

# The $\beta$ Subunit of the Fc $\epsilon$ RI Is Associated with the Fc $\gamma$ RIII on Mast Cells

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## Summary

Fc $\epsilon$ RI is a tetrameric receptor, composed of a ligand recognition subunit,  $\alpha$ , a  $\beta$  chain, and dimeric  $\gamma$  chains. Previous studies have indicated that the dimeric  $\gamma$  chain is associated with Fc $\gamma$ RIIIA (CD16) on natural killer cells and macrophages as well as the clonotypic T cell receptor. Here we show that in mast cells, in addition to the dimeric  $\gamma$  chains, the  $\beta$  subunit is associated not only with Fc $\epsilon$ RI, but also with Fc $\gamma$ RIIIA. Functional reconstitution studies with a mastocytoma cell line indicate that Fc $\gamma$ RIIIA composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits has the capacity for signal transduction. These studies suggest that through the association of alternative ligand recognition subunits ( $\alpha_\epsilon$ ,  $\alpha_\gamma$ ), a common signal transduction complex ( $\beta\gamma_2$ ) mediates similar biochemical and effector functions in response to immunoglobulin G (IgG) and IgE.

Immune complexes trigger diverse inflammatory and immunomodulatory responses by virtue of their interaction with isotype-specific Fc receptors expressed on a wide array of hematopoietic cells. Consistent with this functional heterogeneity, recent studies have elucidated a comparable structural heterogeneity, resulting in a family of membrane proteins that differ in their membrane spanning and intracytoplasmic domains (1–3). Fc $\gamma$ RIIIA (CD16) expressed on NK cells and macrophages mediates antibody-dependent cellular cytotoxicity and phagocytosis. Fc $\epsilon$ RI on mast cells and basophils induces allergic reactions. Previous studies have indicated that Fc $\gamma$ RIIIA and Fc $\epsilon$ RI are closely related both structurally and functionally. Fc $\gamma$ RIIIA- $\alpha$  is associated with the  $\gamma$  chain, which was initially identified as one subunit of Fc $\epsilon$ RI (4–8). Crosslinking of either of these two receptors results in inositol phospholipid hydrolysis and increases in intracellular calcium (1, 9). In mast cells, both IgE and IgG have been shown to trigger similar responses through their interaction with their respective Fc receptors (10). In this study we demonstrate that these two receptors share both  $\beta$  and  $\gamma$  subunits, differing only in their ligand recognition  $\alpha$  subunit.

## Materials and Methods

**Cell Culture and DNA Transfection.** MC9 cells were cultured in DME containing mouse spleen-conditioned medium and 10% FCS. COS-7 cells were cultured in DME containing 10% FCS. Rat Fc $\epsilon$ RI  $\beta$  subunit cDNA was isolated (11) by PCR from the rat basophilic leukemia (RBL) mRNA, and cloned into the pCEXV-3

expression vector. cDNAs (human and mouse Fc $\gamma$ RIIIA- $\alpha$ , human Fc $\epsilon$ RI- $\alpha$ , human TCR/CD3  $\zeta$ , and mouse Fc $\epsilon$ RI- $\gamma$ ) for COS-7 cell transfections were also cloned into pCEXV-3 (1). DNA was transfected into COS-7 cells using DEAE-dextran (150  $\mu$ g/ml) in the presence of 100  $\mu$ M chloroquine followed by 20% glycerol shock. For P815 cell transfections, the plasmid containing human Fc $\gamma$ RIIIA- $\alpha$  and  $\gamma$  cDNAs and neomycin-resistant gene was constructed (pCNeo- $\alpha\gamma$ ), and Fc $\epsilon$ RI- $\beta$  cDNA was cloned into pBabe Puro expression vector (12). P815 cells were transfected with pCNeo- $\alpha\gamma$  alone or a combination of pCNeo- $\alpha\gamma$  and  $\beta$  cDNA by the calcium-phosphate method, and selected in the presence of G418 alone or G418 and puromycin. Clones were screened by flow cytometry using 3G8, and expression of the  $\beta$  subunit was checked by Western blotting analysis.

