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The Src-family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice[1]

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Abstract

Mast cells are critical for various allergic disorders. Mast cells express Src-family kinases which relay positive and negative regulatory signals by antigen. Lyn, for example, initiates activating signaling events but it also induces inhibitory signals. Fyn and Hck are reported to be positive regulators but little is known about the roles of other Src kinases, including Fgr, in mast cells. In this study, we define the role of Fgr. Endogenous Fgr associates with FcεRI and promotes phosphorylation of Syk, Syk substrates which include LAT, SLP76, and Gab2, and downstream targets such as Akt and the MAP kinases in antigen-stimulated mast cells. As a consequence, Fgr positively regulates degranulation, production of eicosanoids, and cytokines. Fgr and Fyn appeared to act in concert as phosphorylation of Syk and degranulation are enhanced by overexpression of Fgr and further augmented by overexpression of Fyn but is suppressed by overexpression of Lyn. Moreover, knockdown of Fgr by siRNAs further suppressed degranulation in Fyn-deficient BMMCs. Overexpression of Fyn or Fgr restored phosphorylation of Syk and partially restored degranulation in Fyn-deficient cells. Additionally, knockdown of Fgr by siRNAs inhibited association of Syk with FcεRIγ as well as the tyrosine phosphorylation of FcεRIγ. Of note, the injection of Fgr siRNAs diminished the protein level of Fgr in mice and simultaneously inhibited IgE-mediated anaphylaxis. In conclusion, Fgr positively regulates mast cell through activation of Syk. These findings help clarify the interplay among Src-family kinases and identify Fgr as a potential therapeutic target for allergic diseases.

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Introduction

Mast cells are responsible for IgE-dependent immediate hypersensitivity and a variety of allergic and autoimmune disorders (1, 2). In allergic conditions, antigen-induced aggregation of high affinity IgE receptors, FcεRI, on mast cells initiates a complex series of signaling pathways (3, 4). Several Src-family kinases including Lyn, Fyn, and Hck are highly expressed and have distinct signaling functions in mast cells. Of these, Lyn is constitutively associated with FcεRI in small amounts and on aggregation of FcεRI is able to transphosphorylate tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs) in adjacent FcεRI β and γ chains. The phosphorylated FcεRIβ is thus able to recruit additional Lyn whereas the phosphorylated FcεRIγ recruits and activates the tyrosine kinase Syk which plays a critical role in the amplification of mast cell signaling. Once activated, Syk phosphorylates key membrane-associated and cytosolic docking proteins such as the linkers for activation of T cells (LAT)[4], LAT1 and LAT2 (also known as NTAL or LAB), Src-homology domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76), and growth-factor-receptor-bound protein 2 (Grb2)-associated binding protein 2 (Gab2). These, in turn, recruit additional docking and signaling proteins to propagate downstream signals for secretion and production of various allergic mediators such as histamine, cytokines, and eicosanoids (3, 4). However, Lyn may also negatively regulate mast cell function (5), especially at high antigen concentrations (6), possibly through activation of inhibitory regulators such as Src homology 2-containing inositol phosphatase (SHIP) and Src homology 2-containing tyrosine phosphatase-1 (SHP-1) (7).

Fyn is reported to initiate signals that complement those of Lyn (8). Fyn activates a Gab2/phosphatidylinositol 3′-kinase (PI3K) pathway which is also necessary for degranulation, synthesis and release of leukotrienes (LT), and production of cytokines (8, 9). Recently, Hck was reported to positively regulate mast cell activation by suppressing the inhibitory actions of Lyn (10). In addition to Lyn, Fyn, and Hck, other Src-family kinases are also expressed in mast cells include c-Src, Fgr, and Yes (11, 12). Previous studies based on overexpression or knockdown of Fgr suggest that Fgr positively regulates activation of phospholipase D and degranulation in mast cells (12, 13) but more detailed studies are lacking.

Here we show that endogenous Fgr positively regulates mast cell activation and is critical for IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. We have also examined the mechanism of this regulation and show that Fgr activates Syk and other downstream signaling molecules to promote degranulation and production of cytokines and eicosanoids in mast cells. Moreover, Fgr acts solely as a positive regulator and appears to act cooperatively with Fyn in the activation of Syk and its downstream targets in a manner that is counteracted by Lyn.

Materials and Methods

Reagents

The sources were as follows: Dinitrophenyl (DNP)-specific monoclonal IgE and DNP-bovine serum albumin (BSA) from Sigma (St. Louis, MO); PP2 from Calbiochem (La Jolla, CA); ATP from ICN Biomedicals (Irvine, CA); siRNAs against Fgr (mouse or rat) from Dharmacon (Lafayette, CO); recombinant tyrosine kinases (Syk, Lyn, and Fgr) and antibodies against phosphotyrosine (pY) (4G10), SLP76, LAT, Gab2, the γ subunit of

[4]Abbreviations used in this paper: LAT, linkers for activation of T cells; SLP-76, Src-homology domain-containing leukocyte-specific phosphoprotein of 76 kDa; Gab2, growth-factor-receptor-bound protein 2-associated binding protein 2; LT, leukotriene; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenol; pY, phosphotyrosine; RBL, rat basophilic leukemia; BMMC, bone marrow-derived mast cells; IP, immunoprecipitation;; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein.

FcεRI, and Syk from Upstate Biotechnology (Lake Placid, NY); antibodies against Lyn, Fyn, Fgr, and myc-tag from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); antibodies against phosphorylated forms of Akt, ERK1/2, p38, JNK, and Syk from Cell Signaling Technology, Inc (Danvers, Mass); cell culture reagents from GIBCO/Invitrogen (Carlsbad, CA).

Culture and stimulation of RBL-2H3 cells and BMMCs

Rat basophilic leukemia (RBL)-2H3 cells were grown as monolayers in minimal essential medium with Earle's salts and supplemented with glutamine, antibiotics, and 15% fetal bovine serum (14). Cells were primed by incubating overnight with 25 ng/ml DNP-specific IgE in the same growth medium and were then stimulated with 25 ng/ml antigen (DNP-BSA) in a PIPES-buffered medium (25 mM PIPES at pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty-acid free fraction V from bovine serum) or in complete growth medium for measurement of production of TNF-α and LTC_4. Bone marrow-derived mast cells (BMMCs) were isolated from male wild type (WT) or $Fyn^{-/-}$ C57BL/6 mice according to the previously reported protocol (15) and were cultured in a 50% enriched growth medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal bovine serum) containing 10 ng/mL of IL-3. BMMCs were primed overnight with 25 ng/ml DNP-specific IgE in complete growth medium and were then stimulated with 25 ng/mL DNP-BSA in a Tyrode-BSA buffer (20 mM HEPES at pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% BSA).

