The FcRβ- and γ-ITAMs Play Crucial but Distinct Roles in the Full Activation of Mast Cells Induced by IgEκ and Protein L

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The FcRβ- and γ-ITAMs Play Crucial but Distinct Roles in the Full Activation of Mast Cells Induced by IgEκ and Protein L

Satoshi Nunomura,* Yuko Kawakami,† Toshiaki Kawakami,† and Chisei Ra*

Previous studies suggested that Protein L (PpL), the bacterial Ig-binding protein, activates mast cells. PpL presumably performs the activation by interacting with membrane-bound IgEκ, but the underlying mechanisms behind the process remain unclear. In the current study, we found that cell-surface FcεRI expression is a critical factor participant in PpL-mediated full activation of murine mast cells, which includes cytokine production, the degranulation response, and leukotriene C4 (LTC4) release, and that engagement of the FcεRI with IgEκ and PpL is enough to induce tyrosine phosphorylation of ITAM in the FcεRIγ and γ-signaling subunits. Introduction of mutations in two canonical tyrosine residues (Y47F/Y58F) of the FcεRIγ and PpL is enough to induce tyrosine phosphorylation of ITAM in the FcεRIγ− and γ-signaling subunits. Engagement of the FcεRI elicits rapid tyrosine phosphorylation of the ITAM of the FcεRIγ and γ-subunits, which in turn leads to activation of subsequent downstream signals, such as Syk, phospholipase Cγ1 (PLCγ1), Grb2-associated binder 2 (Gab2), and MAPK family members, and calcium influx (3). Activation of these intracellular signals is essential for the release of diverse inflammatory mediators, including histamine, arachidonate metabolites, and cytokines from mast cells upon FcεRI cross-linking.

In addition to functions connected with allergic inflammation, previous studies suggested that mast cells act as innate immune cells that protect their host from infections by opportunistic pathogens, such as Candida albicans, Staphylococcus aureus, and Escherichia coli, by expressing a variety of receptors (e.g., TLR2, -4, and CD48) against the cell wall components of these pathogens (4–9). Finegoldia magna (formerly Peptostreptococcus magnus) is one of the major anaerobic bacteria in the oral cavity and the gastrointestinal and urogenital tracts (10). F. magna has also been reported to be an opportunistic pathogen that is frequently isolated from patients with clinical infections. Representative infections associated with F. magna include bacterial vaginosis and wound and bone/joint infections (10, 11). Earlier studies reported that many strains of F. magna isolated from patients with bacterial vaginosis express a surface protein, protein L (PpL), with a high affinity for variable regions of the Igκ L chains (Kd = 1 × 10^9 M−1) (12, 13). Interestingly, purified PpL and F. magna-expressing PpL induce the degranulation response and leukotriene C4 (LTC4) release in Igκκ-sensitized human mast cells but not in nonsensitized cells (14–16). It is thought that PpL probably engages the IgEκ-FcεRI complex in human mast cells, because PpL possesses multiple domains that bind to Igκ L chains (17), which would imply that mast cells can respond to F. magna independent of the typical innate immune receptors responsible for defense against pathogens.

Mast cell activation through FcεRI leads to diverse responses, depending on the quality of the stimulation (18). For example, stimulation of FcεRI with highly cytokinergic IgE elicits considerable cytokine production but not degranulation (19, 20). Like highly cytokinergic IgE, the interaction of a low-affinity Ag with cell surface-bound IgE, or the aggregation of small numbers of FcεRI, results in preferential activation of cellular signaling and cytokine production but not in degranulation (21–24). By contrast, a high-affinity Ag or an aggregation of large numbers of FcεRI induces robust cellular signals that can effectively induce the degranulation response (23, 24). However, it is unclear whether cell-surface FcεRI expression is responsible for mast cell activation via PpL and IgEκ and whether the combination of PpL and IgEκ actually activates FcεRI-dependent signaling. Namely, the biological functions of the FcεRIγ and γ-signaling subunits in PpL-mediated mast cell cytokine production, the degranulation response, and LTC4 secretion are still unknown.

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Abbreviations used in this article: BMMC, bone marrow-derived mouse mast cell; [Ca2+]i, intracellular calcium ion; DNS, dnasyl; Gab2, Grb2-associated binder 2; LTC4, leukotriene C4; MFI, mean fluorescence intensity; PLCγ1, phospholipase Cγ1; PpL, protein L; RBL, rat basophilic leukemia; SCF, stem cell factor; TNF, tumour necrosis factor.

