restoration of SphK2 translocation to the membrane after FcεRI stimulation (Fig. 6C). However, the catalytically inactive mutant (KN-Fyn), which is able to associate with SphK1 and SphK2 (Fig. 5B), was unable to cause significant translocation of SphK2, suggesting a role for the catalytic activity of Fyn in the translocation of SphK2.

**Role of the Gab2-PI3K Signaling Axis in SphK1 and SphK2 Activity**—Our experiments demonstrated that Fyn activity was required for activation of SphKs. Since tyrosine phosphorylation of SphK1 (14) or SphK2 (data not shown) was not observed, demonstrating that these kinases are not direct targets of Fyn kinase activity, we investigated the Fyn-dependent signals involved in SphK activation. We previously found that Fyn-deficient BMMC were defective in PI3K activity (15). Phosphatidylinositol and other acidic phospholipids are known to activate SphK in vitro (31), and PI3K was implicated in the activation of SphK in other cell types (32, 33). Thus, we analyzed the IgE-dependent activation of SphK1 in BMMC in the presence or absence of the PI3K-specific inhibitor, LY294002. Treatment of BMMC with LY294002 inhibited both SphK1 and SphK2 activities (Fig. 7, A–C). However, in contrast to the dramatic inhibition of SphK1 activity (Fig. 7A), LY294002 decreased the extent, but not the onset, of SphK2 activation (Fig. 7B) under conditions where it effectively inhibited Akt phosphorylation (Fig. 7D). This contrasted with the effective abrogation of SphK2 activation observed in
**Fyn Kinase Regulates Sphingosine Kinases in Mast Cells**

Fyn-deficient BMMC (Fig. 2). Therefore, we explored the role of the adapter Gab2, since it is known to regulate PI3K and it binds multiple proteins (17, 34), which could potentially regulate SphK2 activity. BMMC from Gab2-deficient mice showed a pattern of activation of SphK1 and SphK2 almost identical to that observed in LY294002-treated cells (Fig. 7, A–C). Furthermore, treatment of Gab2-deficient BMMC with LY294002 showed no further influence on SphK1 and SphK2 activation (Fig. 7C). This demonstrated a predominant role for the Gab2-associated PI3K pool that is activated by Fyn in the stimulation of SphK1. The results also demonstrated that Fyn-dependent activation of SphK2 requires signals in addition to those generated by activation of the adapter Gab2 and PI3K.

**Functional Impairment of IgE-dependent Responses in Fyn-deficient BMMC Is Partially Restored by Exogenous S1P**—Our previous studies demonstrated the transactivation of S1P receptor family members (S1P1 and S1P2) in Ag-stimulated mast cells (12). SphK-dependent transactivation of these receptors is important for mast cell chemotaxis (S1P1) and degranulation (S1P2) (12). Thus, we analyzed the chemotactic response of Fyn-deficient BMMC relative to that of WT cells. These assays were done in the absence of integrin engagement (such as fibronectin coating of surfaces) to avoid activation of chemotaxis-enhancing signals that might bypass or dampen the effects of Fyn deficiency (35). As shown in Fig. 8A, IgE-sensitized WT BMMC had a greater than 4-fold increase in the chemotactic response to Ag and SCF. The addition of S1P as a chemoattractant to the lower chamber induced the chemotaxis of BMMC at concentrations as low as 4–10 nM (data not shown and Fig. 8A). The extent of migration (about 2-fold), however, was consistently lower than that obtained using Ag as a chemoattractant (Fig. 8A) and was less effective than the response in RBL-2H3 cells (12). In contrast, Fyn-deficient BMMC were defective in chemotaxis toward Ag and SCF, whereas their response to S1P was similar to that of WT BMMC (Fig. 8A). However, when S1P and Ag were used in combination, the chemotactic response of Fyn-deficient BMMC was significantly enhanced, approximating that of FcɛRI-stimulated WT BMMC. Furthermore, as we previously found in RBL-2H3 cells (12), treatment with pertussis toxin, which inhibits Gαi-dependent G-protein-coupled receptor responses, or dimethylsphingosine (DMS), which inhibits SphK activation, reduced by 50% Ag-induced chemotaxis in WT BMMC without affecting the response of Fyn-deficient BMMC (Fig. 8B). This demonstrates that the SphK-S1P-S1P1 chemotactic pathway is inhibited in the absence of Fyn, but exogenous S1P can partly restore the motility defect in combination with signals generated by the addition of IgE/Ag.

