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## Distinct Aggregation of $\beta$ - and $\gamma$ -Chains of the High-affinity IgE Receptor on Cross-Linking

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**SUMMARY** The high-affinity IgE receptor (Fc $\epsilon$ RI) on mast cells and basophils consists of a ligand-binding  $\alpha$ -chain and two kinds of signaling chains, a  $\beta$ -chain and disulfide-linked homodimeric  $\gamma$ -chains. Crosslinking by multivalent antigen results in the aggregation of the bound IgE/ $\alpha$ -chain complexes at the cell surface, triggering cell activation, and subsequent internalization through coated pits. However, the precise topographical alterations of the signaling  $\beta$ - and  $\gamma$ -chains during stimulation remain unclarified despite their importance in ligand binding/signaling coupling. Here we describe the dynamics of Fc $\epsilon$ RI subunit distribution in rat basophilic leukemia cells during stimulation as revealed by immunofluorescence and immunogold electron microscopy. Immunolocalization of  $\beta$ - and  $\gamma$ -chains was homogeneously distributed on the cell surfaces before stimulation, while crosslinking with multivalent antigen, which elicited optimal degranulation, caused a distinct aggregation of these signaling chains on the cell membrane. Moreover, only  $\gamma$ - but not  $\beta$ -chains were aggregated during the stimulation that evoked suboptimal secretion. These findings suggest that high-affinity IgE receptor  $\beta$ - and  $\gamma$ -chains do not co-aggregate but for the most part form homogenous aggregates of  $\beta$ -chains or  $\gamma$ -chains after crosslinking.

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### KEY WORDS

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MAST CELLS and basophils express the high-affinity IgE receptor Fc $\epsilon$ RI on their surface, which plays a pivotal role in the initiation of allergic reactions. Fc $\epsilon$ RI is composed of two kinds of functional subunits: a ligand-binding  $\alpha$ -chain, and signaling chains, a  $\beta$ -chain and disulfide-linked homodimeric  $\gamma$ -chains (Metzger 1992a; Kinet 1999). The  $\alpha$ -chain has an extracellular domain that binds monomeric IgE with high affinity ( $K_a \sim 10^9$ – $10^{10}$ ), while the  $\beta$ - and  $\gamma$ -chains contain unique cytoplasmic domains essential for downstream signaling, called immunoreceptor tyrosine-based activation motifs (ITAMs) (Cambier 1995; Jouvin et al. 1995; Daéron 1997). Be-

cause of homologies in structure and function, Fc $\epsilon$ RI belongs to the family of multichain immunoreceptors that includes B- or T-cell antigen receptors and immunoglobulin G receptors (Fc $\gamma$ R) (Weiss and Littman 1994; Daéron 1997). The engagement of Fc $\epsilon$ RI on mast cells and basophils evokes the activation of multiple signaling pathways, culminating in degranulation, lipid mediator release, and cytokine secretion (Galli 1993; Costa et al. 1998). A panel of inflammatory mediators released from these cells is responsible for the clinical manifestations of allergic reactions. The activation pathways mobilized include the recruitment of protein-tyrosine kinases such as Lyn, Syk, and Btk, tyrosine phosphorylation of various proteins, phosphatidylinositol turnover, and  $Ca^{2+}$  mobilization (Benhamou and Siraganian 1992; Jouvin et al. 1995; Daéron 1997). Fc $\epsilon$ RI engagement also activates mitogen-activated protein kinase which, in turn, regulates cytokine gene expression (Beaven and Ozawa 1996).

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Fc $\epsilon$ RI-mediated signals have been studied extensively in the rat basophilic leukemia cell line RBL-2H3 or its variant sublines. Previous morphological studies employing scanning and transmission electron microscopy demonstrated that the engaged IgE/Fc $\epsilon$ RI  $\alpha$ -chain complexes aggregate at the cell surface and are subsequently internalized through coated pits (Oliver et al. 1988). However, topographical distribution of the signaling chains of Fc $\epsilon$ RI  $\beta$  and  $\gamma$  at the electron microscopic level has not yet been investigated. Current biochemical evidence using chimeric receptor molecules indicates that the  $\gamma$ -chain aggregation alone can evoke cellular responses (Letourneur and Klausner 1991; Jouvin et al. 1994; Wilson et al. 1995), and the  $\beta$ -chain amplifies the  $\gamma$ -chain-mediated signal (Lin et al. 1996). Moreover, the results obtained with mice deficient in  $\beta$ - or  $\gamma$ -chains of Fc $\epsilon$ RI have supported such important functions of these chains (Takai et al. 1994; Dombrowicz et al. 1998). Therefore, physical interactions among the three different subunits of Fc $\epsilon$ RI at the cell surface appear to constitute a crucial event in the initiation of signal transduction. Receptor aggregation occurs within a restricted region of the cell membrane at any given time. Therefore, visualization of topographical alterations of each Fc $\epsilon$ RI subunit at the intact cell level during stimulation will give new insight into the precise links between ligand occupancy and biochemical signal elicitation. In this study we used conventional immunocytochemical methods as well as freeze-fracture replica immunoelectron microscopy (Fujimoto 1995,1997). The two-dimensional distribution of Fc $\epsilon$ RI subunits, particularly signaling  $\beta$ - and  $\gamma$ -chains, on the RBL-2H3 cell membrane has been resolved at the electron microscopic level. We propose that  $\beta$ - and  $\gamma$ -chains aggregate separately at the cell surfaces immediately after crosslinking.