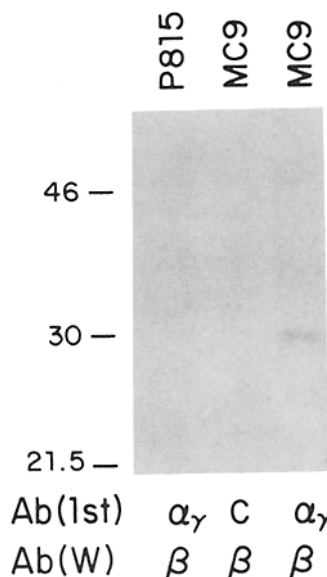
**Antibodies.** The peptide synthesized from the cytoplasmic domain of mouse Fc $\gamma$ RIIIA- $\alpha$  was used to raise the polyclonal antibody. Antibodies against human Fc $\gamma$ RIIIA- $\alpha$ ,  $\gamma$ , and  $\zeta$  chains were already described (13). Antibody against  $\beta$  chain was kindly provided by J.-P. Kinet (National Institutes of Health) (14).

**Immunoprecipitation Analysis.** MC9 cells and transfected COS-7 cells were iodinated with  $^{125}$ I by using 1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenylglycouril (Iodo-Gen; Pierce Chemical Co., Rockford, IL). Cells were solubilized in lysis buffer containing 1% digitonin and 0.12% Triton X-100. Cell lysates were sequentially incubated (2 h, 4°C for each incubation) with antibodies and with protein A-Sepharose or protein A-Sepharose coated with rabbit anti-mouse IgG antibody. For immunoblotting, samples were separated on SDS-polyacrylamide gels and transferred to an Immobilon-P sheet (Millipore Continental Water Systems, Bedford, MA). Membranes were incubated with  $^{125}$ I-labeled anti- $\beta$  antibody or with mAb IgG2bk (antiphosphotyrosine antibody; Upstate Biotechnology Inc., Lake Placid, NY) followed by alkaline phosphatase-conjugated goat anti-mouse IgG.

**Assay for Cytoplasmic-free Calcium ( $[Ca^{2+}]_i$ ) Increase, Inositol Phosphate Generation, and Tyrosine Phosphorylation.** For  $[Ca^{2+}]_i$  measurements, the cells were incubated at 37°C for 45 min in 20 mM Hepes buffer (pH 7.5) containing 5 mM glucose, 0.025% BSA, 1 mM  $CaCl_2$ , and 3  $\mu$ M fura-2/acetoxymethylester. The loaded cells ( $10^6$  cells/ml) were stimulated with 3G8 (0.7  $\mu$ g/ml) and  $F(ab')_2$  of goat anti-mouse IgG (3  $\mu$ g/ml). Fluorescence changes were monitored with an Alphascan spectrofluorimeter (Photon Technology International Inc.) at an excitation wavelength of 340 nm and an emission wavelength of 505 nm.  $[Ca^{2+}]_i$  was calibrated and computed as described (15). For inositol phosphate measurements, transfectants were labeled with  $[^3H]myo$ -inositol (10  $\mu$ Ci/ml) for 6 h in inositol-free RPMI 1640 with 10% dialyzed FCS. The cells ( $5 \times 10^6$  cells/ml) were then stimulated with 3G8 (10  $\mu$ g/ml) in the presence of LiCl. At various time points, the cells were lysed and soluble inositol phosphates were extracted with TCA, and applied to AG1-X8 ion exchange columns (Bio-Rad Laboratories, Richmond, CA) (16). For tyrosine phosphorylation analysis, cells ( $2 \times 10^6$  cells/ml) were stimulated (3 min for each incubation) with 3G8 (5  $\mu$ g/ml) or  $F(ab')_2$  of goat anti-mouse IgG (10  $\mu$ g/ml) in addition to 3G8.