RNA extraction and RT-PCR

Total RNA was isolated from RBL-2H3 cells or BMMCs by use of Trizol Reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR was performed at 94 °C for 45 seconds, 56 °C for 45 seconds, and 72 °C for 60 seconds for 30 cycles. The following primers were used: rat Lyn forward 5′-CCCTCAAGCCTGG CACCATGT-3′, reverse 5′-CCGAAGGACCACACGTCAGA-3′; rat Fgr forward 5′-G CTGAAGCCAGGCACCATGT-3′, reverse 5′-CCAAAGGACCACACGTCTGA-3′; rat GAPDH forward 5′-GTGGAGTCTACTGGCGTCTTC-3′, reverse 5′-CCAAGGCT GTGGGCAAGGTCA-3′; mouse Fgr forward 5′-CGCTGAAGCCGGGCACCATGT-3′, reverse 5′-CCAAAGGACCACACGTCTGA-3′; mouse Lyn forward 5′-CCCTCAAG CCCGGCACCATGT-3′, reverse 5′-CCGAAGGACCACACGTTA GA-3′; mouse GAPDH forward 5′-TGACGTGCCGCCTGGAGAAA-3′, reverse 5′-AGTGTAGCCC AAGATGCCCTTCAG-3′.

Transfection of cells with Lyn, Fgr, and Syk DNA constructs and siRNAs against Fgr

RBL-2H3 cells or BMMCs were transfected with DNA plasmids (10 μg DNA/5 × 10^6 cells, unless stated otherwise in the legend) by electroporating with the Amaxa nucleofector (Lonza Cologne AG, Cologne, Germany). Successful transfection was confirmed by immunoblot analysis and the cells were used within 48 h after transfection. For transfection of siRNAs against Fgr, RBL-2H3 cells or BMMCs (5 × 10^6 cells) were transfected with 10 μg siRNAs by the Amaxa nucleofector. siGENOME ON-TARGETplus SMARTpool targeting Fgr, containing four siRNA duplexes, or ON-TARGETplus siCONTROL non-targeting pool for control were purchased from Dharmacon (Lafayette, CO). The programs used for transfection with the Amaxa nucleofector were T-11 (RBL-H3 cells) or T-5 (BMMCs) in Dulbecco's modified Eagle Medium with 20% fetal bovine serum and 50 mM HEPES at pH 7.5. Cells were harvested 48 h after transfection and successful gene knockdown was confirmed by immunoblot analysis. Under these conditions, no reduction was observed in the level for any other Src-family kinase, apart from Fgr, or actin.

Immunoprecipitation and immunoblot analysis

IgE-primed mast cells were stimulated with 25 ng/ml DNP-BSA for 7 min or as indicated, chilled with ice to terminate stimulation, and then washed twice with ice-cold 1× PBS. The cells were lysed with ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and protease inhibitor cocktail tablet). Lysates were kept on ice for 30 min and then centrifuged 15,000 × g for 15 min at 4 °C. For immunoprecipitation (IP), the supernatant fraction was "precleared" by addition of 50 μl Protein G-agarose. The equal amount of protein was used for immunoprecipitation. Syk, LAT, SLP76, and other proteins were immunoprecipitated by overnight incubation (at 4°C with gentle rocking) with specific antibodies and, in turn, protein G–agarose. The agarose was washed 5 times with a washing buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2.5 mM nitrophenylphosphate, 0.7 μg/mL pepstatin, and protease inhibitor cocktail tablet) and dissolved in 2× Laemmli buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The primary antibodies were incubated at 4 °C overnight and then the immunoreactive proteins were detected by use of horse-radish peroxidase-coupled secondary antibody.

In vitro phosphorylation of Syk

Recombinant Syk (100 ng) was phosphorylated by 100–300 ng Lyn or Fgr in a reaction buffer (30 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 mM Na_3VO_4 and 20 μM ATP). The reaction mixture was incubated at 4 °C for 80 min, and terminated by an addition of 5× SDS sample buffer, and then boiled at 100 °C for 5 min. The supernatants were subjected to immunoblot analysis for detection of phosphorylation of Syk by use of antibody against phosphorylated tyrosine (pY). An equal amount of bovine serum albumin (BSA) was used as the negative control for Lyn and Fgr.

Measurement of degranulation and production of TNF-α and LTC_4

IgE-primed cells were stimulated with 25 ng/ml DNP-BSA for 15 min in the saline buffered solutions as described above for the measurement of degranulation. Otherwise cells were stimulated for 8 h in complete growth medium for measurement of TNF-α and LTC_4. Degranulation was determined by measurement of release of the granule marker, β-hexosaminidase, by use of a colorimetric assay in which release of *p*-nitrophenol from *p*-nitrophenyl-N-acetyl-b-D-glucosaminide is measured. Values were expressed as the percent of intracellular β-hexosaminidase that was released into the medium. TNF-α and LTC_4 were measured in medium by ELISA (Cayman Chemical Co., Ann Arbor, MI).

Confocal microscopy

The RBL-2H3 cells were cultured in Lab-Tek chambered coverslips (Nalge Nunc International, Naperville, IL). The cultures were fixed in 4% formaldehyde in PBS for 10 min, washed, and permeabilized with 0.5% Triton X-100 for 15 min. The fixed cells were blocked for 60 min with 1% BSA in PBS. The coverslips were incubated for 1 h with antibodies against Fgr or Flotillin-1, washed, and then incubated with Rhodamine Red-conjugated goat anti-rabbit IgG for Flotillin-1 or FITC-conjugated goat anti-mouse IgG for Fgr as the secondary antibody (Molecular Probes, Eugene, OR) for 45 min. The coverslips were washed and mounts prepared by use of the ProLonged Antifade Kit (Molecular Probes, Eugene, OR). Confocal images were taken in a Bio-Rad MRC-1024 confocal laser scanning microscope with an Apochromat 60× objective.

Knockdown of Fgr by injection of siRNAs and induction of PCA in mice

Preliminary experiments showed that the Fgr-specific siRNAs was effective both in cell culture and after injection into mice in reducing levels of Fgr. For the injection, the tail was immersed in warm water (40°C) for 10 seconds to dilate veins. Synthetic siRNAs (total 10 μg in 0.2 ml of PBS as indicated in the legend) was injected into the caudal vein in BALB/c mice (n = 7 per group) under mild anesthesia by using a 30G needle. The siRNA injection was repeated after 24 and 48 h, to achieve a significant Fgr gene-silencing effect. Then, PCA was induced as described previously (16). DNP-specific IgE antibody (0.5 μg per mouse) was injected intradermally into the right ear after the final injection of siRNAs and, on the next day, 250 μg DNP-BSA (antigen) in 250 μl PBS containing 4% Evans blue was injected intravenously. The mice were euthanized 1 h after treatment with the antigen, and the treated ear was excised to quantify the amount of dye extravasated into the ear tissue by antigen. The dye was extracted overnight from the ear in 700 μl formamide at 63°C. The absorbance of the dye was measured at 620 nm. The animal study was performed according to the institutional guidelines after obtaining approval from the Institutional Animal Care and Use Committee (IACUC) at Konkuk University.

Histological analysis of mast cells

For histological analysis, the skins excised from ears after the PCA experiments were fixed in 4% paraformaldehyde in PBS. Serial 5-μm-thick paraffin-embedded sections were stained with 0.1% toluidine blue in 1% sodium chloride for 1 min. Mast cell numbers in the ear skin were counted in 10 sections, derived from 5 mice, by three pathologists in a blind manner. Degranulated mast cells were identified from the presence of discharged granules around the cell. The percentage of degranulated cells was assessed on the basis of the number of cells with > 10% extruded granules as previously described (17). The representative images were taken by optical microscope (Olympus DP 70) at 100 × magnification.

