Novel Mechanism for FcεRI-mediated Signal Transducer and Activator of Transcription 5 (STAT5) Tyrosine Phosphorylation and the Selective Influence of STAT5B over Mast Cell Cytokine Production*

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Background: STAT5 is a transcription factor that is vital for mast cell function.

Results: Loss of Fyn kinase prevents, whereas loss of Lyn, Gab2, or SHP-1 enhances, FcεRI-mediated STAT5 tyrosine phosphorylation.

Conclusion: IgE-mediated STAT5 activation in mast cells requires Fyn kinase.

Significance: Elucidating the mechanisms of mast cell activity is essential to understanding and treating allergic pathologies.

Previous studies indicate that STAT5 expression is required for mast cell development, survival, and IgE-mediated function. STAT5 tyrosine phosphorylation is swiftly and transiently induced by activation of the high affinity IgE receptor, FcεRI. However, the mechanism for this mode of activation remains unknown. In this study we observed that STAT5 co-localizes with FcεRI in antigen-stimulated mast cells. This localization was supported by cholesterol depletion of membranes, which ablated STAT5 tyrosine phosphorylation. Through the use of various pharmacological inhibitors and murine knock-out models, we found that IgE-mediated STAT5 activation is dependent upon Fyn kinase, independent of Syk, PI3K, Akt, Bruton’s tyrosine kinase, and JAK2, and enhanced in the context of Lyn kinase deficiency. STAT5 immunoprecipitation revealed that unphosphorylated protein preassociates with Fyn and that this association diminishes significantly during mast cell activation. SHP-1 tyrosine phosphatase deficiency modestly enhanced STAT5 phosphorylation. This effect was more apparent in the absence of Gab2, a scaffolding protein that docks with multiple negative regulators, including SHP-1, SHP-2, and Lyn. Targeting of STAT5A or B with specific siRNA pools revealed that IgE-mediated mast cell cytokine production is selectively dependent upon the STAT5B isoform. Altogether, these data implicate Fyn as the major positive mediator of STAT5 after FcεRI engagement and demonstrate importantly distinct roles for STAT5A and STAT5B in mast cell function.

Asthma and allergic diseases are a tremendous health burden on developed nations. In the United States asthma alone accounts for more than 20 million patients and 500,000 hospitalizations annually, with costs exceeding $15 billion. Nearly half of all asthmatics have poorly controlled disease, supporting an urgency to find better therapies (1).

The best studied mechanism eliciting allergic inflammation is IgE binding to its high affinity receptor, FcεRI, which activates mast cells upon antigen exposure. Within minutes of FcεRI cross-linking, mast cells degranulate, releasing proteoglycans, leukotrienes, and histamine. Hours later, mast cells secrete cytokines such as TNF-α, MIP-1α, IL-13, and various chemokines that collectively elicit leukocyte infiltration and sustained inflammation (2, 3). This clinical importance has focused efforts on understanding IgE-mediated mast cell functions.

Allergic inflammation is known to involve members of the signal transducer and activators of transcription (STAT) family. These transcription factors control key facets of inflamma-
tory progression. For example, STAT6 is required for T_{H2} responses mediated by IL-4 and IL-13 (for review, see Refs. 2, 4). In mast cells, the stem cell factor (SCF) receptor, c-Kit/CD117, activating STATs1, 3, 5, and 6 (2, 5–7), partly through the tyrosine kinase Jak2. Among these, our laboratory previously discovered that STAT5 is requisite for mast cell development and survival (8). We subsequently found that like c-Kit, FceRI rapidly induces STAT5 tyrosine phosphorylation and that STAT5 deficiency greatly reduces early and late mast cell responses (9). The critical role for STAT5 in IgE-mediated inflammation, the most common cause of allergic disease, justifies mechanistic studies. Understanding STAT5 activation could offer novel insight into patient care.