Because Fyn is also required for IgE/Ag-mediated degranulation (15) and S1P2 receptors were demonstrated to contribute to this response (12), we investigated the effects of inhibiting SphK activity on this response. Inhibition of SphK activation by DMS resulted in decreased Ag-dependent degranulation (40–50% inhibition) of IgE-sensitized WT BMMC (Fig. 8C), as previously observed (11, 12). However, the poor degranulation of Fyn-deficient BMMC was not further affected, sug-
Fyn Kinase Regulates Sphingosine Kinases in Mast Cells

gesting the possibility that the lack of Fyn-dependent SphK activation leading to S1P generation might contribute to the defective response in these cells. To bypass the lack of SphK activation in Fyn-deficient BMMC, we added exogenous S1P to IgE/Ag-stimulated cells. Whereas the addition of S1P minimally affected degranulation in maximally activated WT BMMC, it increased degranulation of Fyn-deficient BMMC from less than 5% to more than 32%, achieving approximately one-half of the response observed in WT BMMC (Fig. 8D). Thus, the defective degranulation of Fyn-deficient BMMC can be partly attributed to the lack of SphK activation and S1P receptor transactivation. Consistent with our findings is the previous observation that S1P_2-deficient BMMC showed a decreased (50% inhibited) IgE-dependent degranulation (12).

**DISCUSSION**

Two distinct isofoms of sphingosine kinase (SphK1 and SphK2) catalyze the conversion of sphingosine to S1P in mammalian cells. SphK1 activity is regulated by a variety of stimuli and is involved in diverse cellular functions via the formation of S1P and its intracellular or receptor-mediated effects. Engagement of FcRI was shown to stimulate SphK1 activity, and the ablation of SphK1 expression by antisense oligonucleotides had an effect on several mast cell responses (11, 12). Unlike for SphK1, little was known about SphK2. We now find that in BMMC (and to a lesser extent in HuMC), SphK2 is a prominent activity regulated not only by FcRI but also by c-KIT and IL-3 receptors. The reasons why SphK2 induction in mast cells was not observed in previous studies are not clear but could include differences in the mast cell type or most likely differences in experimental settings, which could influence the location or inducibility of SphK2 activity.

Our findings demonstrate that Fyn kinase is required for the activation of both SphK1 and SphK2 by FcRI. Lyn kinase is also important, as demonstrated in our past (14) and present studies, but it has a lesser role. Lyn is required for early activation of SphKs, but it is not necessary for the late activity of these enzymes. Lyn does not appear to function downstream of Fyn in SphK activation but has a complementary relationship with Fyn in regulating SphK activity (14). However, the participation of Lyn and Lyn in activation of SphKs cannot be generalized to all receptors. IL-3 activation of SphK1 required Lyn and was considerably dependent on Fyn, whereas SphK2 activation by this cytokine was mostly independent of both Src kinases (Fig. 4 and supplemental Fig. 1). Thus, the proposed hierarchy of Fyn and Lyn in activation of both SphKs, following FcRI stimulation, should not be extrapolated to other receptors.

Similar to SphK1 (11, 12), SphK2 was found in the cytosolic fraction of BMMC (85–95%), but a small pool of SphK2 was translocated to cellular membranes and to lipid rafts (data not shown) after FcRI stimulation. Neither SphK1 nor SphK2 have membrane localization signals, but SphK2 contains several predicted transmembrane sequences (36), and thus its direct association with membranes is possible. We demonstrate that in mast cells, the presence of both membrane-localized PTKs, Lyn and Lyn, is necessary for the early translocation of SphK2. Our preliminary experiments suggest that translocation of SphK2 by FcRI appears to be independent of extracellular signal-regulated kinase activation, calcium mobilization, and protein kinase C activation (data not shown), signals known to mediate SphK1 translocation (37–39). Of these, a direct role for extracellular signal-regulated kinase is most unlikely, since this pathway is not impaired in either Fyn or Lyn-deficient BMMC (15, 40). We also demonstrated that both Lyn and Lyn interact with SphK1 and SphK2. Whether the small but measurable interaction of the SphKs with Lyn is important in the overall translocation of these enzymes remains a topic of future exploration.

The direct interaction of SphK1 with Lyn or Lyn in vitro enhanced its activity. In contrast, the interaction of SphK2 with these PTKs did not affect its activity. Lyn did not appear to phosphorylate SphK1 or SphK2, but its activity was crucial for their translocation, activation, and subsequent S1P production. Thus, we explored the effects of known Fyn-dependent signals on the activation of SphK by IgE/Ag. Fyn induces the phosphorylation of the scaffolding adaptor protein Gab2 (15), which then complexes with PI3K, resulting in PI3K activation (17). BMMC lacking Gab2 had a considerable defect in the IgE/Ag-induced activation of SphK1 but only a partial reduction in SphK2 activation. Similarly, experiments using the PI3K-specific inhibitor LY294002 showed that SphK1 was more dependent on PI3K than SphK2. This dissociation in the regulatory pathways governing these isoforms provides several novel insights: 1) it indicates that the two SphK activities detected in our assays reflect two distinctly regulated SphKs, thus suggesting the possibility of distinct cellular functions, and 2) it also implies that Fyn-dependent but Gab2-PI3K-independent signals are generated that are required for SphK2 activation. This defines a previously unrecognized point where Fyn-dependent signals bifurcate. Based on the essential role for Fyn in SphK1 and -2 activation and the noted differences in downstream signals, we propose a model (Fig. 9) in which membrane-localized Lyn, and possibly Lyn, form complexes with SphKs after FcRI engagement. This localizes SphKs to the membrane and to lipid rafts, where their substrate, sphingosine, is enriched. Activated Fyn would provide both Gab2/PI3K-dependent and -independent signals that are key to full activation of SphK1 and SphK2, respectively. Lyn would probably contribute to increasing the kinetics of activation through its role in binding both sphingosine and SphKs (14). This complementary role for Lyn is further supported by the large fraction (50%) of Lyn localized in the sphingolipid-rich lipid rafts of BMMC.