## Materials and Methods

### Cell Culture, Activation, and Histamine Release Assays

RBL-2H3 cells (Barsumian et al. 1981) (American Type Culture Collection; Rockville, MD) were maintained as monolayer cultures in DMEM supplemented with 10% heat-inactivated FCS, penicillin, and streptomycin. Cells ( $2 \times 10^6$  cells/ml) were loaded with 3  $\mu$ g/ml anti-dinitrophenyl (DNP) IgE (Biogenesis; Poole, UK) at 37C for 30 min, washed three times with glucose-saline PIPES buffer (25 mM PIPES, pH 7.2, 110 mM NaCl, 5 mM KCl, 0.4 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, 5.6 mM glucose, and 0.1% BSA) and then stimulated at 37C for the indicated times with dinitrophenyl<sub>30</sub>-BSA (DNP-BSA; Cosmo Bio, Tokyo, Japan) or 15-nm colloidal gold conjugates of it (DNP-BSA-gold; OD<sub>520</sub> = 4) (Oliver et al. 1988) in the PIPES buffer. Histamine in the supernatants of the stimulated cells was measured using an ELISA kit (Shionogi; Tokyo, Japan). Stimulation with 100 ng/ml DNP-BSA or 1:100 diluted DNP-BSA-gold for 30 min caused the maximal net secretion of histamine, about 40%

of the total cell content, while 5 ng/ml of the DNP-BSA caused approximately half the maximal net secretion (data not shown).

### Immunofluorescence Microscopy

RBL-2H3 cells, cultured on 5-mm glass coverslips, were stimulated with anti-DNP IgE and DNP-BSA as described above. Immediately after being rinsed with ice-cold PBS, the cells were fixed with 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4C for 10 min. The cells were permeabilized with acetone at -20C for 10 min and serially rinsed in PBS containing 0.1 M glycine, 50 mM ammonium chloride, and 0.1 M glycine/5% BSA for 10 min at each step. IgE/ $\alpha$ -chain complex,  $\beta$ - or  $\gamma$ -chains of Fc $\epsilon$ RI in the cells were detected using the sheep polyclonal anti-rat IgE antibody (Bethyl Laboratory; Montgomery, AL), the mouse monoclonal antibody JRK for the  $\beta$ -chain (Kinet et al. 1988), or a well-characterized affinity-purified rabbit antibody for the  $\gamma$ -chain (Orloff et al. 1990; Letourneur et al. 1991), respectively, and appropriate fluorochrome-labeled secondary antibodies (Chemicon; Temecula, CA). Each antibody recognized a single protein band after SDS-PAGE and Western blotting (data not shown). Fluorescence images were observed and photographed with a Zeiss Axiophot fluorescence microscope. At least 200 cells were analyzed per coverslip.

### Immunoelectron Microscopy

**Immunolabeling on Ultrathin Cryosections.** After stimulation with DNP-BSA-gold, cells were extensively washed with PBS at 4C, fixed with a mixture of 2% paraformaldehyde/0.02% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4C for 2 days, and then processed for ultrathin cryosectioning and multiple immunogold labeling (Tokuyasu 1997). Briefly, the samples were infiltrated overnight at 4C with 2.3 M sucrose/20% polyvinyl pyrrolidone in 0.1 M phosphate buffer for cryoprotection. Cells were collected by centrifugation, mounted on specimen pins, and then rapidly frozen by plunging into liquid nitrogen. Ultrathin frozen sections (50–100-nm thick) were prepared in a cryo-ultramicrotome (Ultracut E equipped with FC-4; Reichert, Vienna, Austria). The sections were collected on drops of 2.3 M sucrose and placed on glow-discharged, Formvar-coated nickel grids. After a minimum of 30 min in blocking solution (PBS containing 0.1 M glycine/10% FCS), grids were placed on drops of the primary antibodies listed above and monoclonal mouse anti-clathrin antibody (Progen; Heidelberg, Germany) at 4C for overnight. All of these antibodies were mixed in a cocktail and diluted and mixed with the blocking solution. After washing with the blocking solution, grids were incubated with the solution of appropriate colloidal gold-conjugated secondary antibodies (Amersham, Poole, UK; and Chemicon). After washing, the sections were postfixed with 2% glutaraldehyde, stained with 2% uranyl acetate, and embedded in 3% polyvinyl alcohol. The specimens were observed using a JEOL 1200EX electron microscope operated at 80 kV.

**SDS-digested Freeze-fracture Replica Labeling (SDS-FRL).** The procedure for SDS-FRL of the RBL-2H3 cells was the same as described previously (Fujimoto 1995,1997). Briefly, cell pellets were sandwiched with copper foil and then

