Fer and Fps/Fes Participate in a Lyn-dependent Pathway from FcεRI to Platelet-Endothelial Cell Adhesion Molecule 1 to Limit Mast Cell Activation*

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Mast cells express the high affinity IgE receptor FcεRI, which upon aggregation by multivalent antigens elicits signals that cause rapid changes within the mast cell and in the surrounding tissue. We previously showed that FcεRI aggregation caused a rapid increase in phosphorylation of both Fer and Fps/Fes kinases in bone marrow-derived mast cells. In this study, we report that FcεRI aggregation leads to increased Fer/Fps kinase activities and that Fer phosphorylation downstream of FcεRI is independent of Syk, Fyn, and Gab2 but requires Lyn. Activated Fer/Fps readily phosphorylate the C terminus of platelet-endothelial cell adhesion molecule 1 (Pecam-1) on immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and a non-ITIM residue (Tyr685) in vitro and in transfected cells. Mast cells devoid of Fer/Fps kinase activities display a reduction in FcεRI aggregation-induced tyrosine phosphorylation of Pecam-1, with no defects in recruitment of Shp1/Shp2 phosphatases observed. Lyn-deficient mast cells display a dramatic reduction in Pecam-1 phosphorylation at Tyr685 and a complete loss of Shp2 recruitment, suggesting a role as an initiator kinase for Pecam-1. Consistent with previous studies of Pecam-1-deficient mast cells, we observe an exaggerated degranulation response in mast cells lacking Fer/Fps kinases at low antigen dosages. Thus, Lyn and Fer/Fps kinases cooperate to phosphorylate Pecam-1 and activate Shp1/Shp2 phosphatases that function in part to limit mast cell activation.

Mast cells express the high affinity IgE receptor FcεRI, which is composed of an IgE-binding α-chain, a tetramembrane spanning β chain, and a dimeric γ chain (1). Signals are transduced via immunoreceptor tyrosine-based activation motifs (ITAMs)3 that are present in both the β and γ subunits and serve as docking sites for the recruitment of signaling molecules (2). FcεRI signaling is initiated by binding of IgE, which is sufficient for induction of survival pathways as well as cytokine production (3, 4). Some highly cytokinergic IgEs can induce antigen-independent degranulation, survival, adhesion, and chemotaxis of mast cells (5). However, in most cases, aggregation of FcεRI by multivalent antigens is required for a full mast cell response including degranulation, lipid mediator release, increased cell adhesion, and increased motility (6). The Src family protein-tyrosine kinase (PTK) Lyn is constitutively associated with FcεRI (7), and upon antigen-mediated clustering of receptor chains, Lyn phosphorylates ITAMs on β- and γ-chains. The β-chain ITAMs are thought to recruit additional Lyn and Fyn kinases, the p85 subunit of phosphatidylinositol 3-kinase, SH2-containing inositol 5′-phosphatase, and Shp2 phosphatase (8). Phosphorylated ITAMs on the γ-chains recruit the dual SH2 domain-containing PTK Syk (9). Syk activity is essential for signal transduction downstream of FcεRI, because Syk-deficient mast cells fail to degranulate, synthesize leukotrienes, and secrete cytokines following antigen challenge (10). Although FcεRI-induced tyrosine phosphorylation is greatly reduced in Syk-deficient mast cells, phosphorylation of the receptor ITAMs and Lyn are maintained (10). Lyn-deficient mast cells, despite severely reduced tyrosine phosphorylation and delayed calcium flux, are able to degranulate and secrete cytokines (11). In fact, Lyn-deficient mast cells release more of the granule constituent β-hexosaminidase than do wild type mast cells. Further studies have shown that multiple responses to FcεRI aggregation are delayed in Lyn-deficient mast cells, including tyrosine phosphorylation of receptor subunits, calcium flux, and phosphatidylinositol 3,4,5-trisphosphate production but persist far longer than in wild type mast cells (12). Other notable characteristics of Lyn-deficient mast cells are that Fyn kinase is hyperactivate, whereas SH2-containing inositol 5′-phosphatase is completely inactive (12). Thus, in addition to initiating signaling downstream of FcεRI, Lyn is also involved in signal termination at least partly through activation of SHZ-containing inositol 5-phosphatase, which hydrolyzes phosphatidylinositol 3,4,5-trisphosphate and thereby reduces the plasma membrane localization of pleckstrin homology domain-containing proteins. The pleckstrin homology

pY, phosphotyrosine; DNP, dinitrophenyl; FITC, fluorescein isothiocyanate; IL, interleukin; WT, wild type.
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domain-containing adaptor protein Gab2 (Grb2-associated binding-2), is required for phosphatidylinositol 3-kinase activation (13) and, together with Fyn, contributes to activation of RhoA, microtubule formation, and delivery of granules to the plasma membrane (14).