FceRI signaling has been studied in detail and is generally accepted as proceeding via two major pathways. Rapid activation of the Lyn tyrosine kinase allows binding of the kinase Syk, which subsequently activates adapter molecules such as linker of activated T cells (LAT) and SLP-76. A more recently described and somewhat distinct pathway descends from Fyn kinase through the adapters LAT2 and Grb-2 associated binder 2 (Gab2), with robust activation of PI3K. Although distinctions exist (10, 11), these pathways are interrelated. Lyn kinase, through its recruitment of negative regulators, suppresses Fyn function (12, 13). Herein, we explored the hypothesis that STAT5 associates in close proximity with FceRI and is rapidly activated by one of the two major pathways stemming from FceRI. We scrutinized STAT5 tyrosine phosphorylation by perturbing factors such as Fyn and Lyn kinases via biochemical and gene/RNA ablation techniques. Finally, total STAT5 actually accounts for two very similar, but separable, proteins: STAT5A and STAT5B (14). Our prior work demonstrated that the introduction of STAT5A into total STAT5A/B-deficient (KO) mast cells rescues survival, but the importance of STAT5B remained as yet unknown (8, 9). Here, we tested the idea that the two STAT5 proteins play distinct roles in mast cell activation.

In sum, we have found that STAT5 is indeed in close association with FceRI. Moreover, IgE-mediated STAT5 activation proceeds selectively through the Fyn pathway, with negative feedback from Lyn kinase. Gab2 appears to suppress STAT5 tyrosine phosphorylation, which may be due to SH2 domain-containing phosphatase-1 (SHP-1) recruitment. Finally, studies utilizing STAT5A- or B-specific RNA interference demonstrated that STAT5B is the central and selective mediator of cytokine induction, independent of STAT5A. These studies offer a novel mechanistic view of a central pathway controlling allergic disease.

**EXPERIMENTAL PROCEDURES**

Reagents—Cell culture medium (RPMI 1640 medium) was purchased from Invitrogen, fetal bovine serum (FBS) from Quality Biological, Inc. (Gaithersburg, MD), and all other supplements from Mediatech, Inc. (Manassas, VA). Murine IL-3 and SCF were obtained from PeproTech, Inc. (Rocky Hill, NJ). Kinase inhibitors were purchased from EMD Chemicals (Gibbstown, NJ). Methyl-β-cyclodextrin, dinitrophenol-human serum albumin (DNP-HSA), and other basic chemicals not mentioned were purchased from Sigma-Aldrich. Purified mouse IgE (clone C38-2, κ isotype) was purchased from BD Biosciences (Pharmingen division, San Diego, CA). The following primary antibodies were used: rabbit monoclonal anti-total STAT5 (no. 9063), Fyn (no. 4023), Lyn (no. 2732), pJAK2 (Tyr-1007, no. 4406), pSrc (Tyr-416, no. 2113), and pAkt (Ser-473, no.40585) from Cell Signaling Technology (Danvers, MA); rabbit monoclonal anti-Tyr(P)-STAT5 from Epitomics, Inc. (Burlingame, CA); rabbit polyclonal anti-pSyk (Tyr-525, A0092), pBTK (Tyr-222, A0054), STAT5A (no. B7227) and STAT5B (no. B0090) from Assay Biotechnology Company (Sunnyvale, CA); mouse monoclonal anti-total STAT5 (sc-74442), Fyn (sc-71134), and rabbit polyclonal anti-β-actin (sc-44942) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and rabbit polyclonal anti-TRPC1 (ab75322) from Abcam (Cambridge, MA).

Mice—All procedures involving mice were reviewed and approved by the local institutional animal care and use committee. Six- to 8-week-old wild-type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Bruton’s tyrosine kinase (BTK) KO and Gab2 KO (kind gifts of Toshio Hirano, Osaka University) (15) were bred on the C57BL/6 background (16).

Cell Isolation and Culture—Bone marrow-derived mast cells (BMMCs) were differentiated from tibia and femur bone marrow of respective mice. The marrow cultures were maintained in RPMI 1640 medium supplemented with IL-3-containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF was adjusted to 1 ng/ml and 10 ng/ml, respectively, as measured by ELISA. Medium also contained 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM HEPES, 10% (v/v) FBS, and 1 mM sodium pyruvate. Cultures contained purified BMMCs by 4 weeks, and experiments using KO strains were compared with age-matched wild-type controls. Cultures were maintained and sensitized to DNP-specific IgE (0.5 μg/ml for 18 h) in this growth medium. For immunoblotting experiments involving Tyr(P)-STAT5, BMMCs were rinsed free of growth medium and starved for 4 h in complete RPMI 1640 medium (medium without supplementary cytokines). If a chemical treatment (inhibitors or MβCD) was involved, the noted agent was added to the cultures after 3 h of cytokine starvation. After the total 4 h, cultures were stimulated with 100 ng/ml DNP-HSA for 15 min and then immediately lysed.