Presentation of results

The data were presented as the means ± the SEM from 3 or more independent experiments. Statistical analysis was performed by using 1-way ANOVA and the Dunnett test. All statistical calculations (*$P < 0.05$ and **$P < 0.01$) were performed with SigmaStat software (Systat Software, Inc, Point Richmond, CA). All immunoblots are representative images as well as the indicated average density of bands are from at least three independent experiments.

Results

Fgr associates with FcεRI and phosphorylates Syk in antigen-stimulated mast cells

Lyn and Fgr mRNA and protein were expressed in RBL-2H3 cells (Fig. 1A). Fgr was mostly colocalized with flotillin-1 (Fig. 1B), which is a typical lipid raft marker of the plasma membrane (18), and phospholipase D2 (data not shown) in RBL-2H3 cells when viewed by confocal microscopy. Moreover, Fgr co-immunoprecipitated with the β subunit of FcεRI and this apparent association was significantly enhanced when RBL-2H3 cells were stimulated with antigen. Such enhancement was time dependent and reached a maximum within one to three min after the addition of antigen (Fig. 1C).

We next examined whether Syk was a downstream target for Fgr. Antigen-induced tyrosine-phosphorylation of Syk was enhanced in RBL-2H3 cells made to overexpress Lyn and, to an even greater extent, in cells made to overexpress Fgr in a dose-dependent manner (Fig. 1D).

Knockdown of Fgr inhibits Syk, Syk-dependent phosphorylation events, and degranulation in RBL-2H3 cells and BMMCs

Knockdown of Fgr with siRNAs in RBL-2H3 cells (Fig. 2A, the upper panel) resulted in substantially reduced antigen-induced phosphorylation of Syk and LAT as well as other downstream signaling molecules including Akt, ERK1/2, p38, and JNK (Fig. 2A, the lower panel). It should be noted that phosphorylation of Akt is dependent on Syk (19) and the phosphorylation of MAP kinases, at least in part, on LAT (20). Antigen-induced degranulation was also suppressed (Fig. 2B). RBL-2H3 cells express a constitutively activated Kit mutant (21) which facilitates FcεRI-mediated signals (1). For this reason similar studies were conducted with primary cultures of mouse BMMC to eliminate a background Kit activation. BMMC express both Lyn and Fgr mRNA and protein (Fig. 2C). Knockdown of Fgr with siRNAs (Fig. 2D) resulted in a corresponding decrease of antigen-induced phosphorylation of Syk (~70%) as compared to control cells that were transfected with a control siRNAs. Degranulation was also reduced by about 60% (Fig. 2E).

The overexpression of Fgr stimulates Syk, Syk-dependent signaling molecules, and degranulation in RBL-2H3 cells and BMMCs

Overexpression of Fgr or Syk enhanced the antigen-induced phosphorylation of several downstream targets of Syk including LAT, SLP76, and Gab2 as compared to vector transfected RBL-2H3 cells (Fig. 3A). These phosphorylations were further enhanced in an additive or even synergistic manner by coexpression of both kinases (Fig. 3A). These phosphorylations are thought to be essential for degranulation and indeed degranulation was enhanced by overexpression of either Syk or Fgr and more so by overexpression of both kinases (Fig. 3B). In addition, overexpression of either Src kinase enhances the phosphorylation of Syk in antigen-stimulated BMMCs (Fig. 3C). As in RBL-2H3 cells, overexpression of Syk or Fgr augment degranulation and this effect is more pronounced on coexpression of both proteins (Fig. 3D). Collectively, the data strongly suggest that Fgr was critical for propagation of FcεRI-mediated signaling and degranulation.

Knockdown of Fgr reduces secretion of tumor necrosis factor (TNF)-α and leukotriene (LT) C_4

The role of Fgr on production of TNF-α and LTC_4 was investigated in antigen-stimulated RBL-2H3 cells (Fig. 4A and 4B) and BMMCs (Fig. 4C and 4D). The release of both mediators was significantly inhibited by the knockdown of Fgr. These results indicated that Fgr is critical for the production of TNF-α and LTC_4 as well as degranulation.

The positive regulation of mast cell activation by Fgr is negated by Lyn but is reinforced by Fyn

Syk phosphorylation is augmented in antigen-stimulated RBL-2H3 cells by overexpression of Lyn and to a greater extent by Fgr (Fig. 5A). However, co-expression of Lyn and Fgr reduced the extent of phosphorylation to levels that were only slightly greater than that of Lyn alone (Fig. 5A, quantitative data are shown in the lower panel). This counteracting effect of Lyn on Fgr was also apparent in the degranulation response to antigen (Fig. 5B). Overexpression of Fyn also augmented stimulation of Syk phosphorylation by antigen (Fig. 5C). However in contrast to Lyn, Fyn acted cooperatively with Fgr when both were expressed together in RBL-2H3 cells (Fig. 5C, lower panel shows quantitative data). In addition, overexpression of Fyn and Fgr augmented degranulation in antigen-stimulated cells (Fig. 5D).

Restoration of Syk phosphorylation and degranulation in Fyn-deficient BMMCs by overexpression of Fgr or Fyn

Although Fyn is a positive regulator of degranulation, some residual degranulation was evident in Fyn-deficient BMMC (Fig. 6A) as noted in previous reports (8, 22). These findings suggest that another or other enzymes act cooperatively with Fyn in promoting degranulation. To determine whether Fgr is such a candidate, expression of Fgr was reduced by knockdown with the Fgr siRNAs in Fyn-deficient mast cells. Antigen-induced degranulation was further, at least partially, diminished in these cells when compared to the original Fyn-deficient mast cells (Fig. 6A). Fyn-deficient cells also exhibit a partial reduction of tyrosine phosphorylation of Syk and Gab2 by antigen (data not shown) as previously reported (23). Finally, we checked whether the impaired responses to antigen in Fyn-deficient cells could be restored in Fyn-deficient cells. Overexpression of Fyn or Fgr mostly restored phosphorylation of Syk (Fig. 6B) and partially restored degranulation in response to antigen (Fig. 6C). The reason for the partial restoration of degranulation by Fgr, or for that matter Fyn, is unclear as coexpression of both kinases did not fully restore degranulation (data not shown but similar to Fyn and Fgr DNA-transfected cells in Fig. 6C).

Fgr is critical for recruitment of Syk to the phosphorylated γ subunit of FcεRI in antigen-stimulated cells and also phosphorylates Syk in vitro

Finally, to ascertain the mechanism of action of Fgr in mast cells, we tested whether recombinant Fgr directly phosphorylated Syk in vitro and if knockdown of Fgr exerted any effect on the recruitment of Syk to FcεRI on antigen-stimulated mast cells. Knockdown of Fgr by siRNAs drastically suppressed the association of Syk with the γ subunit of FcεRI, most probably, by the inhibition of phosphorylation of the γ subunit of FcεRI (Fig. 7A). *In vitro*, recombinant Lyn and recombinant Fgr appeared to be equi-potent in their ability to phosphorylate Syk in the presence of ATP (Fig. 7B). Some autophosphorylation of Syk was evident when BSA was used as a control but the additional phosphorylation was clearly evident in the presence of these Src-family kinases.