FceRI aggregation also leads to phosphorylation of platelet-endothelial cell adhesion molecule (Pecam-1) on ITIMs that recruit Shp1 and Shp2 phosphatases (15). Both Pecam-1 and Shp1 have been shown to negatively regulate FceRI-triggered degranulation, although the mechanism has not been established (16, 17). Pecam-1 knock-out mice display hypersensitivity to challenge with lipopolysaccharide (or endotoxin) (18–20). This has been attributed to impaired signal transducer and activator of transcription 3 (STAT3) phosphorylation in endothelial cells and lymphocytes and elevated production of inflammatory cytokines in Pecam-deficient mice. Phosphorylation of a non-ITIM tyrosine (Tyr701) in human Pecam-1 allows recruitment of STAT3 or STAT5 via their SH2 domains (19). While tethered to Pecam-1, STAT3 phosphorylation is likely regulated by a Pecam-1-associated kinase or Shp2 phosphatase (21).

We recently showed that Fps and Fer kinases are both activated within 1 min of FceRI aggregation (22). Although the mechanism by which FceRI aggregation leads to Fps and Fer activation is unknown, one or more of Lyn, Fyn, and Syk are likely required, because these are the earliest signaling molecules activated upon FceRI aggregation. Here, we report that FceRI aggregation leads to elevated Fer and Fps kinase activities. Phosphorylation of Fer and Fps downstream of FceRI is independent of Syk, Fyn, and Gab2 but requires Lyn kinase for rapid activation. Once activated, Fer and Fps can phosphorylate both ITIMs and a non-ITIM residue (Tyr703) in Pecam-1. In mast cells devoid of Fer/Fps kinase activities, the overall phosphorylation of Pecam-1 is reduced, whereas recruitment of Shp1/Shp2 is unaffected. In contrast, Lyn-deficient mast cells display a significant defect in Pecam-1 phosphorylation and Shp2 recruitment. Similar to previous studies on Pecam-1- and Shp1-deficient mast cells, we observe a hyperdegranulation response in Fer/Fps-deficient mast cells at low antigen dosages. Thus, a Lyn/Fer/Fps pathway from FceRI to Pecam-1/Shp1 functions in limiting mast cell activation.

EXPERIMENTAL PROCEDURES

Materials—Strain-matched wild type and transgenic mice used in this study have been described: ferDR/DR (DR) (23), fpsKR/KR ferDR/DR (KR/DR) (24), Lyn knock-out (25), Fyn knock-out (26), Gab2 knock-out mice were generated by Gen-Sheng Feng (The Burnham Institute; to be described elsewhere).

Antibodies used in this study include: anti-Fer rabbit polyclonal (FerLA) (27), anti-Fs/Fer/Fes rabbit polyclonal (FpsQE) (27), anti-phosphotyrosine (pY) monoclonal antibody P799 (Santa Cruz), anti-Gab2 (Upstate Biotechnology, Inc.), anti-Lyn (AR/1; kindly provided by Joan Brugge), goat anti-Pecam-1CT (M20, Santa Cruz), rat anti-Pecam-1NT (clone 390, BD Biosciences), rabbit anti-pY686 human Pecam-1 (cross-reacts with Tyr(P)685 of mouse Pecam-1; kindly provided by Peter and Debra Newman), anti-GST monoclonal antibody (Santa Cruz), anti-Phospho-Tyr701 (BD Transduction Labs) and polyclonal antibody (Santa Cruz). Phycoerythrin-conjugated CD117 (c-Kit; BD Biosciences), and fluorescein-conjugated anti-IgE (Southern Biotech) were used along with isotype controls to assess the maturity of BMMCs by flow cytometry.