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Elucidation of the mechanisms involved in the inflammatory responses mediated by mast cells against the superantigen-like protein from *F. magna* could deepen our understanding of mast cells function and the innate immune system. In the current study, therefore, we sought to clarify the underlying mechanisms behind PpL-mediated mast cell activation.

**Materials and Methods**

**Abs and other reagents**

Commercially available IgEκ mAbs were used in this study (BD Biosciences, San Diego, CA, for the 15.3.2, C48-2, and 27.72 clones, antitritriotrophil [TNP], or antidansyl [DNS]; Santa Cruz Biotechnology, Santa Cruz, CA, for the H1-ε-26.82 clone, ant-DNP). The anti-FcRβ mAb (clone JRK, the hybridoma for which was a kind gift from Dr. Juan Rivera, National Institutes of Health) was prepared in our laboratory. The rabbit anti-FcRγ and anti-Gab2 Abs were from Millipore (Billerica, MA). The mouse anti-phosphotyrosine HRP-conjugated mAb (PY20) was purchased from GE Healthcare Biosciences (Little Chalfont, U.K.). The recombinant PpL and PpL conjugated with HRP were purchased from Sigma-Aldrich (St. Louis, MO). The PpL-FITC, TNP-BSA (5 M TNP per mole BSA), and DNS-BSA (6 M DNS per mole BSA) were prepared in our laboratory, and the recombinant murine IL-3 and stem cell factor (SCF) were purchased from PeproTech (Rocky Hill, NJ). All the Abs to phosphorylated proteins and the Abs against Syk, PLCγ1, ERK1/2, and p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA).

**Animals**

FcγRII−/− (C57BL6 background) (25), FcγRII−/− (C57BL6 background) (26), and FcRγ−/−γ−/−mice were bred in the animal facility of the Nihon University School of Medicine under specific pathogen-free conditions. For some experiments, thighbones from FcεRIα−/− and FcεRIα+/+ mice were kindly provided by Dr. Toshiaki Kawakami (La Jolla Institute of Allergy and Immunology). C57BL6/J mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Following the approval of the animal experimentation committee at Nihon University, all of the experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Nihon University.

**FIGURE 1.** 15.3.2, a murine IgEκ clone, is reactive to PpL. (A) Increased vascular leakage of the Evans blue dye by PpL administration. The data are shown as the mean ± SE. **p < 0.01 (Student t test).** (B) The far-Western blotting. The PpL-reactive IgEκ clone was visualized with PpL-HRP. Coomassie brilliant blue (CBB) staining showed comparable sample loading. (C) Binding of PpL-FITC to RBL-2H3 cells sensitized with or without IgEκ clones. IgEκa and IgEκb indicate the Ig allotype of each murine IgEκ clone. Green lines indicate intensity of fluorescence of PpL-FITC. Grey lines indicate intensity of fluorescence of nonlabeled cells. (D) Confocal microscopic analyses for binding of PpL-FITC to RBL-2H3 cells sensitized with 15.3.2 or C48.2. Scale bars, 20 μm. Original magnification ×240 for all panels. (E) Binding of PpL-FITC to BMMCs sensitized with 15.3.2 or without 15.3.2. The cells were analyzed on a flow cytometer. The results shown in (B)–(D) are representative of three independent experiments with similar results. In (E), data shown are the mean ± SD of samples obtained from four independent experiments. **p < 0.01 (Tukey’s test with one-way ANOVA).
Cell culture

Bone marrow-derived mouse mast cell (BMMC) cultures were prepared from the femurs of 4–8 wk-old mice, as previously described (27). The ecotropic retrovirus packaging cell line PLAT-E, which was a kind gift from Dr. Toshio Kitamura (Tokyo University), was maintained in DMEM supplemented with 10% (v/v) FBS, 1 \( \mu \text{g ml}^{-1} \) puromycin (BD Clontech, San Jose, CA), and 10 \( \mu \text{g ml}^{-1} \) blasticidin S (Kaken Pharmaceutical, Tokyo, Japan). Rat basophilic leukemia (RBL)-2H3 cells were maintained in DMEM supplemented with 10% (v/v) FBS.