Besides FcRI, c-KIT, and IL-3, many other receptors expressed in mast cells (9, 36, 41, 42) are known to activate SphKs. Thus, S1P could be generated in the diverse microenvironment in which mast cells reside. Viewed collectively with the inherent signaling versatility of S1P as an intracellular and extracellular messenger, S1P could serve as an important regulator of mast cell responses. We now find that the lack of FcRI-mediated activation of SphK in Fyn-deficient BMMC correlates with the defective degranulation and reduced chemotactic response of these cells. As demonstrated herein, these defects were partially cor-
rected when S1P was supplied exogenously in conjunction with FcεRI stimulation. This provides evidence of a contributory role for SphKs in these responses and demonstrates the presence of functional S1P receptors on FcεRI-deficient BMCC. Together with our previous study (14), the findings provide the first example of cooperation between Fyn and Lyn in the activation of intracellular signals. Of particular note, our findings demonstrate the receptor-inducible nature of SphK2 activity and the distinct regulatory requirements for SphK2 versus the better characterized SphK1. The fact that both are activated in mast cells stresses the importance of the generation of S1P by mast cells and may reflect on the ability of these cells to produce large amounts of S1P relative to other cell types. The findings herein and elsewhere (reviewed in Ref. 13) support the view of an important role for SphK activation in mast cell physiology.