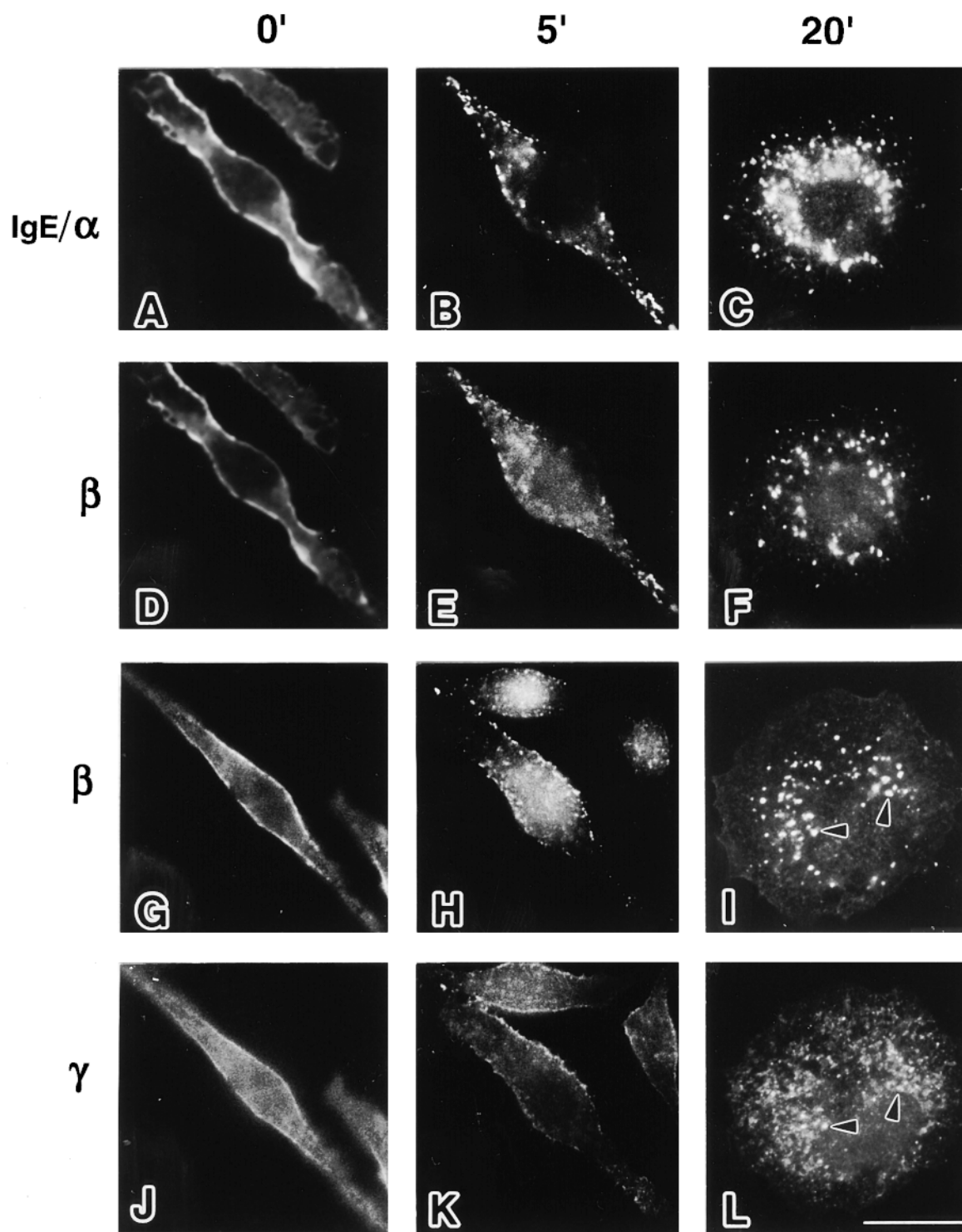
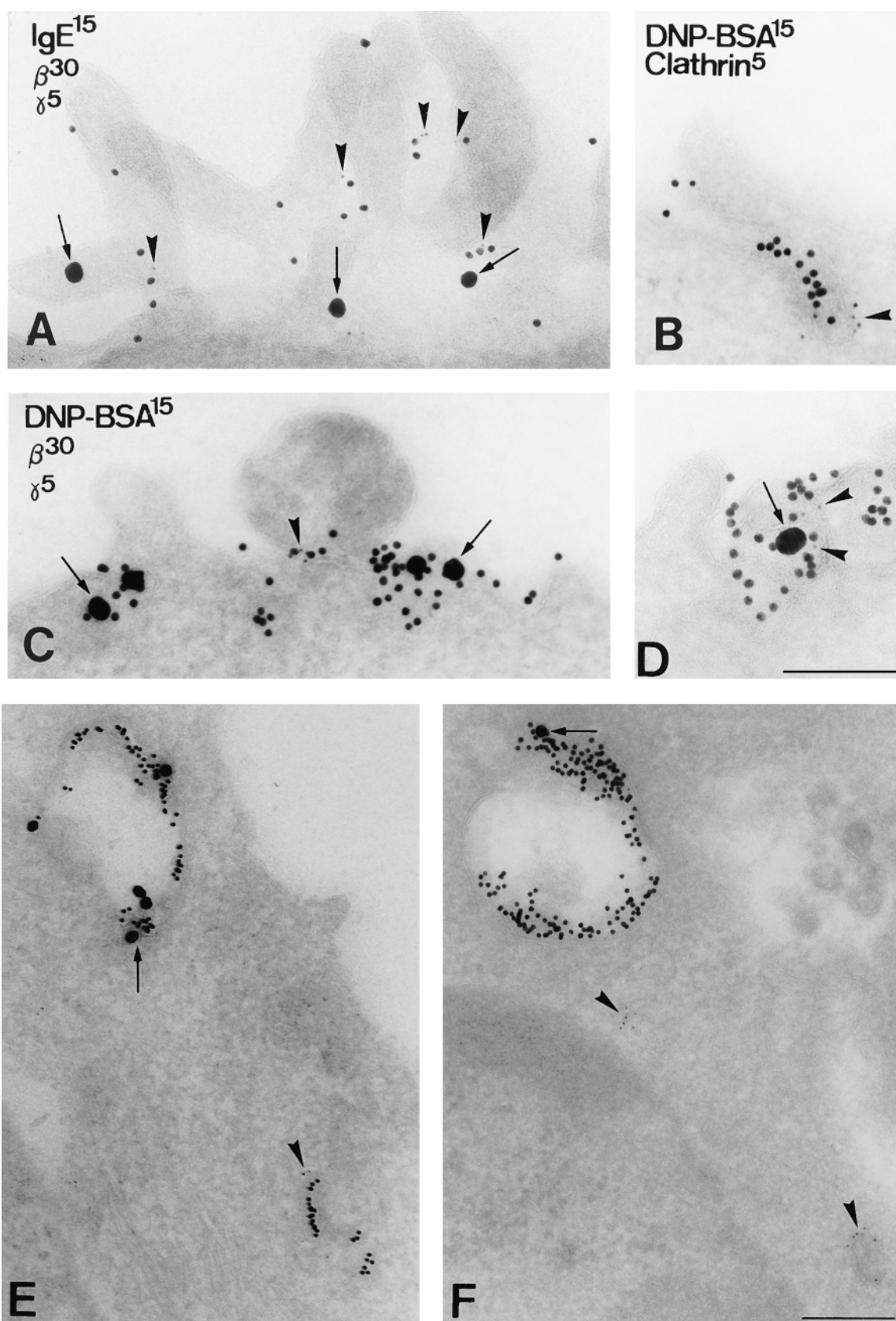


**Figure 1** Immunofluorescent localization of FcεRI chains in RBL-2H3 cells. Cells were sensitized with anti-DNP IgE (A,D,G,J) and then stimulated with 100 ng/ml DNP-BSA at 37°C for 5 min (B,E,H,K) or 20 min (C,F,I,L). Fixed and permeabilized cells were double stained with sheep anti-rat IgE (A–C) and mouse anti-β-chain antibody (D–F), followed by FITC-conjugated donkey anti-sheep and TRITC anti-mouse IgG, respectively. Another set of cells was stained with mouse anti-β (G–I) and rabbit anti-γ-chain antibody (J–L), followed by TRITC-conjugated donkey anti-mouse and FITC anti-rabbit IgG, respectively. Bar = 20 μm.