The knockdown of Fgr by siRNAs inhibits PCA reaction in mice and mast cell degranulation in ear tissues

To assess the relevance of Fgr in mast cell activation in vivo, we tested whether knockdown of Fgr by the injection of siRNAs against Fgr suppressed the IgE-mediated PCA in mice. The protein level of Fgr was substantially diminished in lung, liver, and ear (Fig. 8A) and the IgE-induced PCA reaction was also inhibited in a dose dependent manner (Fig. 8B and 8C). The potency of Fgr siRNA injection at the dose of 10 μg per mouse was comparable to that by oral administration of 20 mg/kg of the antihistamine cetirizine as a reference drug. Next, we tested whether the knockdown by the Fgr-siRNAs suppressed the local degranulation of mast cells in ear tissues. The degranulation of mast cells in ear tissues was significantly suppressed by injection of Fgr siRNAs, but not by injection of control siRNAs, into mice (Fig. 8D and 8E).

Discussion

Lyn was the initial focus of early studies of the Src-family kinases in mast cells where Lyn was found to be associated with FcεRI. It became apparent that following aggregation of FcεRI, the trans-phosphorylation of ITAMs in adjacent FcεRIβ and γ subunits by Lyn results in recruitment of additional Lyn and Syk molecules (11, 24, 25). These kinases then phosphorylated downstream adaptor/docking proteins, including LAT1 and LAT2, which facilitate assembly of other signaling molecules for propagation of signals through several pathways (26). However, it is now recognized that Lyn also exerts a negative regulatory role on some activation mechanisms in mast cells (27 – 30). These are manifested by enhanced

FcεRI-dependent allergic reaction in $Lyn^{-/-}$ mice (5), enhanced cytokine production in $Lyn^{-/-}$ mast cells (27), and reduced degranulation in mast cells expressing constitutively active Lyn (30).

Fyn is reported to initiate additional signals for degranulation that complement those mediated by Lyn. These include the phosphorylation of Gab2 and in turn activation of PI3K (8) as well as the Ca^{2+}-independent microtubule formation that is required for translocation of granules to the membrane (31). However, the positive actions of Fyn are counteracted by Lyn (5, 28) possibly because of the ability of Lyn to phosphorylate the transmembrane Csk-binding protein (Cbp) which can then recruit Csk to the plasma membrane and thereby permit phosphorylation of a C-terminal inhibitory site on Fyn by Csk (32, 33). Nevertheless, other workers have concluded on the basis of siRNA knock down experiments that Fyn has a relatively minor role in regulating FcεRI-mediated reactions (34).

In addition to Fyn, Hck has also been proposed as a positive regulator of FcεRI-mediated mast cell activation as Hck-deficient BMMC exhibit impaired signaling, degranulation, and cytokine production (10). The positive effects of Hck appeared to be mediated in part through suppression of the inhibitory actions of Lyn as indicated by use of various genetic deletion models. Hck, like Lyn, has the capability of associating with Cbp but, contrary to the situation with Lyn and Fyn, Hck is presumed to inactivate Lyn via Csk (10).

The studies described above thus suggest a complex hierarchy of regulatory mechanisms upstream of Syk that involve multiple Src-family kinases and negative feed-back via Csk (10) in which Lyn may inhibit Fyn and Fgr (this paper) and Hck inhibits Lyn. Fgr-mediated phosphorylation of Syk in antigen-stimulated mast cells (Fig. 1D) was significantly reduced by overexpression of Lyn (Fig. 5A). The studies to date point to different, perhaps complementary roles for Fyn, Hck, and Fgr even though the effects of their deletion or knock down on mast cell responses might be similar. These roles could be a necessary reinforcement of common signaling pathways that permit fine tuning of signals towards specific functional end points such as degranulation, cytokine production, and cell survival in a manner that, as in the case of Lyn, might depend on strength of signal (6). Syk in turn appears to play a dominant role in the phosphorylation of adaptor proteins which enable assembly and activation of downstream signaling molecules and pathways (35). However, this topic merits further investigation as no clear differences have emerged from the studies of the Src-family kinases in mast cells except for the dual role of Lyn.

Of note, Fgr substantially enhanced phosphorylation of Syk either when overexpressed in cell cultures (Fig. 1D) or with recombinant protein in an *in vitro kinase* assay (Fig. 7B). Furthermore, knockdown of Fgr with siRNAs markedly inhibited the association of Syk with the γ subunit of FcεRI, most probably, through the inhition of phosphorylation of the γ subunit (Fig. 7A). These results indicate that Fgr could directly phosphorylate Syk or stimulate Syk through phosphorylation of the γ subunit of FcεRI. However, it needs to be determined whether or how Lyn and Fgr differentially phosphorylate the various regulatory tyrosine residues of Syk (15) or relay different signals through the ITAMs of FcεRI. Regardless of these uncertainties, our data clearly demonstrate that Fgr plays an essential positive role in Syk-dependent signaling events in antigen-stimulated mast cells. This is in addition to its previously demonstrated ability to directly phosphorylate and activate phospholipase D which is also critical for mast cell degranulation (15).

Based on the significant effect of Fgr in antigen-stimulated mast cells, we further investigated the effect of Fgr on IgE-mediated hypersensitive reaction in vivo. The PCA has been well established as animal model for IgE-mediated hypersensitivity. To take advantage of this model we used RNA-interference technology to obtain Fgr-knockdown mice because

Fgr-deficient mice are not available. The injection of siRNAs not only substantially reduced the protein levels of Fgr in lung, liver, and ear (Fig. 8A) but also the PCA in a dose-dependent manner (Fig. 8B and 8C). An incidental finding was that the PCA reaction was also associated with an increase in mast cell number in the targeted ear in the mice by stimulating with antigen (Fig. 8E and see reference 36). Although it is unlikely that this could be attributed to formation of additional mast cells from progenitor cells because of the relatively short period after injection of antigen (i.e. 1 h), mast cell migration and survival may be enhanced by antigen activation (37). Infiltration of basophils is also an unlikely confounding factor (38). However, previous studies have shown that mast cells mediate immediate phase reactions to antigen in mouse ear (39, 40) and only the delayed or chronic phase of swelling one or two days later is dependent on the subsequent infiltration of basophils (40).

It is now recognized that mast cells are the primary effector cells for the acute phase of IgE-associated allergic disorders (39, 41). Mast cells also appear to be essential for the acute IgE-induced PCA reaction in mouse ears, the experimental model used in our study, as this reaction does not occur in mast cell-deficient WBB6F1-W/Wv or WCB6F1-S1/S1d mice (39). Our finding that the degranulation of mast cells in ear tissues was suppressed by injection of Fgr siRNAs in mice (Fig. 8D and 8E), suggests that Fgr in mast cells is responsible for mediating the PCA reaction. However, our results do not exclude the possibility that Fgr in other types of cells, such as basophils, in ear also contribute partly to the PCA response.