Preparation of Membrane Sheets and Transmission Electron Microscopy—Detailed methods for preparation and immunogold labeling of membrane sheets have been described (17–19). In brief, BMMCs were primed overnight with DNP-specific IgE, rinsed by centrifugation, and allowed to settle on glass coverslips for 15 min prior to 5-min incubation at 37 °C ± DNP colloidal gold. For the resting condition, cells were primed with DNP-specific IgE conjugated to quantum dots at a 1:1 ratio as in Ref. 19. Reactions were stopped by addition of 0.5% paraformaldehyde. After 10 min, samples were rinsed with PBS, lowered onto poly-l-lysine coated electron microscope grids, and ripped. Membrane sheets on grids were then fixed with 2% paraformaldehyde, rinsed, and sequentially stained with primary and secondary antibodies as in Ref. 18. At least two experiments were performed for each condition, for which at least 10
images were acquired on a Hitachi H-7500 transmission electron microscope.

**Immunoblotting**—Cell cultures were lysed at 1 × 10^5 cells/μl in lysis buffer (Cell Signaling Technology) supplemented with 1.5× ProteaseArrest (G-Biosciences, Maryland Heights, MO). Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific). Proteins were resolved by SDS-PAGE using 50 μg of total protein per sample on 8–16% Tris-glycine gels (Novex system; Invitrogen). Transfer was made onto nitrocellulose membranes subsequently blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) and 5% (w/v) nonfat dried milk. Membranes were rinsed in TBST six times for 5 min and then incubated overnight at 4 °C in TBST containing 5% (w/v) BSA and primary antibody diluted 1:1000. Membranes were rinsed again (same protocol) then incubated at room temperature for 1 h in TBST and 5% milk containing peroxidase-conjugated secondary antibody diluted 1:2500, either goat anti-rabbit or mouse IgG (Jackson ImmunoResearch, West Grove, PA). Membranes went through a final rinsing protocol before exposure to Amersham Biosciences ECL Reagents (GE Healthcare) and film development.

**Immunoprecipitation**—Cell lysates were collected using radioimmunoprecipitation assay buffer (Cell Signaling Technology). Protein A/G beads (Invitrogen) were washed three times with PBS and centrifugation, and then lysates were pre-cleared for 2 h at 4 °C with a 50% slurry of beads added one part for every two parts lysate. The beads were removed by centrifugation, and lysates were incubated for 18 h at 4 °C with monoclonal rabbit anti-STAT5, or anti-Fyn (Santa Cruz Biotechnology). Freshly rinsed beads were added to the lysate antibody mixture and incubated for 2 h at 4 °C, then centrifuged and rinsed three more times with PBS. The final bead pellets were suspended in Tris-glycine sample buffer (Invitrogen) and boiled for 10 min to dissociate the precipitate from the beads. Samples were centrifuged and supernatants immunoblotted.

**ELISA**—BMMCs were sensitized to DNP-specific IgE (0.5 μg/ml, at least 18 h) then antigen-stimulated with DNP-HSA (50 ng/ml) for 18 h. Murine IL-13, MIP-1α, and TNF-α ELISA kits were purchased from PeproTech and performed using culture supernatants according to the manufacturer’s protocols. ELISAs were developed using BD OptEIA reagents from BD Biosciences.

**Nucleofection of siRNAs**—Wild-type C57BL/6 BMMCs were transfected with a Dharmacon ON-TARGET SMARTpool of STAT5A, STAT5B, Lyn, Fyn, or TRPC1 siRNAs or nontargeting negative controls (Thermo Scientific). The Amaxa Nucleofector II and Cell Line Nucleofector kit V (Lonza Cologne GmbH, Cologne, Germany) were utilized according to the manufacturer’s protocol for human monocytes. For the initial nucleofection step, a concentration of 2 μM siRNA and three million cells were used. The cultures were allowed to rest for 72 h in growth medium added to dilute the siRNAs to a final concentration of 57 nM. Efficiency of knockdown was determined through immunoblot analyses.