Pecam-1 Expression Plasmids—Bacterial expression constructs were generated using glutathione S-transferase (GST) fusions to the cytoplasmic tail of Pecam-1 by amplification of the desired sequences from either plasmid DNAs or from cDNA pools from BMMCs generated by reverse transcription-PCR. The full-length Pecam-1 C-tail (with and without ITIM mutations) was amplified from mouse Pecam-1 expression plasmids provided by Andre Veillette (Institut de Recherches Cliniques, Montreal, Canada) (28). The Δ14/15 isoform was derived from an IMAGE clone (Open Biosystems), and the Δ45/15 isoform was cloned from BMMC cDNA. GST fusion proteins were purified from transformed BL21 using glutathione-conjugated beads according to the manufacturer’s instructions (Amersham Biosciences).

A point mutation in exon 15 (Y700F) was made in the Pecam-1 cDNA (wild type and Y662F) using a QuickChange mutagenesis kit (Stratagene) and the following primers: 5′-AATCTCATGAAAAACAGATTCTCGAGAAACGGAAGGC-TCCC-3′ and 5′-AGGCTTTCCGTTCGAGAAATCTGTTTCCATGAGATTAGG-3′.

BMMC Cultures—Femurs were isolated aseptically from 4–8-week-old, strain-matched wild type and transgenic or knock-out mice, and bone marrow cells were isolated by repeated flushing with BMMC medium (Iscove’s modified Dulbecco’s medium, 10% (v/v) fetal bovine serum, 1% (v/v) antimicrobial-antimycotic solution (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1% (v/v) nonessential amino acids (Invitrogen), 1% (v/v) conditioned medium from X63-IL-3 cells (32) (kindly provided by Rob Rotapet, University of Toronto), 50 μM α-monomethylioglycolate (Sigma)). The cultures were maintained at 0.5 × 10⁶ to 1.5 × 10⁶/ml of nonadherent cells, with adherent cells being discarded. After >4 weeks of culturing, the purity of BMMCs was monitored by flow cytometry. For the detection of FceRI, 10⁶ BMMCs were incubated overnight with antibody to α-dinitrophenyl (DNP) IgE (1 μg/ml Sigma) or in some cases 10% (v/v) conditioned medium from SPE-7 cells (kindly provided by Juan Rivera, National Institutes of Health), washed, and then labeled with α-IgE-fluorescein isothiocyanate (FITC; Southern Biotechnology Associates, Inc.) and α-Kit-phycocerythrin (Caltag Labs), or with isotype controls: rat IgG1-FITC (Caltag Labs) and rat IgG2b-phycocerythrin (Caltag Labs) and analyzed by flow cytometry. Prior to experimentation, all of the BMMCs were ≥90% positive for both c-Kit and FceRI, as measured by flow cytometry.

BMMC Stimulations—BMMCs (10⁶/time point) were simultaneously starved of IL-3 and incubated with anti-DNP-IgE (SPE-7 clone; 10% (v/v)) for 18 h, rinsed in Tyrode’s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin), and resuspended in Tyrode’s with or without DNP-HSA (100 ng/ml Sigma) for the times indicated in each figure or figure legend. BMMCs were rinsed with cold phosphate-buffered saline (containing sodium orthovanadate) and lysed in kinase lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v)
Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 10 μg of apro-}


tinin/ml, 10 μg of leupeptin/ml, 1 mM vanadate, 100 μM phen-
yl methylsulfonyl fluoride). After centrifugation, soluble cell
lysates (SCLs) were obtained and subjected to immunoprecipi-
tations (IPs) using Gamma-bind Sepharose (for polyclonals;
Amersham Biosciences) or protein G-agarose (for mono-
clonals; Roche Applied Science).

In Vitro Kinase Assays—The in vitro kinase assays using
[γ-32P]ATP (PerkinElmer Life Sciences) were performed on anti-
DNP-IgE-sensitized BMMCs (WT, DR, and KR/DR) treated with
or without DNP-HSA in kinase reaction buffer, as previously described (27). To assess the preferred sites of
Pecam-1 tyrosine phosphorylation by Fer and Fps, we performed similar in vitro kinase assays, but with unlabelled
ATP (1 μM), and analyzed them by Western blotting with anti-pY.

Transfection of Pecam-1 and Fer Plasmids—COS-7 cells were grown
on 60-mm plates and transfected with the indicated combinations of
c mouse Pecam-1 and Myc-tagged Fer (WT and KR) expression plas-
mids using Lipofectamine (Invitrogen). SCLs were prepared after 48 h
of transfection and subjected to IPs, followed by SDS-PAGE and immu-
noblotting as indicated.