quickly frozen by being slammed against a pure copper block precooled with liquid helium. The frozen samples were fractured at  $-110^{\circ}\text{C}$  in a Balzers Freeze Etching System (BAF 400T; Bal-Tec, Hudson, NH), replicated by the deposition of platinum/carbon. Replicated samples were immersed in a sample lysis buffer containing 2.5% SDS, 10 mM Tris-HCl, and 0.6 M sucrose (pH 8.2) at room temperature for 12 hours. After SDS treatment, the replicas were washed with 10% BSA-PBS. Under these conditions, the integral membrane proteins, i.e., FcεRI subunits, were captured by replicas and their cytoplasmic domains would be accessible to the antibodies. The replicas were labeled with primary antibodies and then with the gold-conjugated secondary antibodies listed above. The samples were collected on Formvar-coated grids and examined with the electron microscope described above operated at 80 kV. For quantitation, the number of gold particles for β- and γ-chains associated with well-preserved regions of the plasma membrane were counted. At least 400 gold particles for each chain were quantified in four independent experiments.

Throughout immunoelectron microscopy, nonimmune, isotype-matched control antibodies did not give any labeling beyond the background level. Specificity of labeling and absence of signaling crossover or competition were also established by examination of single-labeled samples. In addition, a competition between different sizes of immunogold particles for double labeling was examined.

## Results

### Immunofluorescence Microscopy

To clarify the spatial fate of β- and γ-chains of FcεRI in comparison with that of the α-chain during stimulation, we first performed double immunofluorescence staining with two kinds of antibody pairs: anti-IgE antibody for the IgE/α-chain complex and anti-β-chain antibody, and anti-β- and -γ chain antibodies. RBL-2H3 cells sensitized with anti-DNP IgE were generally spindle-shaped and showed homogeneous immunofluorescence staining on the cell membranes for anti-IgE antibody, which represented anti-DNP IgE-bound α-chains (Figure 1A), β-chain (Figures 1D and 1G), and γ-chain antibodies (Figure 1J). Before and after sensitization, staining for β- or γ-chains was essentially of the same pattern (data not shown).

In cells stimulated with 100 ng/ml DNP-BSA (optimal activation) at  $37^{\circ}\text{C}$  for 5 min, the staining for the IgE/α-chain and β-chain (Figures 1B and 1E) and that for the β- and γ-chains (Figures 1H and 1K) consisted of numerous punctate dots outlining the cell surfaces. At 20

min after stimulation, the cells became flattened and spread as reported previously (Oliver et al. 1988), and immunofluorescence for the IgE/α-chain and β-chain staining was localized mainly in large intracellular vacuoles of the cells (Figures 1C and 1F). In contrast, the γ-chain staining revealed a fine granular pattern in the cytoplasm (Figure 1L), and its localization was apparently distinct from that of the β-chains (Figure 1I). However, several co-localized spots for β- and γ-chains were also observed (Figures 1I and 1L, arrowheads).

### Immunogold Electron Microscopy

#### Using Ultrathin Cryosections

To determine the localization of each subunit of FcεRI at the ultrastructural level, immunogold electron microscopy using ultrathin frozen sections was performed. In IgE-sensitized RBL-2H3 cells, immunoreactivity of anti-IgE (15-nm gold particles), anti-β-chain (30-nm particles), and anti-γ-chain (5-nm particles) antibodies were dispersed on the cell surface, including microvilli (Figure 2A) and were not located in any intracellular endosomal structures.

After crosslinking with DNP-BSA-gold (1:100 dilution with the PIPES buffer; optimal activation) at  $37^{\circ}\text{C}$  for 5 min, these particles aggregated on the cell surface, which indicate clustered α-chains. Some of them were localized in the coated pits, which was verified by immunolabeling using anti-clathrin (5-nm particles) antibody (Figure 2B). Almost all the immunogold particles for the β- and γ-chains were associated with the clusters of DNP-BSA-gold particles on cell surfaces. However, in many cases, labeling of either β- or γ-chains was independently co-localized with DNP-BSA-gold particles (Figure 2C), and the association of all three different particles was also observed (Figure 2D).

At 20 min after stimulation, the majority of the DNP-BSA-gold particles were concentrated in the perinuclear endosomal vacuoles. Immunoreactivity for the β-chain was detected almost exclusively on these vacuoles (Figure 2E). Immunolabeling for the γ-chain, however, was present not only at the DNP-BSA-gold-positive endosomes (Figure 2E) but also at the DNP-BSA-gold-negative small vesicles (Figure 2F). Co-localization of both β- and γ-chains on the same endosomes was occasionally found. Gold labeling was essentially absent in the endoplasmic reticulum and Golgi apparatus. These

**Figure 2** Immunolocalization of FcεRI chains and clathrin on ultrathin frozen section of RBL-2H3 cells. Ultrathin cryosections for IgE-sensitized cells were triple labeled with anti-IgE, anti-β (arrows), and anti-γ-chain (arrowheads) antibody (15-nm, 30-nm, and 5-nm immunogold particles, respectively) (A). Sections of the cells stimulated with 1:100 diluted DNP-BSA-15-nm gold at  $37^{\circ}\text{C}$  for 5 min (B,C) or 20 min (E,F) were labeled with anti-β (arrows) and anti-γ-chain (arrowheads) antibody (30-nm and 5-nm particles, respectively). Clathrin (5-nm particles, arrowheads) was labeled on the activated cells with DNP-BSA-15-nm gold for 5 min (D). Bar = 100 nm.