Retroviral transfection

Retroviral gene transduction was performed as previously described (27). Briefly, pMX-puro plasmids harboring FcR\(\gamma\)YY, FcR\(\gamma\)FF, FcR\(\beta\)YYY, or FcR\(\beta\)FFF cDNA were transfected into the PLAT-E cells to generate recombinant retroviruses. Bone marrow cells were infected or coinfected with the retroviruses for 24 h in the presence of 1.2 \( \mu \text{g ml}^{-1} \) polybrene (Sigma-Aldrich). The gene-transduced cells were selected with 1.2 \( \mu \text{g ml}^{-1} \) puromycin for 10 d. The viable cells (10–20% of the bone marrow cells cultured with retroviruses) were expanded for several weeks. The FcR\(\gamma\) transfectants were grown in a medium containing IL-3 (5 ng ml\(^{-1}\)). The FcR\(\beta\)Y cotransfectants were grown in a medium containing IL-3 (5 ng ml\(^{-1}\)) and SCF (10 ng ml\(^{-1}\)) to obtain a sufficient number of FcεRI-positive cells. The puromycin-resistant FcR\(\gamma\) transfectants, which expressed cell-surface FcεRI at comparable levels, were used for the experiments. The FcεRI\(\gamma\) cells (1 to 2%) were purified from the puromycin-resistant FcR\(\beta\)Y cotransfectants using an EasySep FITC Positive Selection kit (StemCell Technologies) and an anti-mouse IgE mAb conjugated with FITC.

Analysis of vascular permeability

PBS containing 0.5% Evans blue dye or PBS containing 100 \( \mu \text{g} \) PpL and 0.5% Evans blue dye was i.v. injected into naive FcR\(\gamma\)+/+ (\(n = 4–6\)) and FcR\(\gamma\)\(-/\) mice (\(n = 4–6\)). The vascular leakage of the Evans blue dye into the skin after 30 min was quantified as the absorbance at 620 nm (A620) of the fluid extracted with formamide.

Assay of degranulation, LTC4 release, and cytokine production

Mast cells (\(1 \times 10^6 \text{ ml}^{-1}\)) were sensitized with each 0.5 \( \mu \text{g ml}^{-1} \) IgE clone overnight. In some experiments, unsensitized mast cells were also analyzed. The IgE-sensitized and unsensitized mast cells (2 \( \times 10^5\))

FIGURE 2. PpL loading induces cytokine production, degranulation, and LTC4 release in BMMCs sensitized with 15.3.2. IgE\(\kappa\) (15.3.2)-sensitized and unsensitized BMMCs were stimulated with or without PpL at the indicated concentrations for 0.5 h (for degranulation and LTC\(_4\)) or 8 h (for cytokine production). IL-6 (A), IL-13 (B), and TNF-\(\alpha\) (C) production. (D) Degranulation. (E) LTC\(_4\) production. Data shown are the mean \( \pm \) SD of quadruplicates. Similar results were obtained in three independent experiments. *\(p < 0.05\), **\(p < 0.01\) (Student \( t \) test).
were washed twice with PBS and then stimulated with or without PpL at the indicated concentrations for 0.5 h (for degranulation and LTC4) or 8 h (for IL-6 and IL-13 production). Ags (TNP5-BSA and DNS5-BSA) were used as immunological controls in place of PpL. IL-6 (A) and IL-13 (B) production. (C) Degranulation. (D) LTC4 synthesis. Data shown are the mean ± SD of quadruplicates. Similar results were obtained in three independent experiments. **p < 0.01 (Student t test).

FIGURE 4. PpL loading elicits tyrosine phosphorylation of the FcRβ and γ-subunits in intracellular calcium mobilization in BMMCs sensitized with 15.3.2. (A) BMMCs sensitized with 15.3.2 were stimulated with 30 nM PpL for 0.5 and 3 min. Total cell lysates were incubated with anti-FcRβ or anti-FcRγ Abs. The immunoprecipitates (IP) were analyzed by immunoblotting with anti-phosphotyrosine HRP-conjugated Ab (PY20). (B and C) PpL-mediated calcium mobilization in the 15.3.2-sensitized BMMCs. Changes in the Fluo-3 fluorescence were determined employing flow cytometry after PpL stimulation in the presence (B) or absence (C) of extracellular Ca2+. The [Ca2+]i mobilization is expressed as the MFI of Fluo-3. The results shown are representative of three (A and B) of four (C) independent experiments with similar results.
nonimmunological positive controls, respectively. Degranulation was determined by β-hexosaminidase release, as described previously (27). The percentage of β-hexosaminidase release was calculated as follows: (supernatant OD of cells)/(total cell lysate OD of cells) × 100. The LTC4 production was analyzed with an LTC4 EIA kit (Cayman Chemical, Ann Arbor, MI). The IL-6, IL-13, and TNF-α productions were analyzed with specific ELISA kits (Invitrogen/BioSource International, Camarillo, CA).