BMMC Degranulation Assay—Degranulation assays using annexin
V-reactivity of cells that have undergone fusion with exocytic ves-
icles were performed as described (29, 30). Briefly, WT and KR/DR
BMMCs were sensitized with anti-TNP-IgE (1 μg/ml; BD Biosciences)
for 18 h and washed once in warm Tyrode’s buffer, and 5 × 10^5 cells
were stimulated in the absence and presence of DNP-HSA (0.1, 1, 10,
and 100 ng/ml) for 15 min at 37 °C. BMMCs were washed once prior to
staining with propidium iodide (2 μg/ml) and FITC-conjugated
annexin V (5 μl/sample). The percentage of annexin V-positive/pro-
idium iodide-negative cells and mean fluorescence intensity (of
annexin V staining) were analyzed
on an EPICS Altra HSS flow cytom-
eter (Beckman Coulter).

RESULTS
Increased Activities of Fer and Fps Kinases upon Aggregation of FcεRI on
Mast Cells—We previously reported that FcεRI aggregation induces phos-
phorylation of Fer and Fps kinases in mast cells (22). The fact that
kinase-defective mutants of Fps and Fer also become phosphoryl-
ated upon FcεRI aggregation (24) suggests that Fer and Fps are
substrates for an upstream kinase. To address potential effects of
FcεRI aggregation-induced phosphorylation on Fer and Fps kinase activities, we cultured BMMCs from WT, fer^DR/DR (DR), and
fps^KR/fer^DR/DR (KR/DR) mice, sensitized with anti-DNP-IgE,
and treated them with or without antigen (DNP-HSA). Soluble cell
lysates were subjected to IP with anti-Fps/Fer antisera (which rec-
nognizes both proteins (27)), and in vitro kinase assays were per-
FIGURE 1. Increased Fer and Fps kinase activity upon aggregation of FcεRI on mast cells. A, wild type (WT),
fer^DR/DR (DR), and fps^KR/fer^DR/DR (KR/DR) BMMCs were sensitized with anti-DNP-IgE and starved of IL-3 for 18 h.
BMMCs were treated with or without DNP-HSA (100 ng/ml) for 2 min, and soluble cell lysates were prepared
and subjected to IP with anti-Fps/Fer, followed by an in vitro kinase assay (containing [γ-32P]ATP) and either
GST or GST-Pecam-1 C-tail (Δ14/15 isoform). The top panel is an autoradiograph, and the bottom panel is a
immunoblot (IB) with anti-Fps/Fer. Positions of Fps, Fer, and GST-Δ14/15 are indicated on the left with arrows.
The positions of molecular weight markers (M), and a partial degradation product of Fer (*) are indicated on the
left. B, GST and GST-Δ14/15 were purified from BL21 transformants using glutathione-Sepharose beads. A
Coomassie Blue-stained gel is shown for whole cell extracts (W) and the eluted fraction (E) for both substrates.
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FIGURE 2. FcεRI-induced phosphorylation of Fer is independent of Syk. RBL-2H3, TB1A2 (B2 (Syk+)), and TB1A2/Syk− cells were sensitized with anti-DNP-IgE without serum for 18 h, stimulated with DNP-HSA for 0, 1, or 5 min, and lysed in the presence of protease/phosphatase inhibitors. The lysates were immunoprecipitated with anti-Fer. SCLs and IPs were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. SCLs were immunoblotted with anti-pY and anti-Fer. Normalized pY indicates the ratio of phosphorylated Fer relative to total Fer, as determined by densitometry.