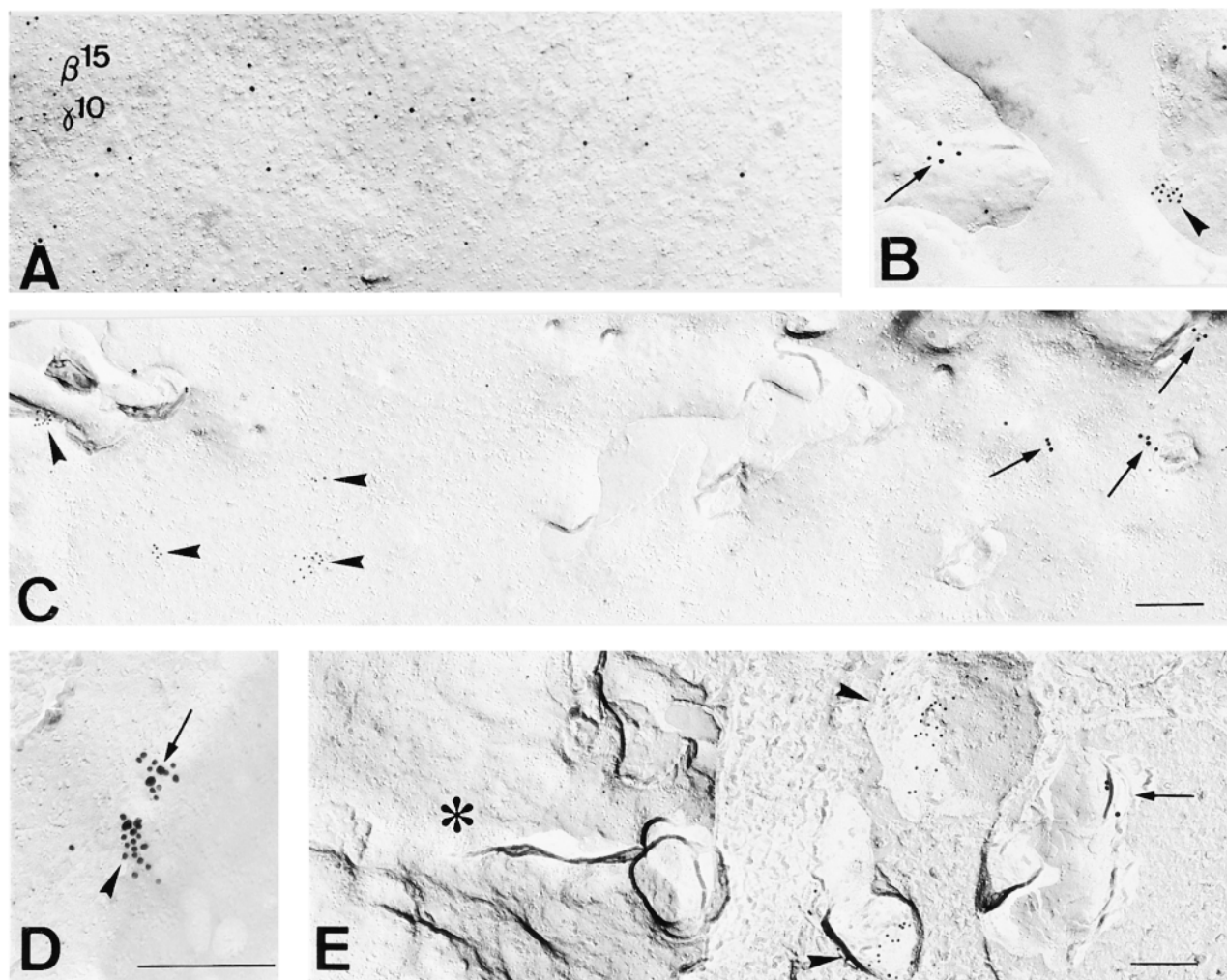


**Figure 3** Immunolocalization of FcεRI chains of RBL-2H3 cells as revealed by SDS-FRL. Cells, just sensitized (A), then stimulated with 100 ng/ml DNP-BSA at 37°C for 10 sec (B), 5 min (C,D), and 20 min (E) were labeled with anti-β (arrows) and anti-γ-chain (arrowheads) antibody (15-nm and 10-nm immunogold particles, respectively). Asterisk shows protoplasmic fracture face. Bars = 200 nm.

results are consistent with immunofluorescence microscopic findings indicating the distinct cellular localization of β- and γ-chains during endocytosis.

#### SDS-digested Freeze-fracture Replica Labeling Electron Microscopy

To further investigate the two-dimensional distribution of β- and γ-chains of FcεRI on RBL-2H3 cell membranes during crosslinking, we next performed SDS-FRL electron microscopic analysis. In platinum/carbon replicas of RBL-2H3 cell membranes, smooth exoplasmic fracture faces were easily distinguished from the protoplasmic fracture faces covered by many intramembrane particles. Because the antibodies used recognize the cytoplasmic domains of these molecules,

the immunogold particles for β- and γ-chains are associated with protoplasmic faces of the cell membrane. Before and after IgE sensitization, homogeneous and dispersed distribution of β- and γ-chain-associated immunogold particles on the protoplasmic faces were observed (Figure 3A), whereas the exoplasmic faces were virtually unlabeled.

When cells were stimulated with 100 ng/ml DNP-BSA (optimal activation) at 37°C for 10 sec, immunolocalization for β- and γ-chains showed remarkable changes. Round clusters (consisting of 4–20 particles about 100–150 nm in diameter) of gold particles were observed (Figure 3B). Interestingly, the results indicated that β- and γ-chains did not co-aggregate but for most part formed homogeneous aggregates of β-chains or γ-chains. Freeze-fracture faces of the immunoreac-