**Image and Statistical Analyses**—Immunoblot films were digitized and analyzed using ImageJ (National Institutes of Health). Optical density (OD) of the immunoreactivity of interest, e.g. Tyr(P)-STAT5, STAT5A/B, was normalized to total STAT5 or β-actin OD. Normalized values were compared among at least three independent experiments and are presented graphically as mean values ± S.D. Differences between pairs were deemed significant for p < 0.05 with Student’s t tests. For electron micrographs, gold particle and quantum dot (QD) distributions were grabbed using a plug-in to ImageJ; the Ripley’s bivariate K function was then applied as a statistical test for co-clustering (20, 21).

**RESULTS**

**STAT5 Co-localization with FcεRI**—Although our past studies established STAT5 as an indispensable component of high affinity IgE receptor signaling, there was additional curiosity with regard to the proximity of the transcription factor with the receptor. To investigate this, membrane sheets were prepared from resting and antigen-stimulated BMMCs followed by immunogold labeling for STAT5 and imaging by transmission electron microscopy. To visualize FcεRI under resting conditions, BMMCs were primed with QD-conjugated IgE (QD-IgE) as in Ref. 19. The electron-dense QDs appear as small rods (some of which are circled) in the transmission electron microscopy image in Fig. 1A, where their distribution is clearly segregated from the 5-nm gold label for STAT5. The lack of co-localization for FcεRI and STAT5 in resting cells was confirmed by the Ripley’s bivariate K because the data line in the plot falls between the two dashed lines, indicating the tolerance interval. To track activated FcεRI, BMMCs were primed with DNP-specific IgE and then stimulated with colloidal gold particles coated with antigen (DNP-gold; Fig. 1B). In this case, STAT5 (marked with 5-nm immunogold) was shown to be recruited to signaling patches that form after FcεRI aggregation. The co-clustering of STAT5 and FcεRI in antigen-stimulated BMMCs is statistically significant, as confirmed by Ripley’s bivariate K analysis, i.e. the data line is above the confidence interval.

**Disruption of Lipid Rafts Diminishes IgE-mediated STAT5 Activity**—Having observed that STAT5 and FcεRI co-localize in activated mast cells (Fig. 1, A and B), we explored the dependence of STAT5 with respect to membrane subdomain stability. Field et al. established that FcεRI aggregates into lipid rafts upon ligation with antigen-IgE complexes (22). The cholesterol-depleting, chelating agent MβCD has been utilized for the disruption of lipid rafts (23, 24). BMMCs were sensitized to IgE (0.5 μg/ml) for 18 h in their normal growth medium. After sensitization, the cultures were washed twice and starved for 4 h in complete RPMI 1640 medium to extricate the effects of IL-3 because this cytokine potently stimulates STAT5 phosphorylation independent of FcεRI ligation (25). MβCD was added to the cultures (final concentration of 10 mM) 1 h prior to antigen stimulation, which was performed for 15 min at 37 °C with DNP-HSA (100 ng/ml). Immunoblotting for Tyr(P)-STAT5 and total STAT5 protein was performed to assess the effect of raft depletion on signaling. Detection of Tyr(P)-STAT5 is expected to increase in antigen-stimulated (cross-linked) samples, indicative of an intact FcεRI signaling apparatus. Indeed, this is the case for control-stimulated BMMCs (Fig. 1C, left two lanes). However, MβCD treatment reduced Tyr(P)-STAT5 lev-
**FIGURE 1.** **STAT5** is in close association with FcεRI. **A**, upper, electron micrograph of a membrane sheet prepared from resting BMMC; STAT5 is labeled with 5-nm gold, FcεRI with QD-IgE. **A**, lower, Ripley’s bivariate K graph, a spatial statistics test for co-clustering between the labeled particles, showing the lack of co-clustering as the data plot (solid line) falls within the tolerance interval (dashed lines). **B**, upper, electron micrograph of membrane sheet from BMMCs stimulated with 10-nm gold-labeled DNP-HAS. STAT5 was labeled again with 5-nm gold. **B**, lower, another Ripley’s graph. **C**, sample immunoblot of cell lysates that were antigen-stimulated in the absence or presence of lipid raft disruptor MβCD. **D**, graphical representation of **C** based upon Tyr(P)-STAT5:total STAT5-fold stimulation over respective unstimulated samples from three independent experiments. *, p < 0.05 DNP-HSA + MβCD compared with DNP-HSA. Error bars, S.D.
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Syk with ATP-competitive Syk inhibitor II (SykiII, 2 μM) also had no effect on STAT5 activation (Fig. 2B). Additionally, targeting of Akt either upstream at PI3K with LY294002 (20 μM) or directly with Akt inhibitor VIII (1 μM) did not prevent STAT5 tyrosine phosphorylation following receptor cross-linking (Fig. 2, C and D). Similarly, inhibiting the common STAT5-activating kinase JAK2, through use of AG490 (50 μM), had no effect on IgE-mediated STAT5 activation (Fig. 2E). In contrast, the ATP-competitive inhibitor of the Src family of protein kinases, PP2 (10 μM), ablated Tyr(P)-STAT5 to an undetectable level in our immunoblots (Fig. 2F). Immunodetection of the respective phosphorylated targets of each inhibitor was performed to determine inhibitor efficacy, showing either lack of target activation or complete ablation of the signal. A statistical summary of inhibitor effects showed that of those examined here, only inhibition of Src family kinases significantly reduced Tyr(P)-STAT5 (Fig. 2G).