Measurement of mobilization of intracellular calcium ions
Mast cells (1 × 10⁶ ml⁻¹) were sensitized with 0.5 μg ml⁻¹ 15.3.2 overnight. The IgE-sensitized cells (1 × 10⁶ ml⁻¹) were washed twice with PBS and then loaded with 4 μM Fluo-3-AM (Dojindo, Kumamoto, Japan) for 30 min at 37°C. The cells were resuspended in 1× Tyrode’s buffer and stimulated with or without PpL. In some experiments, assays were performed in Ca²⁺-free 1× Tyrode’s buffer containing 1 mM EGTA for complete depletion of extracellular Ca²⁺. A23187 (1 μM) was used as a nonimmunological positive control in place of PpL. Changes in dye fluorescence upon addition of stimuli were monitored at 200-ms intervals by a flow cytometer (FACSCalibur; BD Biosciences). The mobilization of intracellular calcium ions ([Ca²⁺]i) was expressed as the mean fluorescence intensity (MFI). The graph was generated by plotting values of MFI.

Immunoblotting
Mast cells (1 × 10⁶ ml⁻¹) were sensitized with 0.5 μg ml⁻¹ 15.3.2 overnight. The IgE-sensitized cells were washed twice with PBS and then stimulated with 30 nM PpL for the indicated time periods. The stimulated mast cells (1–5 × 10⁶) were washed twice with ice-cold PBS and lysed for 30 min on ice in a lysis buffer (TBS containing 1% Nonidet P-40, 2 mM PMSE, 10 μg ml⁻¹ aprotinin, 2 μg ml⁻¹ leupeptin and pepstatin A, 50 mM NaF, and 1 mM sodium orthovanadate). The lysates were centrifuged for 15 min at 15,000 × g. For immunoprecipitation assays, the cell lysates were incubated with Ab-bound Protein G Sepharose for 3 h on ice. The samples were then boiled, separated on a 12% SDS-PAGE gel, and transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with a primary Ab and an appropriate secondary HRP-conjugated Ab. The signals were detected by ECL (GE Healthcare Biosciences). The immunoreactive bands were scanned to produce digital images that were quantified using the SCION Image software, and then the phosphorylation was calculated from the amount of phospho-protein relative to the corresponding non-phospho–loading control.

Far-Western blotting
Four mouse IgEκ proteins (5 μg/lane) were separated on a 12% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore). The membrane...
was incubated with PpL-HRP (1:2000) for 1 h. The signals were detected by ECL. Coomassie brilliant blue staining showed comparable sample loading.

Confocal microscopy

RBL-2H3 cells \( \left(2 \times 10^5 \text{ ml}^{-1}\right) \) were sensitized with 0.5 \( \mu \text{g ml}^{-1}\) 15.3.2 or C48-2 on culture coverglass for 1 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min and then permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature. The cells were washed twice with 1 ml PBS and stained with FITC-conjugated PpL (1:1000) for 60 min in the dark. The coverglasses were washed with PBS and then mounted with Vectashield mounting medium containing DAPI (Molecular Probes/Invitrogen Life Technologies). Confocal microscopy was performed using a FV1000 system and Fluoview software (Olympus, Tokyo, Japan).

Flow cytometric analyses

For analyses of PpL-FITC binding to mast cells, RBL-2H3 cells \( \left(2 \times 10^5 \text{ ml}^{-1}\right) \) and murine mast cells \( \left(2 \times 10^5 \text{ ml}^{-1}\right) \) were sensitized with or without each 0.5 \( \mu \text{g ml}^{-1}\) IgE clone. These cells were washed twice with PBS and labeled with PpL-FITC (1:1000) at 4˚C for 30 min. The labeled cells were analyzed with FACSCalibur (BD Biosciences). The receptor internalization was defined as changes in cell-surface FcɛRI expression after PpL stimulation. Briefly, mast cells \( \left(1 \times 10^6 \text{ ml}^{-1}\right) \) were sensitized with 0.5 \( \mu \text{g ml}^{-1}\) 15.3.2 overnight. The cells were washed twice with PBS and stimulated with or without 30 nM PpL for 20 min. The stimulated cells were labeled with 0.1 \( \mu \text{g ml}^{-1}\) anti-mouse IgE mAb-FITC for 15 min on ice. The labeled cells were analyzed with FACSCalibur (BD Biosciences).

Statistical analysis

The data shown are the mean \( \pm \) SE or SD. The statistical analyses were performed using the Student t test or Tukey’s test in a one-way ANOVA model. The \( p \) values \(<0.05\) were considered to indicate statistically significant differences.