## Results and Discussion

The mouse mast cell line MC9 expresses the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of Fc $\epsilon$ RI and the  $\alpha$  subunit of Fc $\gamma$ RIIIA, as assessed by Northern blotting (data not shown). Previous studies have indicated that  $\gamma$  associates with Fc $\gamma$ RIIIA (6–8). To determine if the  $\beta$  chain of Fc $\epsilon$ RI also associates with Fc $\gamma$ RIIIA, MC9 cells were surface labeled with  $^{125}I$  in the presence of 0.01% digitonin, solubilized, and immunoprecipitated using anti-Fc $\gamma$ RIIIA- $\alpha$  antibody. Anti-Fc $\gamma$ RIIIA- $\alpha$  coimmunoprecipitated the ligand binding subunit  $\alpha$ , the  $\gamma$  chain, and in addition, a 30-kD protein. This 30-kD protein migrated with the same apparent molecular mass as the Fc $\epsilon$ RI  $\beta$  subunit, shown by using anti- $\beta$  antibody (Fig. 1 A). The association of the 30-kD protein was not the result of cross reactivity

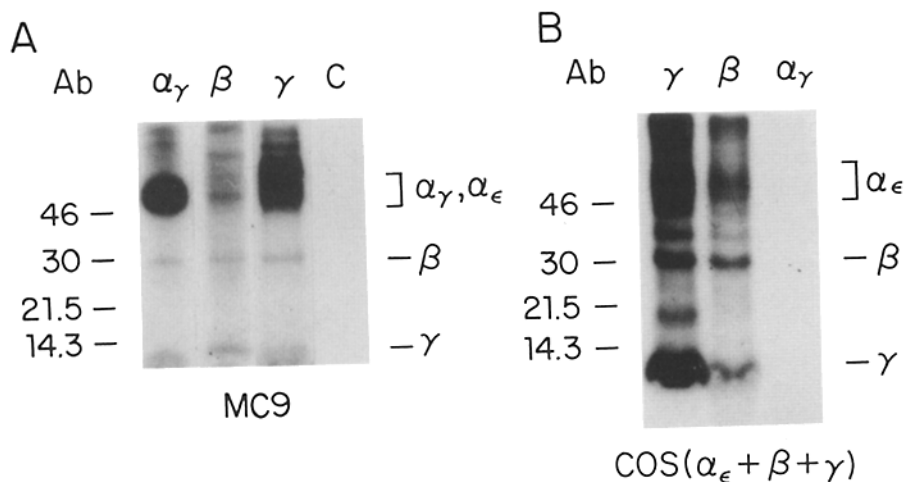


**Figure 2.** Identification of 30-kD protein as Fc $\epsilon$ RI  $\beta$  subunit. Digitonin lysates of MC9 cells and P815 cells were immunoprecipitated with anti-mouse Fc $\gamma$ RIIIA- $\alpha$  or control antibodies. These immunoprecipitated samples were separated on 12.5% SDS-polyacrylamide gels, transferred to Immobilon-P sheet, and incubated with  $^{125}I$ -labeled anti- $\beta$  antibody.

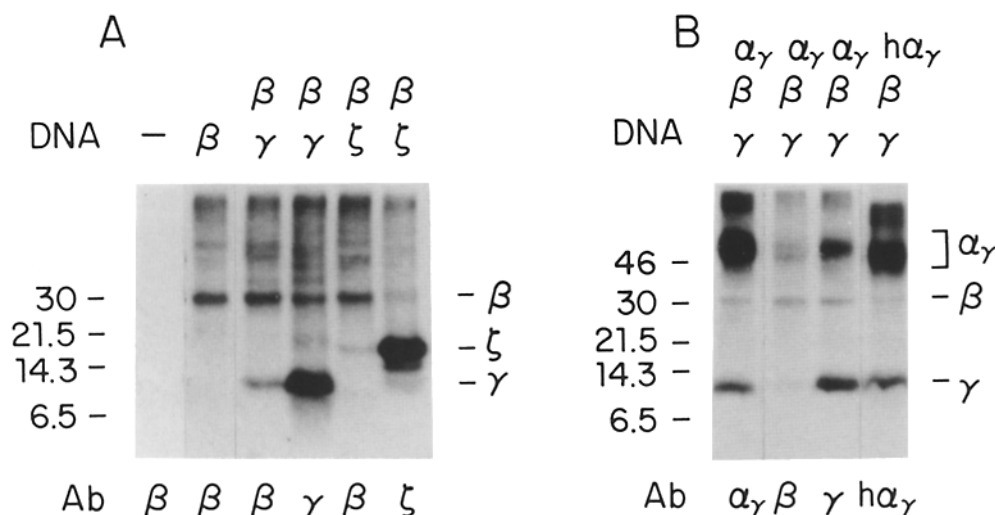
of the anti-Fc $\gamma$ RIIIA- $\alpha$  antibody with Fc $\epsilon$ RI- $\alpha$ , as demonstrated by Fig. 1 B. COS cells transfected with Fc $\epsilon$ RI- $\alpha$ , - $\beta$ , and - $\gamma$  chains were surface labeled and immunoprecipitated with specific antibodies. Anti- $\beta$  and anti- $\gamma$  antibodies coimmunoprecipitated these three chains, whereas anti-Fc $\gamma$ RIIIA- $\alpha$  antibody did not (Fig. 1 B).