**Fyn Is Required for FcεRI-mediated STAT5 Activity**—Having narrowed our search for the link between STAT5 and FcεRI to Src kinases, we assessed the importance of Lyn and Fyn because these Src family members are essential for the homeostasis of FcεRI signals. First, we addressed Fyn by growing Fyn KO BMMCs. Fyn KO BMMCs cross-linked for 5, 15, and 30 min revealed a profound and sustained abrogation of STAT5 tyrosine phosphorylation, indicating Fyn as the essential STAT5 regulator (Fig. 3A). It should be noted that total STAT5 resoloves at a lower molecular mass in Fyn KO samples versus respective WT, an interesting phenotype, possibly due to impaired phosphorylation and other post-translational modifications, that requires further investigation.

Lyn deletion has been shown to enhance IgE signaling, partly due to Fyn hyperactivity. These effects have been most overt on the 129/Sv genetic background, which appears to have increased Fyn expression (12). To address the importance of Lyn and Fyn under controlled conditions, we depleted each kinase through siRNA transfection, using C57BL/6 BMMCs. We obtained nearly complete depletion of each kinase. This approach confirmed the importance of Fyn, as Fyn depletion reduced STAT5 tyrosine phosphorylation. Interestingly, Lyn depletion enhanced IgE-induced Tyr(P)-STAT5 nearly 3-fold of control levels (Fig. 3B), fitting the description of Lyn as a negative regulator of Fyn function.

Because active Fyn is likely recruited to FcεRI complexes (26), and considering our previous observations that STAT5 and FcεRI aggregate during antigen stimulation (Fig. 1, A and B), we assessed STAT5 association with Fyn. Wild-type BMMCs were untreated or IgE-sensitized and then exposed to antigen for 5 or 15 min, after which total STAT5 was immunoprecipitated. Immunoblots for Fyn demonstrated that the two proteins co-precipitated and that this association appeared to decrease following receptor ligation. When normalized to STAT5 levels, Fyn association significantly decreased in activated versus unstimulated cells according to STAT5-specific immunoprecipititates (Fig. 3, C and D). The reverse co-immunoprecipitation was also attempted wherein Fyn was the pulldown target. There was a striking reduction in the ability to immunoprecipitate Fyn in cross-linked cultures regardless of time (Fig. 3E). However, total Fyn is readily detected in whole cell lysates to approximately those of unstimulated cells (Fig. 1, C and D). Although MβCD is a broad acting cholesterol depletion agent, these experiments support the EM observations that STAT5 associates with FcεRI.