Results

A reactive murine IgEκ clone to PpL (15.3.2 clone)

First, we employed FcRγ\(^++\) and FcRγ\(^--\) mice to examine whether PpL elicits an in vivo inflammatory response through the murine Igs and FcRs. We i.v. injected PpL (100 \( \mu \text{g}\) and Evans...
blue dye into naive FcRγ<sup>+/+</sup> and FcRγ<sup>−/−</sup> mice to visualize changes in vascular permeability. As shown in Fig. 1A, administration of PpL increased leakage of the Evans blue dye. Compared to the FcRγ<sup>+/+</sup> mice, the FcRγ<sup>−/−</sup> mice showed less dye leakage. These data suggest that the murine endogenous IgEs and FcRs are sufficiently reactive to PpL and that murine IgE can elicit PpL-triggered, IgE-dependent mast cell activation. Thus, we examined the bindability of murine IgE clones to PpL. Far-Western blotting showed that PpL preferentially binds to the light chains of 15.3.2 clone among the four IgE clones studied (15.3.2; anti-TNP, C48.2; anti-TNP, 27.74; anti-DNS, H1-e-26.82; anti-DNP) (Fig. 1B). Two clones, 27.74 and H1-e-26.82, were slightly reactive to PpL. Conversely, no significant binding between PpL and C48.2 was detected. Next, we used RBL-2H3 cells to investigate the ability of PpL to bind to the intact IgE protein. RBL-2H3 cells were incubated with or without the IgE clones and then labeled with FITC-conjugated PpL. Fig. 1C shows that PpL-FITC binds to 15.3.2 but not the other IgE clones. Confocal microscopy analyses indicated that PpL localizes to the cell surface of RBL-2H3 cells sensitized with 15.3.2 (Fig. 1D). Similarly, 15.3.2 was essential for the binding of PpL to BMMCs (Fig. 1E). Together, these results indicate that 15.3.2 is the most reactive murine IgE clone to PpL.

15.3.2 clone mediates mast cell activation upon PpL loading

To examine whether murine mast cells produces inflammatory mediators in response to PpL in a 15.3.2-dependent manner, BMMCs were sensitized with or without 15.3.2. As shown in Fig. 2, PpL loading elicited cytokine production (IL-6, IL-13, and TNF-α), the degranulation response, and LTC4 release in the

**FIGURE 7.** Introduction of mutations in tyrosine residues of the FcRγ–ITAM decreases FcεRI-dependent proximal and distal signals elicited by 15.3.2 and PpL. (A) Wild-type FcRγ (FcRγ<sup>YY</sup>) and mutant FcRγ–ITAM (FcRγ<sup>FF</sup>), with two tyrosine residues in the ITAM replaced with phenylalanine. (B) Introduction of the FcRγ genes into the FcRγ<sup>−/−</sup> mast cells restored their defective PpL binding. (C) FcRγ–ITAM transfectants sensitized with 15.3.2 were stimulated with 30 nM PpL for the indicated times. In place of PpL, TNP<sub>5</sub>-BSA was used as an immunological positive control. The total cell lysates were analyzed by immunoblotting with anti–phospho-Syk (Tyr<sup>352</sup>), PLCγ1 (Tyr<sup>783</sup>), Gab2 (Tyr<sup>145</sup>), ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), and p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>) Abs. The blots were reprobed with each anti–nonphospho-Ab. The results shown are representative of three or four independent experiments with similar results. The densitometric data (right panels) are shown as mean ± SD.
BMMCs sensitized with 15.3.2. The PpL concentration for inducing the degranulation response differed from that for other cellular responses. A degranulation response required 3 nM of PpL. Next, we examined the effect of two other IgE clones (C48.2, 27.74) on PpL-mediated mast cell activation (Fig. 3). We omitted H1-e-26.82 from our analyses because this highly cytokinergic IgE clone itself has the ability to activate mast cells in the absence of Ags (20, 28, 29). Fig. 3 shows that neither C48.2 nor 27.74 induced PpL-dependent mast cell activation but that these IgE clones became capable of mast cell activation upon loading of their specific Ags (TPNp-BSA or DNS6-BSA).

PpL loading elicits tyrosine phosphorylation of the FcεRI signaling subunits and calcium influx in mast cells sensitized with 15.3.2

To elucidate whether PpL triggers aggregation of the IgE–FcεRI complex, we examined the tyrosyl phosphorylation state of the FeRβ- and γ-subunits in 15.3.2-sensitized mast cells. As shown in Fig. 4A, PpL induced rapid and significant tyrosine phosphorylation of both of the FcR signaling subunits of the BMMCs sensitized with 15.3.2. We next examined the mobilization of [Ca²⁺], which is an essential second messenger for cytokine production and chemical mediator release. Fig. 4B shows that PpL induced increases in [Ca²⁺], mobilization in a dose-dependent manner. This [Ca²⁺] mobilization was considerably reduced under calcium-free extracellular conditions, suggesting that calcium influx was activated by the PpL loading (Fig. 4C).