formed (Fig. 1A). The lysates from either untreated or DNP-HSA-treated BMMCs are indicated at the top. Recombinant, purified GST, or a GST fusion to the cytoplasmic tail of Pecam-1 (Δ14/15 isoform, containing only one ITIM residue, Tyr662) were added as potential substrates. In WT cells, IPs recovered both Fer and Fps kinases (lanes 1–3, lower panel). Fer and Fps displayed increased autophosphorylation and substrate phosphorylation when recovered from cells treated with antigen (lanes 1–3, top panel). To address whether substrate phosphorylation was indeed carried out by Fer and Fps kinases, we performed a parallel experiment with KR/DR BMMCs (which lack Fer kinase activity and express very low levels of FerDR because of protein instability (23)). Both autophosphorylation of Fps and substrate phosphorylation were significantly elevated upon antigen treatment (compare lanes 5 and 6, with lane 4 in top panel). To address whether substrate phosphorylation was indeed carried out by Fer and Fps kinases, we performed a parallel experiment with KR/DR BMMCs (which lack both Fer and Fps kinase activities (24)). Despite recovering similar amounts of Fer and Fps (lanes 7–9, bottom panel), we detected no signals for autophosphorylation or substrate phosphorylation. Coomassie staining of our substrate preparations indicated that the proteins were relatively pure and ran at their expected molecular masses (Fig. 1B).

FceRI-induced Phosphorylation of Fer Is Independent of Syk—Because Syk kinase plays a central role in signaling pathways emanating from the FcεRI (9), we wished to address its potential involvement in Fer kinase activation. Using rat basophilic leukemia cells (RBL-2H3), a Syk-deficient subline (B2), and B2 cells that were stably transfected with a Syk expression plasmid (B2-Syk+), we sensitized the cells with anti-DNP-IgE and stimulated them with antigen (DNP-HSA) for various times (Fig. 2). Antigen-induced changes in tyrosine phosphorylation were noted in all cell lines (top panel), but the B2 cells were defective in phosphorylation of many proteins, including a prominent 70-kilodalton protein that co-migrated with Syk (data not shown). We tested Fer phosphorylation in each cell line and found that Fer phosphorylation downstream of FceRI aggregation does not require Syk (compare lanes 1–3 with 4–6). Although the basal amount of Fer phosphorylation was higher in B2-Syk+ cells, a similar increase in Fer phosphorylation was observed upon antigen treatment (compare lanes 7–9 with lanes 1–3). Similar results were obtained using Fps/Fer antisera; however, we have not been able to convincingly resolve endogenous Fer and Fps proteins in RBL-2H3 cells. These experiments, together with picetannol treatment of BMMCs (data not shown), suggest that Fer activation downstream of FceRI is Syk-independent.

FceRI-induced Phosphorylation of Fer Is Independent of Fyn Kinase and the Gab2 Adaptor—Recently, Fyn kinase and the adaptor protein Gab2 were shown to regulate FcεRI aggregation-induced degranulation (31). Studies in RBL-2H3 cells and
BMMCs have shown potential involvement of Gab2 in FcεRI aggregation-induced p38 MAPK activation (13, 32). Because we have observed defects in p38 MAPK activation in Fer-deficient BMMCs (22), we wanted to address whether Fer may signal in the Fyn/Gab2 pathway. We generated BMMCs from wild type lyn+/− mice, sensitized them with anti-DNP-IgE, and treated them with antigen for various times (Fig. 3A). Analysis of Fer phosphorylation revealed that peak phosphorylation of Fer was similar between genotypes (compare lanes 1–4 with lanes 5–8). A similar experiment using wild type (genotype lyn+/+) and lyn−/− BMMCs, revealed no involvement of Gab2 for FcεRI aggregation-induced phosphorylation of Fer (Fig. 3B).

**Rapid FcεRI-induced Phosphorylation of Fer Requires Lyn kinase**—To assess whether the upstream kinase acting on Fer is potentially FcεRI-associated Lyn kinase, we generated BMMCs from wild type lyn+/− and lyn−/− mice, sensitized them with anti-DNP-IgE, and treated them with antigen for various times (Fig. 4). SCLs were prepared, and immunoblotting confirmed the absence of both Lyn isoforms (p51/p53) in lyn−/− cells. Characterization of Fer phosphorylation revealed that the rapid phosphorylation of Fer, which was maximal at 1 min in wild type cells, was lost in lyn−/− cells (compare lanes 1–4 with lanes 5–8). This difference was not due to the amounts of Fer recovered, because we have recovered more Fer in lyn−/− samples (bottom panel). The phosphorylation of Fer increased gradually in Lyn-deficient cells and did not reach the levels of phosphorylation that occurred within 1 min in lyn+/+ cells. Comparable results were obtained for two independent cultures of lyn+/+ and lyn−/− BMMCs and suggest that Lyn is required for rapid phosphorylation of Fer but that another kinase, or perhaps Fer itself, can partially compensate for Lyn in mediating Fer activation.