Src Kinase Family Members Are Likely Intermediaries of FcεRI-induced Tyr(P)-STAT5—Next, we dissected the machinery responsible for IgE-mediated STAT5 activation by employing chemical inhibitors or gene-deficient BMMC cultures. Following 3 h starvation from WEHI/BHK-containing medium, BMMC cultures were pretreated with each of the listed inhibitors for 1 h at 37 °C before cross-linking. BTK KO BMMCs or treatment of WT with the substrate-competitive BTK inhibitor LFM-A13 (at 100 μM) showed no significant decrease in Tyr(P)-STAT5 detection (Fig. 2A; KO not shown). Targeting of

![Figure 2. Mapping of STAT5 activation pathway reveals Src kinases as likely intermediaries in FcεRI-dependent Tyr(P)-STAT5. Representative immunoblotting for Tyr(P)-STAT5 was performed with lysates from various BMMCs treated with inhibitors to certain signaling factors, with or without antigen stimulation for 15 min.](image-url)
using a harsher, standard immunoblotting lysis buffer, e.g. Fig. 3B. We posit that the lack of Fyn pulldown in activated cells is due to epitope masking through protein associations that are intentionally preserved by the lysis conditions of immunoprecipitation.

**Lack of Transient Receptor Potential Channel 1 (TRPC1) Does Not Prevent Early IgE-mediated Tyr(P)-STAT5**—Recently, Suzuki et al. have demonstrated that the nonselective cation channel TRPC1 is involved in Ca\(^{2+}\) influx, F-actin depolymerization, and subsequent degranulation in activated mast cells (27). Considering that STAT5 tyrosine phosphorylation is an early response to Fc\(\alpha\)RI engagement, it was important to determine whether TRPC1 has any input in this activation. BMMCs were transfected with pools of siRNA against TRPC1, and efficiency was assessed 3 days after treatment (Fig. 4A). These cells were sensitized to DNP-specific IgE for 18 h and then assayed for Tyr(P)-STAT5 after 15 min of receptor cross-linking. No significant difference in Tyr(P)-STAT5 level was observed between cells with TRPC1 knockdown and those that received nontargeting siRNA (Fig. 4B). Furthermore, siTRPC1 BMMCs were subjected to a longer term (18 h) exposure of DNP (50 ng/ml), and no difference in secreted cytokines was seen (Fig. 4C).

**SHP-1 and Gab2 Deficiencies Enhance Tyr(P)-STAT5 Detection**—Fyn is linked to PI3K signaling through an intermediate, Gab2 (26). To map STAT5 regulation downstream of Fyn, we examined Fc\(\alpha\)RI-mediated STAT5 phosphorylation in Gab2 KO BMMCs. Unlike Fyn deletion, loss of Gab2 increased STAT5 activation 2.2-fold (5\(^{\text{min}}\) cross-linked) and 4.4-fold (15\(^{\text{min}}\) cross-linked) respective of WT controls (Fig. 5, A and B). In addition to its link to PI3K, Gab2 is known to associate with negative regulatory proteins, including Lyn and SHPs. Having already observed enhanced Tyr(P)-STAT5 in Lyn-depleted BMMCs, we addressed the role of SHP-1. SHP-1-deficient BMMCs cultured from Motheaten mice (me) (28) demonstrated modestly enhanced STAT5 tyrosine phosphorylation upon activation compared with their respective controls. This
increased over time during FcεRI cross-linking, peaking at 5 min and still significantly greater at 15 min after ligation (Fig. 5, C and D). Together, these data indicate that IgE-mediated STAT5 activity is under exquisite control of not just Fyn kinase but also negative regulators such as SHPs and other proteins associated with Gab2.