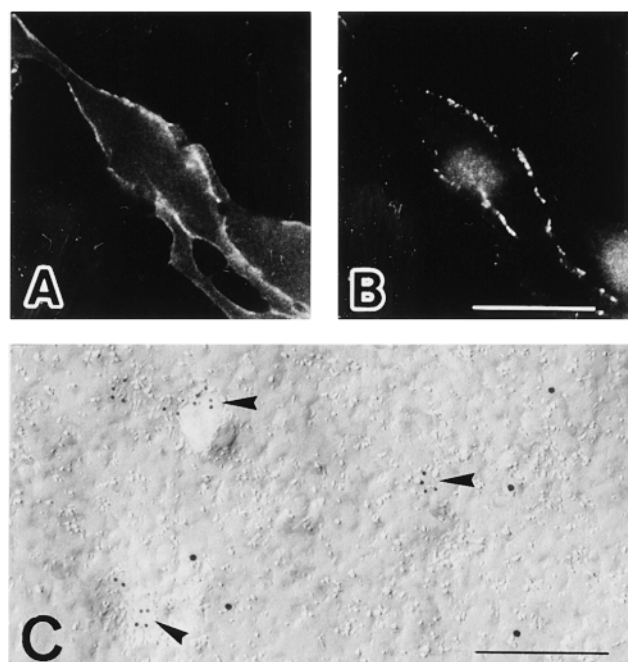


**Figure 4** Immunolocalization of FcεRI chains in suboptimally stimulated RBL-2H3 cells revealed by immunofluorescence (A,B) and SDS-FRL (C). Cells, sensitized and then stimulated with 5 ng/ml DNP-BSA at 37°C for 5 min, were double stained with anti-β (A) or anti-γ-chain antibody (B). Bar = 20 μm. The suboptimally stimulated cells were also double labeled with anti-β and anti-γ-chain (arrowheads) antibody (15-nm and 10-nm immunogold particles, respectively) (C). Bar = 10 nm.

tion-positive regions of the cell membranes were morphologically indistinguishable from those of the surrounding membranes. After crosslinking for 5 min, patchy immunolabeled clusters became more conspicuous (Figure 3C). However, as observed by cryoimmunoelectron microscopy, co-localization of the two chains was also observed (Figure 3D). At 20 min after crosslinking, immunolabeling for either chain was observed not on protoplasmic faces of the cell membranes but in intracellular membranes of vesicles or vacuoles (Figure 3E). Aggregated immunogold particles for β- and γ-chains were found in the distinct vesicles.

#### Immunofluorescence Microscopy and SDS-FRL Electron Microscopy in Suboptimally Stimulated Cells

To clarify the relationships between the signaling intensity and spatial distribution of β- and γ-chains, we performed immunofluorescence (Figures 4A and 4B) and SDS-FRL immunogold electron microscopy (Figure 4C) in the cells stimulated with 5 ng/ml of DNP-BSA that evoked a suboptimal net histamine secretion, for 5 min at 37°C. In contrast to the cells stimulated with 100 ng/ml DNP-BSA (optimal activation), β-chains

showed homogeneous immunofluorescence staining on the cell membranes (Figure 4A) like cells before crosslinking, while γ-chains had a punctate staining pattern outlining the cell surfaces (Figure 4B). In SDS-FRL electron microscopy, consistent with the results of immunofluorescence, only γ-chains were observed to be aggregated on the protoplasmic face of the cell membrane, while almost all the gold particles for β-chains remained dispersed on the membrane (Figure 4C).

Figure 5 summarizes quantitative analysis of the distribution of β- and γ-chains on the cell membrane during stimulation, as revealed by SDS-FRL labeling. The mean labeling density of immunogold particles for β- (15-nm gold particles) and γ-chains (10-nm gold particles) was 53.4 and 43.1 particles/μm<sup>2</sup>, respectively. Before crosslinking (Figure 5A), almost all the immunogold particles for both β- and γ-chains were dispersed, and no patchy clusters were observed. After suboptimal crosslinking with 5 ng/ml DNP-BSA at 37°C for 5 min (Figure 5B), however, 65.8% of a total of 421 gold particles for γ-chains were aggregated and 31.8% were dispersed, whereas 94.0% of a total of 597 particles for β-chains remained dispersed and only 4.2% were aggregated. Thus, 38 (80.9%) of 47 clusters counted contained γ-chains alone, and only five (10.6%) and four (8.5%) clusters contained β-chains alone and co-aggregated β- and γ-chains, respectively. After optimal stimulation with 100 ng/ml DNP-BSA at 37°C for 5 min (Figure 5C), 58.4% of 515 particles for β-chain and 57.9% of 466 particles for γ-chains became aggregated, without co-aggregating with each other. Under these conditions, only four (5.0%) of 80 clusters consisted of immunogold particles for both β- and γ-chains, while 34 (42.5%) consisted of β-chains alone and 42 (52.5%) of γ-chains alone. The relative density of immunolabels for β- and γ-chains did not change when the two primary antibodies were linked to a different-sized gold particle (10-nm gold particle for β-chain and 15-nm gold particle for γ-chain). These findings indicate that γ-chains alone, but not β-chains, aggregate during weak stimulation, whereas both β- and γ-chains aggregate mostly separately during optimal stimulation.

#### Discussion

Our present findings on the dynamics of the FcεRI chain distribution on crosslinking not only are consistent with the importance of the aggregation mechanism in transmembrane signaling (Metzger 1992b) but also demonstrate a new aspect of the FcεRI signaling system. SDS-FRL electron microscopy has enabled us to visualize the topographical locations of β- or γ-chains and their changes occurring in a restricted cell membrane region after FcεRI stimulation. This technique demonstrated the uniformly dispersed localization of