**Fer and Fps Phosphorylate ITIM and Non-ITIM Sites in Pecam-1**—Several putative substrates of Fer have been identified in endothelial cells, including Pecam-1, Shp2, and Gab1 (33). The cytoplasmic tail of Pecam-1 contains several sites of phosphorylation, including ITIM tyrosines (Tyr662 and Tyr685 in mouse Pecam-1) that bind Shp1 and Shp2 phosphatases. Phosphorylation of Tyr700 in mouse Pecam-1 (Tyr701 in human) has been shown to promote recruitment of STAT3 or STAT5 (18). To address the sites in Pecam-1 preferred by Fer or Fps kinases isolated from activated BMMCs, we performed parallel in vitro kinase assays for Fer and Fps with a series of Pecam-1 cytoplasmic tail substrates (expressed as GST fusion proteins; Fig. 5). Kinase assays were performed with unlabeled GST-FceRI fusion protein or with GST alone (10, 11) in the presence of [γ-33P]ATP (1 μCi). One reaction was performed with Fer IPs from IgE/DNP-treated DR BMMCs and incubated with GST-Pecam-1 as a negative control (lane 2, top two panels). The kinase reactions were fractionated by SDS-PAGE and immunoblotted sequentially with anti-pY and mouse monoclonal anti-GST antibodies. Assays of Fps kinase activities were performed in a similar fashion, except that DR BMMCs were employed (to remove any effects caused by cross-reactivity of anti-Fps with Fer). One control reaction was performed with KR/DR BMMCs and GST-Pecam-1 as a negative control (lane 2, bottom two panels). The substrates added to each reaction are indicated at the top. The positions of Fer, Fps, Gab2, GST-Pecam-1, and GST proteins are indicated on the right. The ratio of phosphorylated GST-Pecam-1 relative to total GST-Pecam-1, was determined by densitometry.

**Summary**

Fer and Lyn kinase have been shown to play important roles in mast cell activation and proliferation. Lyn kinase is known for its ability to phosphorylate ITIM sites, which are negatively charged tyrosines that facilitate recruitment of signaling proteins like STATs. Fer, another Src-family kinase, has also been implicated in these processes, although its specific roles are not well understood.

**Figure 4**

**Rapid FcεRI-induced phosphorylation of Fer requires Lyn kinase.** Wild type and Lyn−/− BMMCs were sensitized with anti-DNP-IgE and starved of IL-3 for 18 h, stimulated with DNP-HSA for 0, 1, 5, or 15 min, and lysed in the presence of protease/phosphatase inhibitors. SCLs were IB with anti-Lyn and subjected to IPs with anti-Fer, followed by sequential IBs with anti-pY and anti-Fer. The ratio of phosphorylated Fer relative to total Fer was determined by densitometry.

**Figure 5**

**Evidence that Fer and Fps phosphorylate ITIM and non-ITIM sites in Pecam-1 in vitro.** The lysates were prepared from WT BMMC after FcεRI aggregation (as in Fig. 1), and Fer IPs were incubated with the indicated GST-Pecam-1 fusion protein or with GST alone (1 μg), in the presence of unlabeled ATP (1 μM). One reaction was performed with Fer IPs from IgE/DNP-treated DR BMMCs and incubated with GST-Pecam-1 as a negative control (lane 2, top two panels). The kinase reactions were fractionated by SDS-PAGE and immunoblotted sequentially with anti-pY and mouse monoclonal anti-GST antibodies. Assays of Fps kinase activities were performed in a similar fashion, except that DR BMMCs were employed (to remove any effects caused by cross-reactivity of anti-Fps with Fer). One control reaction was performed with KR/DR BMMCs and GST-Pecam-1 as a negative control (lane 2, bottom two panels). The substrates added to each reaction are indicated at the top. The positions of Fer, Fps, Gab2, GST-Pecam-1, and GST proteins are indicated on the left. The ratio of phosphorylated GST-Pecam-1 relative to total GST-Pecam-1, was determined by densitometry.
ATP and analyzed by immunoblotting with anti-pY to avoid potential spurious signals caused by serine/threonine phosphorylation. Fer was found to phosphorylate the full-length Pecam-1 C-tail, but not GST (top panel, lanes 1 and 3). A control reaction from lysates of DR BMMCs shows that the activity being measured was solely that of Fer (lane 2). Mutation of either ITIM (Y662F or Y685F) resulted in a 60% decrease in substrate phosphorylation but not autophosphorylation of Fer (compare lanes 3–5, upper panel). Using a substrate derived from the exon 15-deleted isoform of Pecam-1 (Δ15), we observed a significant reduction in phosphorylation by Fer (compare lanes 3 and 6). A parallel experiment to examine preferred Fps phosphorylation sites in Pecam-1 using DR BMMCs (to avoid recovery of active Fer kinase in our IPs) was carried out. The results showed that Fps has higher activity toward Tyr685 than Tyr662 (60% versus 30% reduction because of mutation; third panel, lanes 3–5) and showed a significant difference in phosphorylation of the Δ15 isoform (70% reduction; lane 6). Exon 15 contains one tyrosine (Tyr700) that has been implicated in the recruitment of STAT3/STAT5 (18).