REFERENCES

1. Spiegel, S., and Milstien, S. (2003) Nat. Rev. Mol. Cell. Biol. 4, 397–407
2. Olivera, A., and Spiegel, S. (2001) Prostaglandins 64, 123–134
3. Olivera, A., Rosenfeld, H. M., Bektas, M., Wang, F., Ishii, I., Chun, J., Milstien, S., and Spiegel, S. (2003) J. Biol. Chem. 278, 46452–46460
4. Sanchez, T., and Hla, T. (2004) J. Cell. Biochem. 92, 913–922
5. Spiegel, S. (2000) Annu. N. Y. Acad. Sci. 905, 54–60
6. Liu, H., Sugiyama, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000) J. Biol. Chem. 275, 19513–19520
7. Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) J. Biol. Chem. 273, 23722–23728
8. Saha, J. D., and Hla, T. (2004) Circ. Res. 94, 724–734
9. Hatt, N. C., Sarker, S., Le Stunff, H., Mikami, A., Maceyka, M., Milstien, S., and Spiegel, S. (2005) J. Biol. Chem. 280, 29462–29469
10. Choi, O. H., Kim, J.-H., and Kinet, J.-P. (1996) Nature 380, 634–636
11. Melendez, A. J., and Khaw, A. K. (2002) J. Biol. Chem. 277, 17255–17262
12. Jolly, P. S., Bektas, M., Olivera, A., Gonzalez-Espinoza, C., Proia, R. L., Rivera, J., Milstien, S., and Spiegel, S. (2004) J. Exp. Med. 199, 959–970
13. Olivera, A., and Rivera, J. (2005) J. Immunol. 174, 1153–1158
14. Urta, N., Olivera, A., Boffi-Cardona, E., Csonga, R., Bilsch, A., Mechtcheriakova, D., Bornancin, F., Wosietzlager, M., Rivera, J., and Baumrucker, T. (2004) Mol. Cell. Biol. 24, 8765–8777
15. Pavaricini, V., Gadina, M., Kovalova, M., Odorn, S., Gonzalez-Espinoza, C., Furumoto, Y., Saioto, S., Samelson, L. E., O'Shea, J. J., and Rivera, J. (2002) Nat. Immunol. 3, 741–748
16. Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gellifan, A. M., Tkaczyk, C., Kuehn, N., Gray, A., Giddings, J., Peskett, E., Fox, R., Bruce, I., Walker, C., Sawyer, C., Okkenhaug, K., Finan, P., and Vanhaesebroeck, B. (2004) Nature 431, 1007–1011
17. Gu, H., Saioto, K., Klamann, L. D., Shen, J., Fleming, T., Wang, Y., Pratt, J. C., Lin, G., Lim, B., Kinet, J. P., and Neel, B. G. (2001) Nature 412, 186–190
18. Allende, M. L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., van Echten-Deckert, G., Hajdu, R., Rosenbach, M., Kooheane, C. A., Mandala, S., Spiegel, S., and Proia, R. L. (2004) J. Biol. Chem. 279, 52487–52492
19. Saioto, S., Arudchandran, R., Manetz, T. S., Zhang, W., Sommers, C. L., Love, P. E., Rivera, J., and Samelson, L. E. (2000) Immunity 12, 525–535
20. Kirshenbaum, A. S., Goiff, J. P., Semere, T., Foster, B., Scott, L. M., and Metcalfe, D. D. (1999) Blood 94, 2333–2342
21. Olivera, A., Kohama, T., Tu, Z., Milstien, S., and Spiegel, S. (1998) J. Biol. Chem. 273, 12576–12583
22. Edsall, L., Vann, L., Milstien, S., and Spiegel, S. (2000) Methods Enzymol. 312, 9–16
23. Olivera, A., Rosenthal, J., and Spiegel, S. (1994) Anal. Biochem. 230, 306–312
24. Prieschi, E. E., Csonga, R., Novotny, V., Kikuchi, G. E., and Baumrucker, T. (1999) J. Exp. Med. 190, 1–8
25. Timokhina, I., Kissel, H., Stella, G., and Besmer, P. (1999) EMBO J. 17, 6250–6262
26. Burton, E. A., Hunter, S., Wu, S. C., and Anderson, S. M. (1997) J. Biol. Chem. 272, 16189–16195
27. Hobson, J. P., Rosenfeld, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S., and Spiegel, S. (2001) Science 291, 1800–1803
28. Olivera, A., and Spiegel, S. (1993) Nature 365, 577–580
29. Xia, P., Wang, L., Moretti, P. A., Albaneese, N., Chai, F., Pitson, S. M., D’Andrea, R. J., Gamble, J. R., and Vadas, M. A. (2002) J. Biol. Chem. 277, 7996–8003
30. Yoshimoto, T., Furuhata, M., Kamiya, S., Hisada, M., Miyaji, Y., Magami, Y., Yarnamoto, K., Fujihara, H., and Mizuguchi, J. (2003) J. Immunol. 171, 1532–1539
31. Olivera, A., Rosenthal, J., and Spiegel, S. (1996) J. Cell. Biochem. 60, 529–537
32. Smith, B. E., Patel, V., Seatter, S. D., Deehan, M. R., Brown, M. H., Brooke, G. P., Goodridge, H. S., Howard, C. J., Rigley, K. P., Harnett, W., and Harnett, M. M. (2003) Blood 102, 2532–2540
33. Hanna, A. N., Berthiaume, L. G., Kikuchi, Y., Begg, D., Bourgoin, S., and Brindley, D. N. (2001) Mol. Biol. Cell 12, 3618–3630
34. Rivera, J. (2002) Curr. Opin. Immunol. 14, 688–693
35. Tan, B. L., Yacizciglu, M. N., Ingram, D., McCarthy, J., Borneo, J., Williams, D. A., and Kapur, B. (2003) Blood 101, 4725–4732
36. Liu, H., Chakravarty, D., Maceyka, M., Milstien, S., and Spiegel, S. (2002) Proc. Natl. Acad. Sci. U. S. A. (2003) 100, 52487–52492
37. Pitson, S. M., Moretti, P. A., Zebol, J. R., Xia, P., Vadas, M. A., and Wattenberg, B. W. (2003) EMBO J. 22, 5491–5500
38. Young, K. W., Willets, J. M., Parkinson, M. J., Bartlett, P., Spiegel, S., Nahorski, S. R., and Challiss, R. A. (2003) Cell Calcium 33, 119–128
39. Johnson, K. R., Becker, K. P., Facchinetti, M. M., Hannun, Y. A., and Obeid, L. M. (2002) J. Biol. Chem. 277, 35257–35262
40. Kawakami, Y., Kitaura, J., Sattherlhwate, A. B., Kato, R. M., Aso, K., Hartman, S. E., Maeda Yarnamoto, M., Lowell, C. A., Rawlings, D. J., Witte, O. N., and Kawakami, T. (2000) J. Immunol. 165, 1210–1219
41. Melendez, A., Floto, R. A., Gillooly, D. J., Harnett, M. M., and Allen, J. M. (1998) J. Biol. Chem. 273, 9393–9402
42. Gordon, J. R., Zhang, X., Stevenson, K., and Cosford, K. (2000) Cell. Immunol. 205, 128–135