Distinct Roles for STAT5A and STAT5B—Our earlier work found that STAT5 deficiency had some inhibitory impact on IgE-mediated degranulation, but the most significant effects were in cytokine secretion (9). Because we and others have found that STAT5A is critical for mast cell survival (8, 29), we sought to determine whether STAT5A and B have distinct roles in IgE-mediated cytokine induction. We assessed this through the use of siRNA pools targeting STAT5A or STAT5B. After

FIGURE 5. Gab2 or SHP-1 deficiency enhances FcεRI-mediated STAT5 tyrosine phosphorylation. A, representative immunoblot of Gab2 KO BMMCs and their respective wild-type controls stimulated over time with antigen. B, graphical representation of A based upon Tyr(P)-STAT5:total STAT5 densitometry. *, p < 0.05 comparing KO with WT. Error bars, S.D. C, representative immunoblot for Tyr(P)-STAT5 in wild-type and me (SHP-1-deficient) BMMCs that were stimulated over time with antigen. D, graphical representation of C based upon Tyr(P)-STAT5:total STAT5 densitometry from three independent experiments. p < 0.05 comparing me with respective wild-type, time point controls.

IgE sensitization and antigen exposure, conditioned media were assayed by ELISA for MIP-1α and IL-13 production. There were no remarkable differences between nontargeting siRNA-treated BMMCs and siSTAT5A with respect to MIP-1α and IL-13 production (Fig. 6A, middle and right panels). This is in the context of an average of 66% STAT5A knockdown and 22% decrease of STAT5B. Nucleofection of siSTAT5B pools into wild-type BMMCs ablated STAT5B protein (Fig. 6B, left panel, immunoblots). STAT5A expression was also slightly decreased, but when adjusted for protein loading in separate experiments not nearly to the extent of STAT5B silencing, demonstrating higher selectivity for STAT5B targeting. BMMC treated with siSTAT5B prior to IgE stimulation displayed significantly less MIP-1α and IL-13 production than cells treated with control siRNA (Fig. 6B, right panels). Because siSTAT5A-treated BMMCs produced cytokines normally, these data collectively pointed to STAT5B as the regulator of IgE-mediated cytokine secretion.

DISCUSSION

It is estimated that allergic disease affects nearly one quarter of the developed human population (30). Chronic asthma, just one of these disorders, was most recently predicted by the Global Initiative for Asthma to affect ~300 million people (31). This estimate is conservative, considering the age of the report and factors such as cryptic, or missed diagnoses in older populations, which account for >two-thirds of asthma-related mortality (32). At the crux of allergic pathologies, and best studied in asthma, is the activated mast cell. Better understanding the intricacies of mast cell biology is critical to discovering new avenues for targeted treatment of mast cell-mediated pathologies. To this end we have established that STAT5 is essential for mast cell development, vitality, and function. STAT5 is one of the more promiscuous members of the STAT family, expressed ubiquitously and with pleiomorphic
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effects (2, 33). However, over the past decade it has come to the front that tissue niche and/or cell lineage play a large role in determining STAT5 effects (4, 33). This is especially true in the context of myeloid cells, including mast cells. There, dogma indicates that STAT5 activity can be largely dependent upon JAK2 activation (4). Indeed, JAK2 is the major intermediary between SCF/c-Kit and activated STAT5 in mast cells (2, 33).

The present data indicate STAT5 apposition with the high affinity IgE receptor, FceRI. This is novel in light of several lines of thought regarding how STAT5 is typically activated. First, under circumstances of more steady-state STAT5 activation, or perhaps “JAK-skewed” pSTAT5, there is potent cross-talk with signaling cascades such as the PI3K-Akt pathway. Here, we report that inhibition of JAK2 or the PI3K-Akt pathways, as well as other signaling factors such as BTK and Syk, has no effect on IgE-mediated STAT5 tyrosine phosphorylation. Second is the potential for regular cycling of STAT5 between the cytosol and nucleus as a result of chronic activation by means other than FceRI engagement, e.g. due to c-Kit (33). Our analyses indicate that IgE-induced STAT5 tyrosine phosphorylation is dependent upon Fyn kinase and furthermore, that unphosphorylated STAT5 associates with Fyn. As Fyn is suspected of associating with FceRI, this supports the suggestion of a STAT5 store sequestered outside the nucleus in a juxta-FceRI compartment. Interestingly, pSTAT5 cytoplasmic accumulation has been reported in a cancerous setting (34), raising the question of whether or not a similar situation could arise in noncancerous persistently STAT5-active conditions, e.g. IgE-induced inflammation. Our inability to co-immunoprecipitate Fyn and STAT5 after stimulation could likely be due to additional protein modifications and interactions that require further detailed studies.