To identify the 30-kD protein as  $\beta$ , we prepared cell lysates from MC9 cells and immunoprecipitated with anti-Fc $\gamma$ RIIIA- $\alpha$  antibody. The immunoprecipitated samples were separated on 12.5% SDS-polyacrylamide gels, transferred to membrane filters, and incubated with  $^{125}I$ -labeled anti- $\beta$  antibody. Isotype-matched control antibody precipitates of MC9 cells and anti-Fc $\gamma$ RIIIA- $\alpha$  precipitates of P815 cells were run in parallel as controls. The mouse mastocytoma cell line P815 expresses mouse Fc $\gamma$ RIIIA- $\alpha$  and  $\gamma$  chains, but not  $\beta$  chain, as determined by both RNA and protein analysis (6, 22).  $\beta$  chain was only found in the MC9 lysates precipitated with anti-Fc $\gamma$ RIIIA- $\alpha$  specifically, but not in either control (Fig.

<sup>1</sup> Abbreviation used in this paper:  $[Ca^{2+}]_i$ , cytoplasmic-free calcium.



**Figure 1.** Association of 30-kD protein with Fc $\gamma$ RIIIA. Immunoprecipitation analysis of MC9 cells (A) and COS cells transfected with Fc $\epsilon$ RI- $\alpha$ , - $\beta$ , and - $\gamma$  subunits (B). Cell lysates were prepared with digitonin lysis buffer, were incubated with indicated antibodies (C, control antibody), and immune complexes were separated on 10% Tricine-SDS-polyacrylamide gels.



**Figure 3.** Complex formation of various combinations of Fc $\gamma$ RIIIA- $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\zeta$  chains. COS cells transfected with various combinations of indicated cDNAs were labeled with  $^{125}$ I and immunoprecipitated with indicated antibodies.  $\alpha_\gamma$  and  $h\alpha_\gamma$  indicate mouse Fc $\gamma$ RIIIA- $\alpha$  and human Fc $\gamma$ RIIIA- $\alpha$ , respectively.

2). These results indicate that in mast cells, Fc $\epsilon$ RI- $\beta$  is not only one component of Fc $\epsilon$ RI but is also complexed with Fc $\gamma$ RIIIA- $\alpha$  and  $\gamma$  chains.