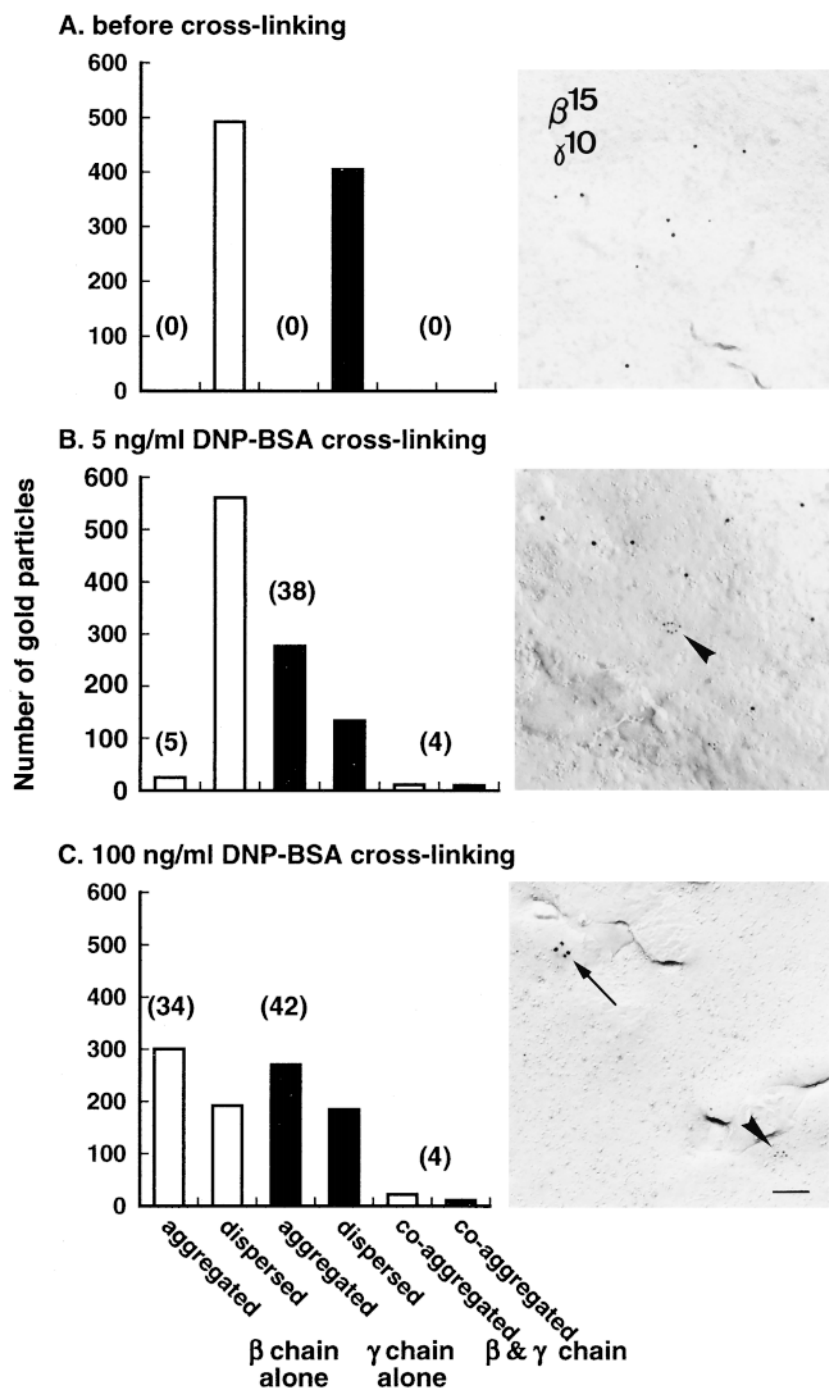


**Figure 5** Quantitative analysis of the distribution of gold labeling on the plasma membrane of RBL-2H3 cells analyzed by SDS-FRL. Histograms show the number of immunogold particles (A) before crosslinking, (B) after stimulation with 5 ng/ml DNP-BSA for 5 min (C), and with 100 ng/ml DNP-BSA for 5 min. Open and closed columns represent the number of gold particles for the  $\beta$ - and  $\gamma$ -chains, respectively. At least 400 gold particles for each chain were quantified on the well-preserved regions of the plasma membrane. The numbers of clusters are shown in parentheses. Typical micrographs of two-dimensional distribution of  $\beta$ - and  $\gamma$ -chains are also shown. Bar = 100 nm.

$\beta$ - and  $\gamma$ -chains on unstimulated RBL-2H3 cell surfaces. On antigen stimulation, the distribution of these chains changed markedly to distinctly aggregated forms. Conventional immunogold electron microscopy using ultrathin cryosections also showed that most of the  $\beta$ - and  $\gamma$ -chains are independently co-localized with clustered DNP-BSA-gold/IgE/ $\alpha$ -chain complexes on the cell surface. We therefore propose that crosslinking

with multivalent antigen leads to the distinct aggregation of  $\beta$ - and  $\gamma$ -chains on the cell membrane and subsequently to independent sorting of these molecules along the endocytic pathway.

Several hypothetical models could explain our observations on the predominant formation of complexes consisting of  $\alpha$ - and  $\beta$ -chains or  $\alpha$ - and  $\gamma$ -chains: One would be that all receptors on the cell surfaces exist as

an  $\alpha\beta\gamma_2$  tetramer before stimulation and then dissociate to generate  $\alpha\beta$  and  $\alpha\gamma_2$  partial complexes after crosslinking. Alternatively,  $\gamma$ -chains dissociate from other subunits at some point during the internalization process. Figure 2F provides some evidence for this possibility. The other possibility is that receptors before stimulation exist in various forms, such as partial complexes including  $\alpha\beta$  or  $\alpha\gamma_2$  (or even as unassociated  $\alpha$ ,  $\beta$ , or  $\gamma_2$  forms) as well as  $\alpha\beta\gamma_2$  complete tetramers, and independently aggregate after crosslinking.

Our hypothesis on the existence of partial Fc $\epsilon$ RI complexes on RBL-2H3 cell surfaces presents a striking contrast to the current tetramer model of the receptor (Metzger 1992a; Jouvin et al. 1995; Daëron 1997; Kinet 1999), which was established by affinity chromatographic analysis (Prez-Montfort et al. 1983) or immunoprecipitation of these subunits from RBL cells (Rivera et al. 1988; Blank et al. 1989), and transfection studies on COS cells (Blank et al. 1989; Ra et al. 1989). However, during purification, the  $\alpha\beta\gamma_2$  tetrameric complex is also known to be easily dissociated even in mild detergent (Kinet et al. 1985). Co-transfection of human  $\alpha$ - and  $\gamma$ -chains is sufficient to achieve expression of the receptor (Miller et al. 1989).  $\alpha\gamma_2$  complexes have been shown to be capable of inducing cell activation (Alber et al. 1991) and to exist naturally in several human hematopoietic cells, such as monocytes (Maurer et al. 1994), dendritic cells (Maurer et al. 1996), Langerhans cells (Bieber et al. 1992; Wang et al. 1992), and eosinophils (Gounni et al. 1994). Bio-synthetic labeling studies employing RBL cells suggest that the  $\alpha$ -chain associates first with the  $\beta$ -chain and  $\alpha\beta$  complex with the  $\gamma$ -chain later (Blank et al. 1989; Donnadieu et al. 2000). Complete Fc $\epsilon$ RI complexes are considered to be assembled simply to mask the retention signal and to escape degradation in the endoplasmic reticulum before surface expression (Letourneur et al. 1995). Therefore, we could not simply apply the biochemical results concerning the molecular compositions of Fc $\epsilon$ RI, which were obtained by analyzing the cell lysates or artificial transfectants, to the natural existence of Fc $\epsilon$ RI on intact RBL-2H3 cells.