To determine whether Fer contributes to Pecam-1 phosphorylation on ITIM and non-ITIM residues in cells, we performed co-transfections of COS-7 cells with Myc-tagged Fer (WT and kinase-dead (KR)), and various full-length Pecam-1 expression plasmids (Fig. 6). SCLS were prepared, and Western blotting showed comparable expression of Myc-Fer<sup>WT</sup> and Myc-Fer<sup>KR</sup> between samples (top panel, alternating lanes), and high level expression of Myc-Fer compared with endogenous Fer in all of the samples except those lacking Pecam-1 (lanes 1 and 2). Pecam-1 phosphorylation by Fer was reduced upon mutation of Tyr<sup>700</sup> (30% reduction, middle panel, lanes 3 and 5), which is consistent with a previous study (33). A single mutation at Tyr<sup>700</sup> did not significantly reduce the overall phosphorylation (lanes 3 and 7). However, a double mutation at Y662F and Y700F resulted in a 50% reduction in Pecam-1 phosphorylation compared with mutation of the Tyr<sup>662</sup> site alone (lanes 5 and 9). These studies suggest that Fer and Fps kinases can phosphorylate Pecam-1 ITIMs and a non-ITIM residue in vitro and in vivo.

**FccR1-induced Tyrosine Phosphorylation of Pecam-1 Is Reduced in the Absence of Fps and Fer kinase activities.** A WT, DR, and KR/DR BMMCs were sensitized with anti-DNP-IgE and starved of IL-3 for 18 h, stimulated with DNP-HSA (100 ng/ml) for 0, 10, or 20 min, and lysed in the presence of protease/phosphatase inhibitors. SCLS were IB with either a phospho-specific antibody to Tyr<sup>p</sup> position of Pecam-1 or with a goat anti-mouse Pecam-1 (Pecam-1<sub>CT</sub>). IPs were carried out. The results showed that Fps has higher activity toward Tyr<sup>685</sup> than Tyr<sup>662</sup> (60% versus 30% reduction because of mutation; third panel, lanes 3–5) and showed a significant difference in phosphorylation of the Δ15 isoform (70% reduction; lane 6). Exon 15 contains one tyrosine (Tyr<sup>700</sup>) that has been implicated in the recruitment of STAT3/STAT5 (18).
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Tyr\(^{686}\) of human Pecam-1 (which cross-reacts with Tyr\(^{685}\) of mouse Pecam-1; Tyr\(^{685}\)), we observed no differences in Pecam-1 phosphorylation between genotypes (Fig. 7A). However, we did observe reduced overall tyrosine phosphorylation of Pecam-1 in KR/DR BMMCs following IgE/DNP treatment (Fig. 7A; \( \approx 30\% - 40\% \) reduction). Despite the reduced phosphorylation, there is no defect in Shp2 recruitment to Pecam-1 in KR/DR BMMCs following FceRI aggregation (Fig. 7A, arrow indicates position of Shp2). Similar results were obtained for Shp1 recruitment (data not shown). Using an antibody raised against the extreme C terminus for immunoprecipitation that should preferentially recover the full-length Pecam-1 isoform harboring both ITIMs and Tyr\(^{700}\), we observed a more profound reduction in Pecam-1 tyrosine phosphorylation in Lyn-deficient BMMCs compared with WT (Fig. 7A, \( \% \) reduction). Thus, Fer and Fps play redundant roles in promoting Pecam-1 phosphorylation at positions other than Tyr\(^{685}\). Given our \textit{in vitro} kinase assay results, Fer and Fps may phosphorylate Pecam-1 at positions Tyr\(^{662}\) and/or Tyr\(^{700}\).