It is important to contrast these findings to recent results from another group. By using RNA interference in cell lines and BMMCs, Barbu et al. reported that the contributions of Fyn and Gab2 with respect to FceRI signaling are not as relevant as that from Syk (35). Although our results show that Syk is not required for early FceRI-related STAT5 activity, these data certainly do not preclude Syk as one of the major mediators of FceRI signaling. Our findings do, however, support an important role for Fyn. This complements other work demonstrating Fyn dependence for chemokine, cytokine, and eicosanoid production (36). Parravicini et al. originally presented the FceRI-Fyn link and showed that Fyn, not Lyn, is required for mast cell degranulation (26). Suzuki et al. examined the role of Fyn in degranulation further and discovered that loss of TRPC1, a nonspecific cation channel, contributes to the degranulation-impaired phenotype of Fyn-deficient BMMCs (27). In the present study, silencing of TRPC1 had no effect on IgE-mediated Tyr(P)-STAT5 detection. This demonstrates separability between the physiological events behind degranulation and IgE-mediated STAT5 signaling discussed here. Interestingly, we observed that Gab2 deficiency enhanced STAT5 phosphorylation in response to FceRI. This was a surprise considering the various positive regulators of signaling that associate with this scaffold protein. Barbu et al. assert that Gab2 is most important for late phase mast cell responses, rather than processes of degranulation (35). Indeed, in our experience total STAT5-KO BMMCs show only limited, yet significant, reduction in histamine and leukotriene B4 release (9). It is notable, however, that the protein phosphatases SHP-1 and SHP-2 also associate with Gab2 (37), and that in another system, osteoclast development, Lyn complexes with Gab2 and SHP-1 (38). We hypothesize that the assemblage of these negative regulators with Gab2 contributes to the increase in Tyr(P)-STAT5 observed under the setting of a Gab2, SHP-1, or Lyn deficiency. Lyn depletion likely enhances Fyn activity, as the inhibitory C-terminal Src kinase (also known as CSK) should be recruited poorly without Lyn (12). We also documented a modest enhancement of Tyr(P)-STAT5 in me mast cells, possibly due to the distal position of SHP-1 in this transduction pathway, whereas Lyn and Gab2 occupy more apical positions. Other possible negative regulators remain unidentified, and our current summary of this transduction pathway is illustrated in Fig. 7.

STAT5 expression is required for the development of the various hematopoietic lineages (2, 4, 33). Mice totally deficient in STAT5 are most often unviable by the perinatal period, and those few that survive present with severe combined immunodeficiency characterized by defective B cell, T cell, and lymphoid development (39). However, STAT5 is duplicated in many metazoans as STAT5 A/B (14). Work in our laboratories has demonstrated the importance of STAT5 in mast cell survival, development, and cytokine production, the hallmark of the late phase mast cell response. Specific use of a dominant negative
STAT5A reduced viability, whereas STAT5A transfection conferred survival to STAT5 A/B KO BMMCs (8, 9). Ikeda et al. confirmed this and additionally showed that STAT5A KO mast cells have enhanced apoptosis with reduced Bcl-xL expression (29). Li et al. further dissected the role of STAT5A in survival, determining that the N-domain is required for bcl-2 induction (40). Together, these data suggest that STAT5A could be the key to mast cell survival. Using siRNA pools to STAT5A or STAT5B we found that cytokine production in the context of STAT5B removal was significantly impaired. Therefore, considering accumulating data regarding the separable STAT5 proteins, STAT5A appears to be most critical for mast cell development and survival, whereas the B isoform influences cytokine regulation.

This separation of duties between STAT5A and B warrants further study. Tools are now becoming available to examine STAT5 proteins, particularly with regard to their various phosphorylation states. In general, the importance of STAT5 serine phosphorylation is becoming more recognized and explored with time, as in a recent report by Friedbichler et al. demonstrating that Ser(P)-STAT5A is required for hematopoietic phosphorilation states. In general, the importance of STAT5 serine phosphorylation is becoming more recognized and explored with time, as in a recent report by Friedbichler et al. demonstrating that Ser(P)-STAT5A is required for hematopoietic

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