In summary, we report that in addition to the Src-family kinases Lyn, Fyn, and Hck, mast cells also express Fgr which is functionally responsive to antigen stimulation. Although the actions of Fgr resemble those of Fyn, both kinases appear to play necessary roles in mast cell activation. Endogenous Fgr not only associates with FcεRI in stimulated cells but also regulates the phosphorylation of Syk and its downstream targets as indicated by knockdown or overexpression of Fgr in RBL-2H3 cells and BMMCs. Direct targets of Syk include LAT, SLP76 and Gab2 and indirect targets may include Akt and the MAP kinases. Fgr also appears to have functional impact on degranulation and the production of eicosanoids and cytokines. Therefore, Fgr positively regulates mast cell through direct or indirect stimulation of Syk and is critical for IgE-mediated allergic response and degranulation of mast cells in vivo. The emerging complexity in the interplay of Src-family kinases in mast cells may provide therapeutic discrimination if this interplay among the kinases differs significantly from that found in other types of immunological cells.

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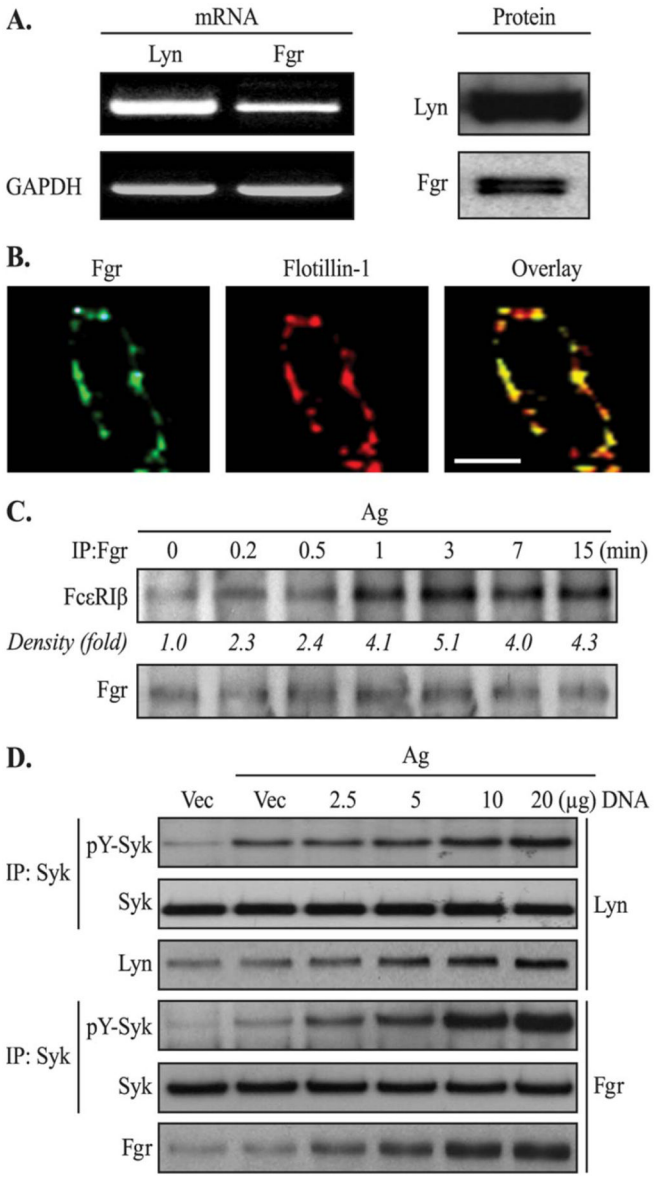

FIGURE 1.

Fgr is associated with the plasma membrane, interacts with FcεRI, and phosphorylates Syk in RBL-2H3 cells. *A*, *left panel*, Expression of message for Lyn, Fgr, and GAPDH was determined by RT-PCR. *A*, *right panel*, Whole cell lysates were subjected to immunoblot analysis. *B*, Fgr and Flotillin-1 were visualized in RBL-2H3 cells by confocal microscopy: scale bar, 10 μm. *C*, Fgr was immunoprecipitated (IP) and subjected to immunoblot analysis in RBL-2H3 cells stimulated with antigen (Ag). *D*, RBL-2H3 cells were transiently transfected with DNA constructs encoding vector (Vec), Lyn or Fgr as indicated. The cells were stimulated by antigen (Ag) or not. The level of overexpressed Lyn, Fgr or immunoprecipitated (IP) Syk from whole cell lysates were subjected to immunoblot analysis. Representative results from three or more independent experiments are shown.

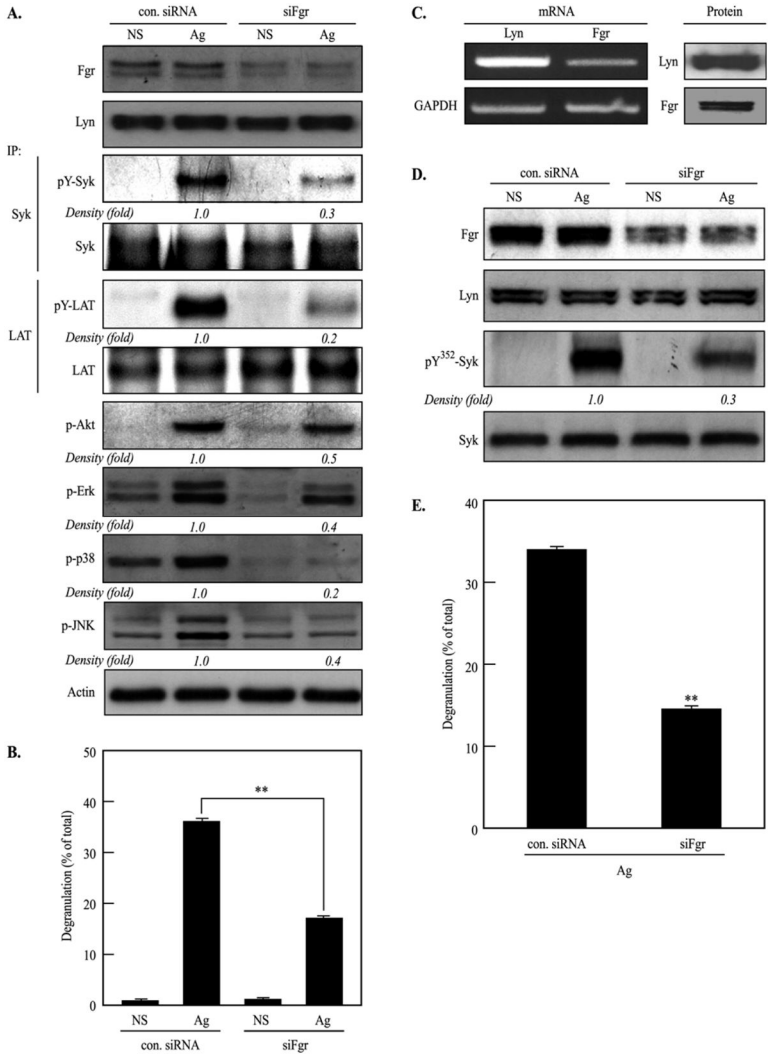

FIGURE 2.