\textit{Lyn Is an Initiator Kinase for Pecam-1 and Is Essential for Shp2 Recruitment}—Because Lyn is also a candidate kinase for Pecam-1 (33) and is required for rapid activation of Fer kinase (Fig. 4), we addressed the phosphorylation status of Pecam-1 in Lyn-deficient mast cells. Wild type (\( lyn^{+/+} \)) and \( lyn^{-/-} \) BMMCs were starved and sensitized with anti-DNP-IgE prior to exposure to antigen for the times indicated (Fig. 8). Immuno-

FIGURE 8. Pecam-1 phosphorylation is greatly reduced in Lyn-deficient mast cells following FceRI aggregation. Wild type (\( lyn^{+/+} \)) and \( lyn^{-/-} \) BMMCs were sensitized with anti-DNP-IgE and starved of IL-3 for 18 h and stimulated with DNP-HSA for 0, 2, 10, or 20 min, and SCLs were prepared. SCLs were IB with either a phospho-specific antibody to Tyr(P)\(^{685}\) position of Pecam-1 or with a goat anti-mouse Pecam-1 (Pecam-1\(_{CT}\)). IPs were also performed using anti-Pecam-1\(_{NT}\), followed by IBs of duplicate gels with either anti-pY or anti-Pecam-1CT. The control blot was stripped and reprobed with annexin V staining was significantly higher at low doses of antigen in KR/DR BMMCs compared with WT (Fig. 9A, base; \( p < 0.01 \)). At increasing antigen dosages there was no difference in the percentage of cells that underwent degranulation. However, the mean fluorescent intensity of annexin V staining was significantly higher at low doses of antigen in WT and KR/DR BMMCs compared with WT (Fig. 9A, 0.1 and 1 ng/ml; \( p < 0.05 \)). Taken together, these results suggest that Fer/Fps kinases function to limit the extent of granule mobilization in IgE-sensitized mast cells. At higher intensity stimulation, this function of Fer/Fps kinases may be compensated for by other kinases.

\textbf{DISCUSSION}

Mast cell activation via clustering of FceRI elicits rapid changes in protein phosphorylation and localization, cytoskeletal organization, calcium mobilization, degranulat-
play some hyperdegranulation phenotypes associated with Pecam-1- or Shp1-deficient mast cells (Fig. 9B and Refs. 16 and 17). Future studies will attempt to identify the sites of Pecam-1 phosphorylation in mast cells and their involvement in downstream signaling. In endothelial cells, Pecam-1 phosphorylation elicits Shp2 recruitment and regulation of cell adhesion and migration (21). It will be interesting to determine whether Pecam-1/Shp2 plays similar roles in mast cells.

Although mice lacking Pecam-1, Fps, and Fer kinases are viable (23, 36, 37), they all share phenotypes associated with hypersensitivity to lipopolysaccharide-induced inflammation (19, 20, 37, 38). A recent study also showed that Pecam-1-deficient endothelial cells and lymphocytes are partially defective in STAT3 activation in response to lipopolysaccharide treatment. This is thought to involve recruitment of STAT3 to a non-ITIM tyrosine (Tyr700) of Pecam-1 (19). We show in this study that Fer and Fps kinases can phosphorylate Tyr700 of Pecam-1. It is worth noting that the peptide sequence surrounding Tyr700 of Pecam-1 is similar to the C-terminal phosphorylation site of STAT3 (Tyr705) and that previous studies have implicated Fer and Fps as STAT3 kinases in some cell types and conditions (39, 40). Although STAT3 phosphorylation downstream of FcεRI has not been reported, it was shown to occur downstream of the T cell receptor (which also signals via Src family kinases and ITAMs) (41). Activated mast cells also produce the STAT3 target gene vascular endothelial growth factor (42, 43), a known angiogenic factor and chemotactic factor for mast cells (44). Interestingly, a high proportion of lung adenocarcinomas contain infiltrating mast cells that express vascular endothelial growth factor, and this correlates with poor prognosis (45). Therefore, defining the pathway that controls vascular endothelial growth factor production by mast cells, could provide therapeutic targets to limit tumor-associated angiogenesis.

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