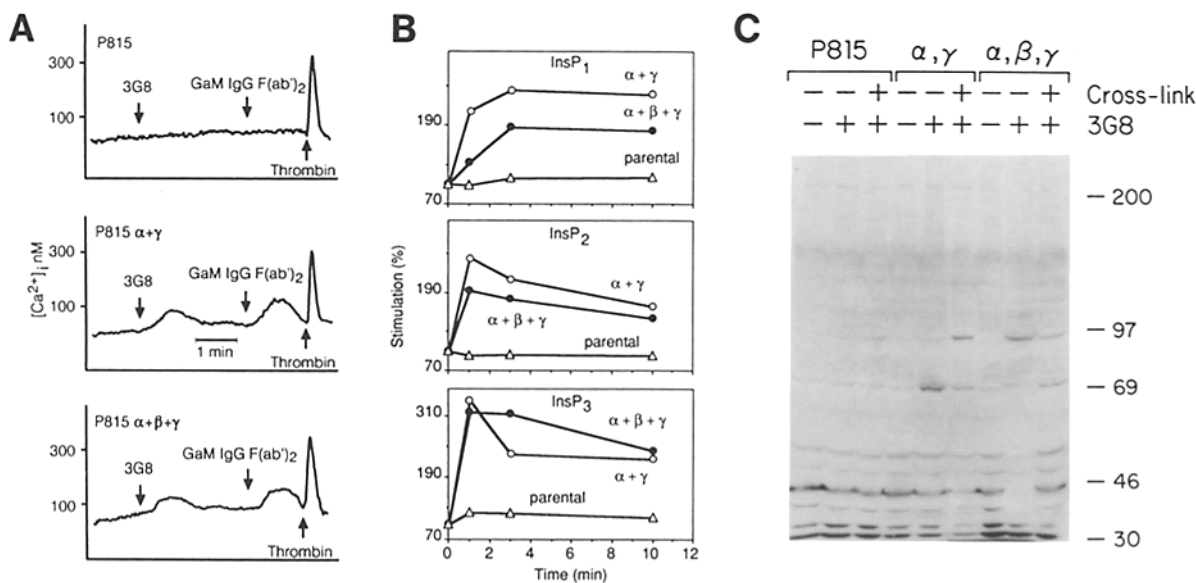
To gain further insight into the nature of the association of  $\beta$  with Fc $\gamma$ RIIIA, we used a transient cDNA expression system in COS cells. We have previously shown that Fc $\gamma$ RIIIA- $\alpha$  interacts directly with  $\gamma$  or TCR/CD3  $\zeta$  chain in the endoplasmic reticulum. In the absence of  $\gamma$  or  $\zeta$  chains, the  $\alpha$  subunit is rapidly degraded (13). One function of the  $\gamma$  subunit is in the assembly of the Fc $\gamma$ RIIIA complex by protecting the  $\alpha$  subunit from degradation. Therefore, we studied the requirement of  $\beta$  chain for surface expression of Fc $\gamma$ RIIIA- $\alpha$  by flow cytometry analysis. As has been reported (6–8), cotransfection of the  $\alpha$  subunit with  $\gamma$  resulted in a 20-fold stimulation of surface expression of Fc $\gamma$ RIIIA- $\alpha$ . The  $\beta$  subunit was unable to substitute for the  $\gamma$  chain to enhance surface expression of Fc $\gamma$ RIIIA- $\alpha$ . The efficiency of the cell surface expression of COS cells transfected with  $\alpha$ ,  $\beta$ , and  $\gamma$  was nearly identical to the cell surface expression observed with  $\alpha$  and  $\gamma$  (data not shown). No difference in the kinetics of  $\alpha$  degradation was observed in pulse-chase experiments of COS cells transfected with either  $\alpha$ ,  $\beta$ ,  $\gamma$  or  $\alpha$ ,  $\gamma$ . These results are similar to what has been observed for human Fc $\epsilon$ RI, in which  $\gamma$  is sufficient for cell surface expression of human Fc $\epsilon$ RI- $\alpha$  (17).

The  $\gamma$  chain is closely related to the  $\zeta$  chain of TCR/CD3 complex (17–19) and is found complexed with the Fc $\gamma$ RIIIA- $\alpha$  chain in NK cells (20, 21). Association of  $\beta$  with Fc $\gamma$ RIIIA- $\alpha$ ,  $\gamma$ , or  $\zeta$  in COS cells was therefore examined by coimmunoprecipitation experiments. The  $\beta$  subunit was complexed with the  $\gamma$  or  $\zeta$  subunit (Fig. 3 A). COS cells transfected with Fc $\gamma$ RIIIA- $\alpha$ ,  $\beta$ , and  $\gamma$  chains formed the complex observed in MC9 cells (Fig. 3 B), suggesting that the interaction of these three chains do not require cell type-specific proteins. Similar results were obtained when these subunits were translated *in vitro* in the presence of microsomal membranes (data not shown). We have consistently observed in both MC-9 cells (Fig. 1 A) and COS cell transfectants (Fig.

3) that the stoichiometry of  $\beta$  in complexes with  $\alpha$ ,  $\gamma$ , or  $\zeta$  subunits varies depending on the antibody used for coimmunoprecipitation. In Fig. 1 A, the antibodies against  $\alpha_\gamma$  or  $\gamma$  chain are far more efficient in precipitating  $\alpha$  chain than is the anti- $\beta$  antibody. Similarly, in Fig. 3 A, anti- $\beta$  antibody precipitates the  $\beta$ - $\gamma$  complex more efficiently than the  $\beta$ - $\zeta$  complex. These differences might be related to the stability of these various complexes in the presence of the antibodies used, partial dissociation of the receptors after solubilization, or may reflect the distribution of these complexes *in vivo*.