Knockdown of Fgr suppresses Syk, Syk-dependent signals, and degranulation. RBL-2H3 cells (*A* and *B*) or BMMCs (*D* and *E*) were transiently transfected with siRNAs directed against Fgr (siFgr), or with the control siRNAs (con. siRNA), respectively. The cells were stimulated with antigen (Ag) or not stimulated (NS) for immunoblot analysis and measurement of degranulation. Immunoprecipitates (IP) for Syk and LAT or whole cell lysates were subjected to immunoblot analysis (*A* and *D*). *C*, *left panel*, Expression of message for Lyn, Fgr, and GAPDH was determined by RT-PCR in BMMCs. *C*, *right panel*, Whole cell lysates from BMMCs were subjected to immunoblot analysis. Representative images from three independent experiments are shown. (*B* and *E*) Cells were stimulated with antigen (Ag) for 15 min or not (NS) to measure release of the granule marker, β-hexosaminidase. Values for degranulation are the means ± SEM of values from three independent experiments: $^{**}P < 0.01$.

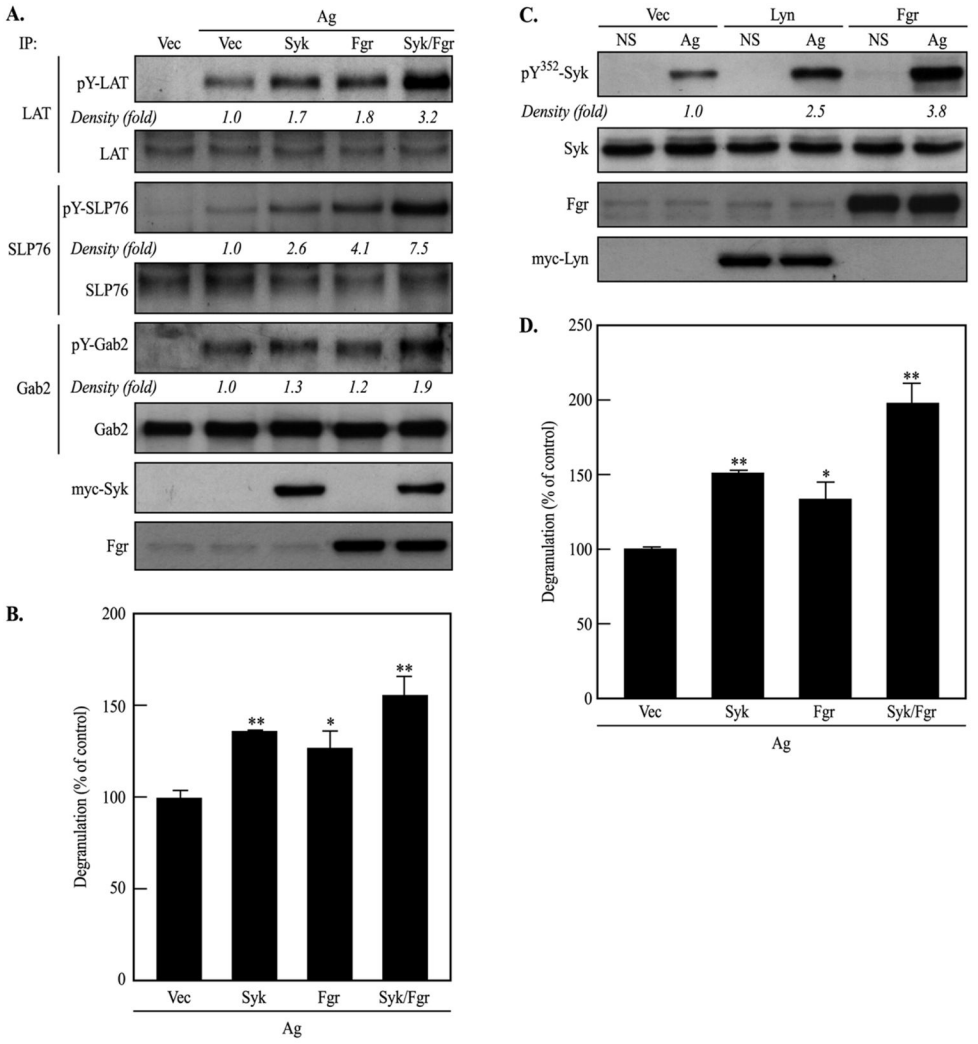

FIGURE 3.

Overexpression Fgr stimulates Syk-dependent signals and degranulation. RBL-2H3 cells (*A–B*) or BMMCs (*C–D*) were transiently transfected with DNA constructs encoding myc-Syk, Lyn, Fgr, or vector (Vec). *A* and *C*, Cells were stimulated with antigen (Ag) for 7 min or not stimulated (NS). Immunoprecipitates (IP) for LAT, SLP76, and Gab2 or whole cell lysates were subjected to immunoblot analysis. Representative results from three independent experiments are shown. *B* and *D*, Degranulation was determined. Values are the means ± SEM of values from three independent experiments: $*P < 0.05$ and $**P < 0.01$.

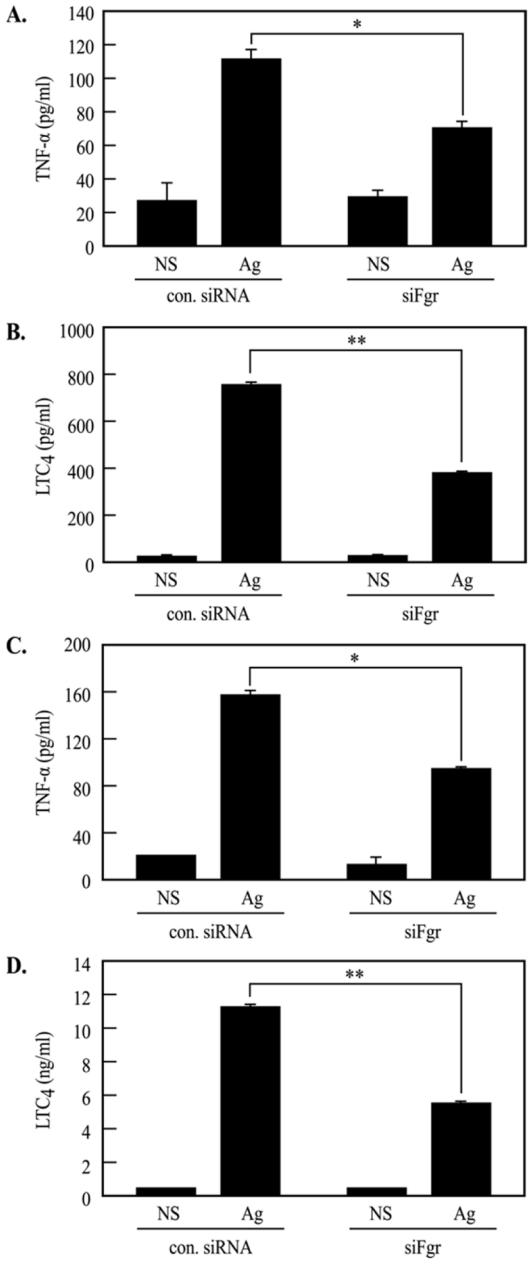

FIGURE 4.