Cell-surface FcεRI is required for 15.3.2- and PpL-induced mast cell activation

To clarify the role of cell-surface FcεRI in the mast cell activation induced by 15.3.2 and PpL, we prepared FcRγ⁻/⁻ BMMCs. The FcRγ⁻/⁻ and FcRγ⁺/+ BMMCs were incubated with 15.3.2. Flow cytometric analyses showed no binding of PpL to the 15.3.2-

![Figure 8](http://www.jimmunol.org/)  
**Figure 8.** FcRγ⁺/⁺ mast cells can release LTC₄. (A) PpL-mediated calcium mobilization in 15.3.2-sensitized FcRγ⁺/⁺ and FcRγ⁺/⁺ mast cells was analyzed as shown in Fig. 4B. In place of PpL, A23187 (1 μM) was used as a nonimmunological positive control for the calcium response. The results shown are representative of four independent experiments with similar results. The 15.3.2-sensitized FcRγ⁺/⁺ and FcRγ⁺/⁺ mast cells were stimulated with or without 30 nM PpL for 0.5 h (for degranulation and LTC₄) or 8 h (for IL-6 production). In place of PpL, TNP₅-BSA and A23187 were used as immunological and nonimmunological positive controls, respectively. (B) IL-6 production. (C) Degranulation. (D) LTC₄ production. (E and F) The net LTC₄ production. The 15.3.2-sensitized FcRγ⁺/⁺ mast cells were stimulated with or without 30 nM PpL in the presence or absence of extracellular calcium (E) or in the presence or absence of 5 μM U0126 (F). In (B)–(F), data shown are the mean ± SD of quadruplicates. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.01 (Student *t* test).
sensitized FcRε/FcRγRII BMMCs, suggesting that cell-surface FcεRI expression is a critical element in the binding of PpL to BMMCs (Fig. 5A). Consistent with this result, FcRε/FcRγRII BMMCs sensitized with 15.3.2 failed to induce [Ca2+]i mobilization (Fig. 5B), cytokine production (IL-6 and IL-13), the degranulation response, and LTC4 secretion upon PpL loading (Fig. 5C–F). Like FcRε/FcRγRII BMMCs, FcRε/FcRγRII BMMCs sensitized with the 15.3.2 lacked the bindability to PpL and failed to induce PpL-dependent cytokine production, the degranulation response, and LTC4 secretion (Fig. 6). These data indicate that cell-surface FcεRI is responsible for mast cell activation mediated by IgEκ and PpL.

FcRγFF mast cells exhibit decreased activation of proximal and distal signaling molecules elicited by 15.3.2 and PpL

Fig. 4 raises the possibility that tyrosine phosphorylation of the FcRγ subunit plays an important role in PpL-mediated mast cell activation. We therefore prepared BMMCs with or without mutations (Y47F/Y58F) in two canonical tyrosine residues of the FcRγ–ITAM by using retroviral gene transfer to introduce the FcRγ–ITAM constructs into the FcRγFF BMMCs (Fig. 7A). Introduction of the FcRγ-subunit genes restored the defective PpL binding seen in the FcRγFF BMMCs (Fig. 7B). Next, we examined the activation of representative proximal and distal signaling molecules in the FcRγYY and FcRγFF BMMCs. Fig. 7C shows that PpL induced tyrosine phosphorylation of Syk, PLCγ1, and Gab2 in the FcRγYY BMMCs sensitized with 15.3.2. In contrast, the FcRγFF BMMCs had decreased phosphorylation of these proximal signaling molecules. Phosphorylation of the distal signaling molecules ERK1/2 and p38 MAPK was also induced following FcεRI stimulation with 15.3.2 and PpL. Consistent with our proximal signaling data, the level of p38 MAPK phosphorylation was significantly decreased in the FcRγFF BMMCs compared with FcRγYY BMMCs. To our surprise, we observed apparent ERK1/2 phosphorylation in the FcRγFF BMMCs.