Fc $\gamma$ RIIIA on NK cells and macrophages is composed of at least three polypeptide chains: a ligand-recognition subunit (Fc $\gamma$ RIIIA- $\alpha$ ) and the associated dimeric  $\gamma$  and  $\zeta$  chains (5–8, 20, 21), whereas on mast cells it is composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  chain. To characterize potential functional differences between these Fc $\gamma$ RIIIA isoforms ( $\alpha_\gamma$  and  $\alpha_\beta\gamma$ ), these complexes were reconstituted in transfected cells. Fc receptors for IgE and IgG are incapable of evoking proximal or distal signals in heterologous cells, such as fibroblasts. To study the signaling capacity of these isoforms, we used the mouse mastocytoma cell line P815 (22) for transfection of the human Fc $\gamma$ RIIIA- $\alpha$  and  $\gamma$  subunits or, alternatively, the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. Stable transfectants were obtained, and the density of cell surface expression for these two isoforms was quantitated by flow cytometry using the mAb 3G8. Comparable levels of Fc $\gamma$ RIIIA- $\alpha$  were observed for each isoform.

Immunoprecipitation experiments confirmed the association of  $\alpha$  with the  $\gamma$  subunit, or alternatively with the  $\beta$  and  $\gamma$  subunits, respectively (data not shown). The ability of 3G8 to activate an increase in  $[Ca^{2+}]_i$  and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) hydrolysis in these transfected cells was evaluated. In both transfectants, a substantial increase in  $[Ca^{2+}]_i$  was seen in response to 3G8, which was further enhanced by crosslinking using F(ab')<sub>2</sub> of goat anti-mouse IgG (Fig. 4 A). Stimulation of Fc $\gamma$ RIIIA with 3G8 resulted in the generation of inositol phosphates in both transfectants (Fig. 4 B). Since activation of a tyrosine kinase



**Figure 4.** Signal transducing properties of P815 cells expressing  $\alpha,\gamma$  and  $\alpha,\beta,\gamma$ .  $[Ca^{2+}]_i$  increase (A), inositol phosphate generation (B), and tyrosine phosphorylation (C) after stimulation of  $\alpha,\gamma$  and  $\alpha,\beta,\gamma$  transfectants. Each lane of tyrosine phosphorylation patterns represents lysates from  $\sim 2 \times 10^6$  cells.

pathway by stimulation of TCR/CD3 (23), Fc $\epsilon$ RI (24), and Fc $\gamma$ R1IIIA (25) has been established, we sought to determine whether 3G8 would stimulate tyrosine kinase activation in both transfectants. Western blot analysis using a mAb against phosphotyrosine revealed a 70-kD protein whose phosphorylation was stimulated by 3G8 in the  $\alpha,\gamma$  transfectant, whereas in the  $\alpha,\beta,\gamma$  transfectant, tyrosine phosphorylation of this protein required additional crosslinking of the receptor. In contrast, tyrosine phosphorylation of a 90-kD protein in the  $\alpha,\beta,\gamma$  transfectant was stimulated with 3G8 only (Fig. 4 C). These results indicate that both the  $\alpha,\gamma$  complex and the  $\alpha,\beta,\gamma$  complex have the capability for transmitting early responses. Antibodies to endogenous murine Fc $\gamma$ Rs elicit similar signals in P815 cells (22, 29).

The demonstration that Fc $\gamma$ R1IIIA on mast cells is com-

posed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits raises the possibility that NK cells and macrophages, the other two cell types known to express Fc $\gamma$ R1IIIA, express a tetrameric form of this receptor. Although by RNA and protein analysis  $\beta$  expression is restricted to mast cells, structurally related molecules have been described on other hematopoietic cell types (26–28) and may be candidates for association with Fc $\gamma$ R1IIIA. Indeed, the presence of such molecules may account for the ability of P815 cells to signal in the absence of a transfected  $\beta$  chain. The identification of these related molecules and their functional dissection will certainly shed light on the complexities of these receptors. In mast cells, however, Fc-mediated signaling has converged so that structurally distinct ligands result in a common signaling pathway by virtue of the exchange of ligand recognition subunits in a tetrameric receptor complex.

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