Transfection of the Fgr siRNAs impairs production of TNF-α and LTC_4. RBL-2H3 cells (*A* and *B*) or BMMCs (*C* and *D*) were transfected with Fgr siRNAs (siFgr), or the control siRNAs (con. siRNA). IgE-primed cells were stimulated with antigen (Ag) for 8 h or not stimulated (NS) for analysis of TNF-α or LTC_4 in culture media by ELISA. Values are the means ± the SEM of values from three independent experiments: $*P < 0.05$ and $**P < 0.01$.

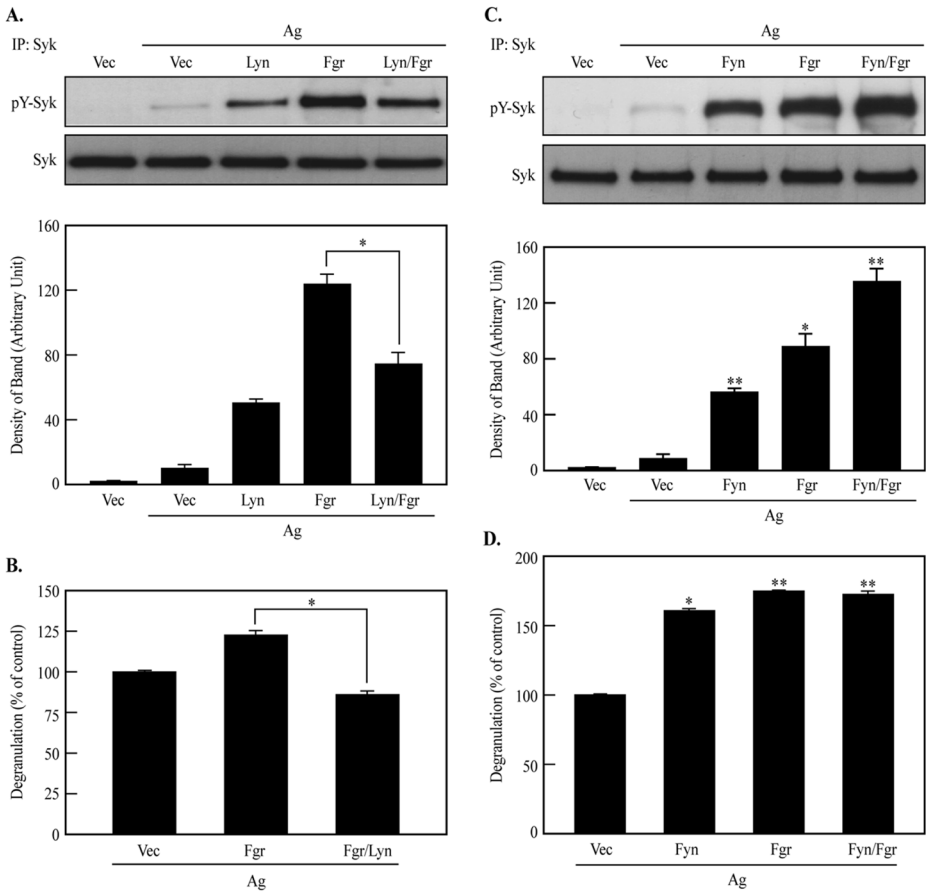

FIGURE 5.

Positive role of Fgr on phosphorylation of Syk is negated by overexpression of Lyn, but reinforced by Fyn. RBL-2H3 cells were transfected with individual or a combination of plasmids at suboptimal dose (3 μg each) encoding Lyn, Fgr, Fyn or Vector (Vec). *A* and *C*, IgE-primed cells were stimulated with antigen (Ag) for 7 min or not stimulated. Immunoprecipitates (IP) for Syk were analyzed by immunoblot analysis. Representative images (upper panels) and density of bands for phoshorylated Syk (lower panels) are the mean ± SEM from three independent experiments. *B* and *D*, IgE-primed cells were stimulated with antigen (Ag) for 15 min to measure degranulation. Values are the means ± the SEM of values from three independent experiments: **P* < 0.05 and ***P* < 0.01.

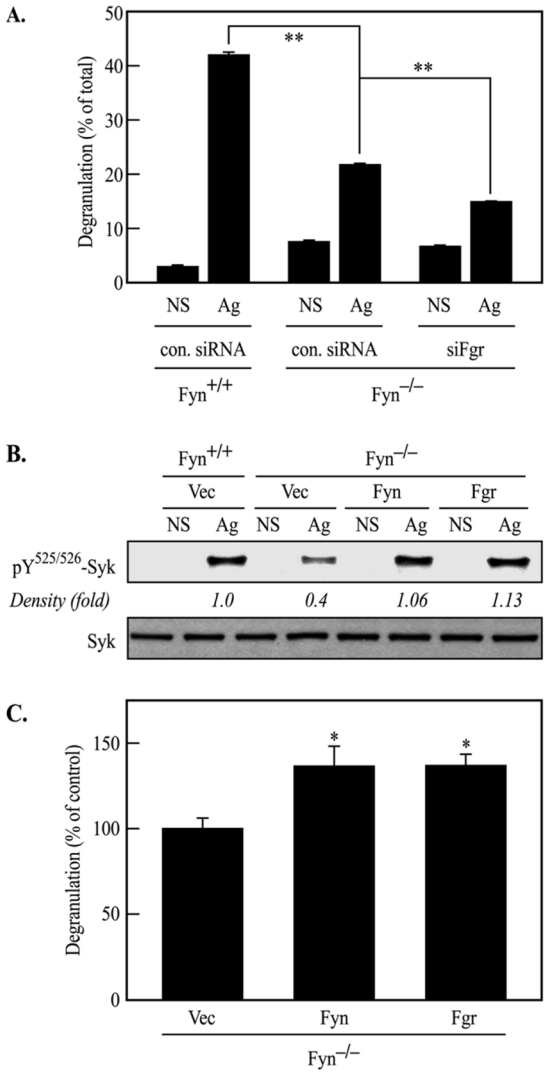

FIGURE 6.

Overexpression of Fgr or Fyn restores phosphorylation of Syk and partly so degranulation in Fyn-deficient cells. *A*, BMMCs isolated from $Fyn^{+/+}$ or $Fyn^{-/-}$ C57BL6 mice were transfected with Fgr siRNAs (siFgr) or the control siRNAs (con. siRNA). IgE-primed cells were stimulated with antigen (Ag) for 15 min or not stimulated (NS) to measure degranulation. *B*, $Fyn^{+/+}$ or $Fyn^{-/-}$ BMMCs were transfected with vector (Vec), Fgr, or Fyn DNA as indicated. Cells were primed with IgE and then stimulated with antigen (Ag) for 7 min or not (NS). Whole cell lysates were subjected to immunoblot analysis for detection of phosphorylated forms of Syk. Representative results from three independent experiments are shown. *C*, $Fyn^{-/-}$ BMMCs were transfected with vector (Vec), Fgr or Fyn DNA. IgE-primed cells were stimulated with antigen for 15 min to measure degranulation. Values are the means ± the SEM of values from three independent experiments: $*P < 0.05$ and $**P < 0.01$.