LTC4 secretion, but not cytokine production and the degranulation response, is inducible in FcRγFF BMMCs

As demonstrated in Fig. 7C, the phosphorylation of FcεRI-dependent signaling molecules was reduced in the FcRγFF BMMCs but did not completely disappear. Therefore, we examined whether PpL-mediated activation of the FcεRI-dependent signal in the 15.3.2-sensitized FcRγFF BMMCs was sufficient to induce mast cell activation. Upon PpL loading, detectable [Ca2+]i mobilization was observed in the 15.3.2-sensitized FcRγFF BMMCs, although the level of mobilization was much lower than

FIGURE 9. Preparation of the FcRβγ–ITAM cotransfectants. (A) Defective expression of the FcRβ and -γ proteins in BMMCs from the FcRβγ double-knockout mice. (B) Wild-type FcRβ (FcRβYYY) and mutant FcRβ–ITAM (FcRβFFF), with three tyrosine residues in the ITAM replaced with phenylalanine. (C) The FcεRI+ cells were purified from puromycin-resistant FcRβγ cotransfectants. In (A) and (C), similar results were observed in three independent experiments.
in the 15.3.2-sensitized FcRγYY BMMCs (Fig. 8A). Fig. 8B and 8C show that introducing mutations into the FcRγ-ITAM completely abolished IL-6 production and the degranulation response in the mast cells stimulated with 15.3.2 and PpL. In contrast, LTC4 release was sufficiently induced, even in the FcRγYY BMMCs (Fig. 8D). Taken together, PpL-mediated activation of the FcεRI-dependent signaling in the 15.3.2-sensitized FcRγFF BMMCs was able to induce LTC4 release but not cytokine production and the degranulation response. Fig. 8E shows that calcium influx plays a crucial role in LTC4 release in FcRγFF BMMCs sensitized with 15.3.2. U0126, a potent inhibitor of MEK1/2, also inhibited LTC4 release (Fig. 8F), suggesting that MEK1/2-dependent ERK1/2 activation contributed to the release of this lipid mediator.

FcRb is a signal transducer responsible for LTC4 release in the absence of a functional FcRγ–ITAM

A previous study employing a chimera of the IL-2Rα subunit (Tac) and FcRb suggested that the FcRb ITAM lacks signaling capacity (30). However, we re-evaluated the possibility that FcRb itself acts as a signal transducer that leads to LTC4 secretion in the absence of a functional FcRγ–ITAM. To prove this hypothesis, we generated mice deficient in both FcRb and FcRγ. The FcRb−/−

**FIGURE 10.** The FcRb–ITAM regulates LTC4 secretion in an FcRγ–ITAM-independent manner. FcRbYYFF and FcRbFFFF mast cells were sensitized with 15.3.2 and stimulated with or without PpL at the indicated concentrations. In (A) and (B), A23187 (1 μM) was used as a nonimmunological positive control in place of PpL. (A) The net LTC4 production. Data shown are the mean ± SD of quadruplicates. Similar results were obtained in three independent experiments. (B) PpL-mediated calcium mobilization was analyzed in the 15.3.2-sensitized FcRbYYFF and FcRbFFFF mast cells, as shown in Fig. 4B. The results shown are representative of three independent experiments with similar results. (C) The ERK1/2 phosphorylation was analyzed by immunoblotting with anti–phospho-ERK1/2 (Thr202/Tyr204) Ab. The blot was reprobed with anti–nonphospho-Ab. Densitometric data obtained from three independent experiments (bottom panel) are shown as mean ± SD. (D) The changes in cell-surface FcεRI expression in 15.3.2-sensitized FcRbYYFF and FcRbFFFF mast cells by PpL stimulation. Data obtained from three independent experiments (bottom panel) are shown as mean ± SD. The statistical analysis was performed using Student t test. ***p < 0.001.
BMMCs failed to express the FcRβ and FcRγ proteins (Fig. 9A). We then prepared two cotransfectants (FcRβYYγFF and FcRβFFγFF) that harbored mutations in the tyrosine residues of each ITAM (Fig. 9B, 9C). Because both FcRβ and FcRγ are indispensable for cell-surface FcεRI expression (26, 31), we recognized the FcεRI-positive cells as mast cells with the exogenous FcRβ and FcRγ genes and enriched this cell population (Fig. 9C). As shown in Fig. 10A, the 15.3.2-sensitized FcRβFFγFF mast cells did not produce LTC4 in response to PpL loading. Consistent with this finding, [Ca2+]i mobilization and ERK1/2 activation in response to PpL were entirely abolished in the 15.3.2-sensitized FcRβFFγFF mast cells (Fig. 10B, 10C). Conversely, cell-surface FcεRI was downregulated in a comparable manner in the 15.3.2-sensitized FcRβYYγFF and FcRβFFγFF mast cells following PpL stimulation (Fig. 10D), suggesting that FcεRI cross-linking-induced receptor internalization is unaltered by introducing mutations into the FcRβ–ITAM. Fig. 11 illustrates a model of mast cell activation triggered by 15.3.2 and PpL.