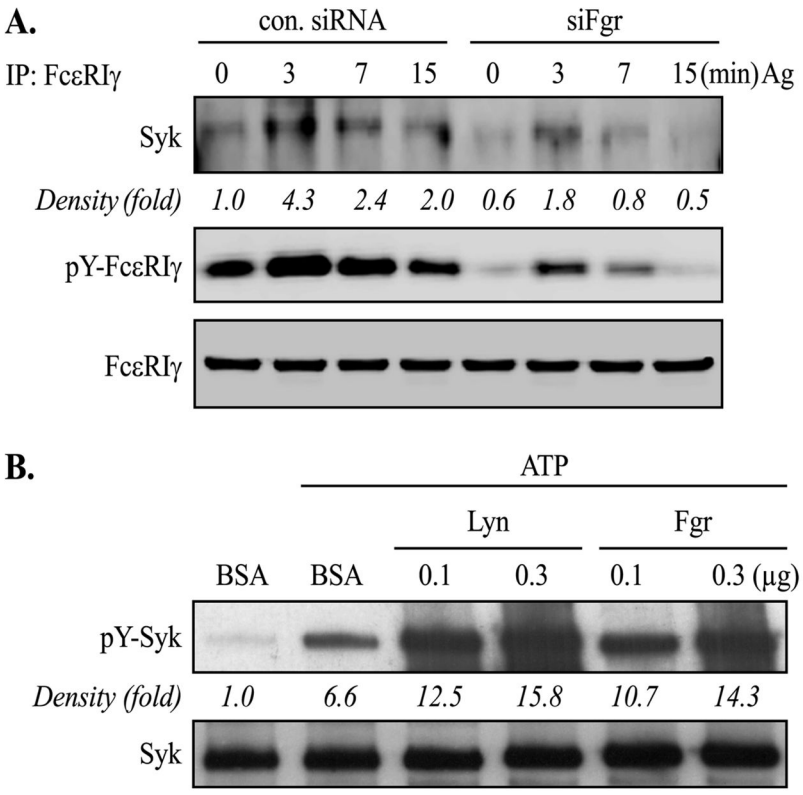

FIGURE 7.

Fgr phosphorylates Syk in vitro and is also critical for recruitment of Syk to the γ subunit of FcεRI in antigen-stimulated cells. *A*, BMMCs were primed with IgE and then stimulated with antigen (Ag) for indicated times. The γ subunit of FcεRI (FcεRIγ) was immunoprecipitated (IP) from cell lysates with anti-FcεRIγ antibody, and precipitated proteins were subjected to immunoblot analysis with antibodies against Syk, FcεRIγ, and phosphotyrosine residues (pY) (4G10). *B*, Recombinant Syk was incubated with 100–300 ng of Lyn, Fgr, or 300 ng of bovine serum albumin (BSA) as a control protein in a kinase reaction buffer. Phosphorylated Syk was measured by immunoblot analysis. Representative images were obtained from three independent experiments.

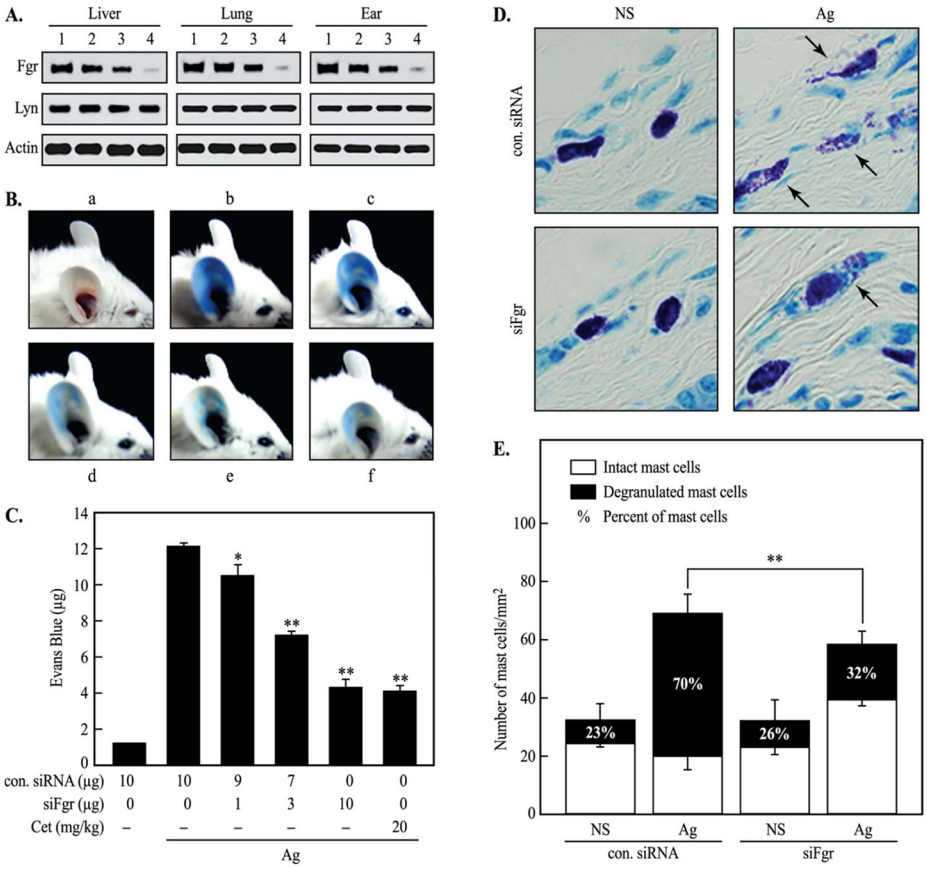

FIGURE 8.

Fgr is critical for IgE-mediated PCA reaction and degranulation in mice. The Fgr siRNAs (siFgr) that were used in vitro (Fig. 2D) were injected into BALB/c mice three times every 24 h before assessing the levels of Fgr protein in tissues and the PCA reaction as described in “Materials and Methods”. *A*, Extracts from liver, lung, and ear were subjected to Western blotting analysis: 1, control (con.) siRNAs (10 μg); 2, siFgr (1 μg) + con. siRNAs (9 μg); 3, siFgr (3 μg) + con. siRNAs (7 μg); 4, siFgr (10 μg). Results are typical from at least three independent experiments. *B*, Representative photographs of ears (a, con. siRNAs (10 μg) without Ag; b, con. siRNAs (10 μg) with Ag; c, siFgr (1 μg) + con. siRNAs (9 μg) with Ag; d, siFgr (3 μg) + con. siRNAs (7 μg) with Ag; e, siFgr (10 μg) with Ag; f, Cetrizine (Cet, 20 mg/kg) with Ag). *C*, Quantitative data for ear-tissue content of Evans blue are expressed as the mean ± SEM of values from three independent experiments, each with 10 mice. The asterisks indicate significant difference from antigen (Ag)-stimulated controls with con. siRNAs (*$p < 0.05$; **$p < 0.01$). Cetirizine was orally administered 1 h before the antigen injection as a typical anti-histamine reference drug. *D*, Control siRNA or siFgr were administered (10 μg of each) as for panel B and the ear skins were prepared for histological examination as described in “Materials and Methods”. Representative histological images are shown: arrows indicate degranulated mast cells. *E*, The histograms show the percentage of degranulated mast cells in the given number of mast cells in ear skin sections. Values for degranulated mast cells are expressed as percent of total number of mast cells and are the means ± the SEM of values from three independent experiments: **$P < 0.01$.