**Discussion**

Mast cells are resident in various tissues, where they act as innate immune cells against pathogens. Previous studies demonstrated that PpL activates human mast cells by interacting with IgEx (14, 15), suggesting that FcεRI, the high-affinity receptor for IgE, is a critical participant in PpL-mediated mast cell activation. Consistent with these studies, we also found that sensitization of mast cells with IgEx (15.3.2) is required for cytokine production, the degranulation response, and LTC4 release in murine mast cells upon PpL loading. Like FcεRI, other Fc receptors (such as FcγRI, FcγRIII, and FcγRIIV) also require FcγRI for their cell-surface expression. It has been reported that FcγRIIV acts as a low-affinity receptor for mouse IgE (32, 33). However, murine mast cells do not express FcγRIIV (33), and 15.3.2 does not bind to FcγRI, FcγRIII, or FcγRIIV (32). In addition, we showed that FcγRIIV and FcεRI−/− mast cells lack PpL-dependent cell responses. Therefore, we conclude that FcεRI definitely contributes to the activation of murine mast cells induced by IgEx (15.3.2) and PpL.

In this study, the murine IgEx clones that were examined, with the exception of 15.3.2, showed little or no binding to PpL, which was similar to the previous finding that out of six murine IgEx clones, at least three could not bind to PpL (34). Murine IgE clonal clones have been divided into two allotypes, IgEa and IgEb (35). Because 15.3.2, 27.74, and H1-ε-26.82 are IgEa allotypes, and C48-2 is an IgEb allotype, we believe that the specific IgE allotype does not determine the ability of IgEx to bind to PpL. As demonstrated in the current study, the avidity between 15.3.2 and PpL is sufficient to allow FcεRI-dependent cytokine production, the degranulation response, and LTC4 secretion by mast cells. However, we think that the avidity between 15.3.2 and PpL may be much lower than that between human polyclonal IgEx and PpL for the following reasons: 1) the interaction between human polyclonal IgEx and PpL elicits a robust degranulation response (36); 2) the interaction between 15.3.2 and PpL elicited an apparent but weak degranulation response; and 3) high-avidity interactions between IgE and multivalent ligands are required for robust degranulation (21). We speculate that the avidity between 15.3.2 and PpL may explain why stimulation of FcεRI with 15.3.2 and PpL does not induce a strong degranulation response.

In the case of stimulation of FcεRI by IgE and its specific Ag, FcRγ–ITAM is a well-known signal transducer responsible for FcεRI-dependent mast cell function. By contrast, FcRβ–ITAM is a known modulator of FcγRI–ITAM-dependent signaling (31, 37, 38). Sakurai et al. (39) and Yamashita et al. (40) have independently reported that FcRγ–ITAM is indispensable for cytokine production, the degranulation response, and PGD2 secretion in mast cells upon FcεRI stimulation. With respect to IL-6 production and the degranulation response, our data supported these previous studies. Interestingly, our results on LTC4 secretion were not consistent with the current theory that all aspects of mast cell activation depend on the FcγRI–ITAM functions. The discrepancy in the secretion of LTC4 and PGD2 may be explained by a previous report, which demonstrated that BMMCs predominantly synthesize LTC4, not PGD2, upon FcεRI cross-linking (41), suggesting that LTC4 is a more highly detected lipid mediator than PGD2. Our experiments employing cotransfectants (FcRβYYγFF and FcRβFFγFF) clearly revealed that the FcRβ–ITAM contributes to FcγRI–ITAM-independent LTC4 production from mast cells stimulated with 15.3.2 and PpL by activating ERK1/2 and [Ca2+]i mobilization. To our knowledge, this is the first report demonstrating a novel role for the FcRβ–ITAM as a functional signal transducer in FcεRI-mediated mast cell activation. Currently, it is unclear how the FcRβ–ITAM itself regulates ERK1/2 and calcium signaling in the absence of a functional FcγRI–ITAM. Because the tyrosine-phosphorylated FcRβ–ITAM peptide can bind to Lyn, Syk, and PLCγ1 (31, 42, 43), these signaling molecules are likely to play pivotal roles in the FcRβ–ITAM-dependent regulation of ERK1/2 and calcium signaling. Recently, it was reported that the internalization of FcεRI indepen-
dently activates calcium signaling in mast cells (44). We previously reported that the FcRβ–ITAM is essential for FcεRI internalization upon the stimulation of FcεRI by highly cytophilic IgE (20); we therefore expected that FcεRIβγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma}