Our SDS-FRL electron microscopic study revealed only the distribution of  $\beta$ - and  $\gamma$ -chains but nothing about the topographical relationship between  $\alpha$ - and  $\beta$ - or  $\gamma$ -chains. Although immunogold electron microscopy using ultrathin cryosections showed that most of the immunogold particles for  $\beta$ - and  $\gamma$ -chains are independently associated with clustered DNP-BSA-gold/IgE/ $\alpha$ -chain complexes on the cell surface, the resolution is not great enough to examine the molecular composition of each individual Fc $\epsilon$ RI complex. In addition, particular care should be taken in how precisely our immunogold labeling procedure reflects virtual number and aggregation status of membrane protein molecules. The possibility cannot be com-

pletely ruled out that the oligomerization status of molecules concerned may affect the efficiency of antibodies used in immunocytochemical experiments, and that more than one gold-labeled antibody molecule can bind to a single primary antibody on specimens. Our result in the present study appears to reflect relative distribution and oligomeric states, but because of a certain number of technical issues and limitations, we could not discard the current established concept that the receptor exists as an  $\alpha\beta\gamma_2$  complete tetramer after crosslinking stimuli.

If crosslinking stimuli uniformly affect each receptor complex on the cell surface, an additional mechanism should exist in either model to favor the aggregation of the same kinds of signaling chains over that to form  $\alpha\beta\gamma_2$  tetramers. A possible mechanism for differential aggregation would be that phosphorylated  $\beta$ - and  $\gamma$ -chains may undergo conformational changes leading to oligomerization of the same kind of subunits, as shown in cytokine receptor dimerization and transphosphorylation (Lemmon and Schlessinger 1994) or in T-cell receptor molecule oligomerization (Reich et al. 1997). Another possibility would be that a cellular component(s) other than the receptor subunits themselves, independently interacting with  $\beta$ - or  $\gamma$ -chain, could participate in the receptor aggregation. Recently, Fc $\epsilon$ RI on the cell surface was shown to be surrounded by a detergent-insoluble lipid membrane domain (Holowka and Baird 1996), and therefore these specialized membrane compositions around the receptor may affect the lateral movement of each chain in such homo-type oligomerization during stimulation.

In contrast to the distinct aggregation of  $\beta$ - and  $\gamma$ -chains in cells stimulated with an optimal concentration of antigen (Figures 3C and 5C), only  $\gamma$ -chains were aggregated on surfaces of cells stimulated with a suboptimal concentration of antigen, while  $\beta$ -chains remained dispersed (Figures 4C and 5B). These results are consistent with earlier studies on chimeric receptors (Letourneur and Klausner 1991; Jouvin et al. 1994; Wilson et al. 1995) and provide direct evidence at the intact cell level that the aggregation of  $\gamma$ -chains alone can evoke a cellular response. Moreover, these results are also consistent with the current model on the role of  $\beta$ -chains as a signaling amplifier of the Fc $\epsilon$ RI system (Lin et al. 1996). This model indicates that Lyn, a protein-tyrosine kinase that is constitutively associated with  $\beta$ -chains, phosphorylates the tyrosine residues of ITAMs of  $\beta$ - and  $\gamma$ -chains, and phosphorylated ITAM motifs of  $\gamma$ -chains in turn recruit another protein-tyrosine kinase, Syk, which phosphorylates downstream targets. It is not clear in this model how  $\alpha\gamma_2$  complexes generate the activation signal.

Our present data might suggest that  $\beta$ -chains can elicit a signal in a  $\gamma$ -chain-independent manner. Interactions between  $\beta$ -chains and Lyn might be involved in this

potential  $\beta$ -chain-dependent signaling. In this regard, Btk is shown to be activated by Lyn, and in turn activates downstream pathways such as  $\text{Ca}^{2+}$  mobilization and mitogen-activated protein kinase cascades (Rawlings et al. 1996; Kawakami et al. 1997). On the other hand, partial activation can be induced by only  $\gamma$ -chain-Syk aggregation without mobilization of the  $\beta$ -chain-Lyn systems. The topographical segregation of Lyn and Syk is consistent with recent data (Wilson et al. 2000). In any event, synergy between  $\beta$ - and  $\gamma$ -chains may be required for full activation of downstream pathways.

Fc $\epsilon$ RI belongs to the family of multichain immunoreceptors, such as B- or T-cell receptors and Fc $\gamma$ R, because of homologies in structure and function (Weiss and Littman 1994; Daéron 1997). Several cytokine receptors also assume such multichain structures (Kishimoto et al. 1994). Recent studies show that B-cell receptor (Bonnerot et al. 1995; Vilon et al. 1999), T-cell receptor-CD3 complex (Kishimoto et al. 1995), and IL-2 receptor (Hémer et al. 1995) are dissociated after stimulation and are sorted separately along the endocytic pathway. Our present findings on Fc $\epsilon$ RI, as well as these studies, suggest that multichain receptor subunits can easily dissociate from each other and function independently after ligand engagement. Figures 2E, 2F, and 3C provide some evidence that  $\beta$ - and  $\gamma$ -chains may segregate into distinct invaginations. However, it is not yet possible to determine whether these chains enter the cell via separate endocytotic vesicles or whether the  $\gamma$ -chains are selectively sorted to another vesicular compartment for recycling. Further experimentation is needed to validate these possibilities.

Finally, we speculate that different partial receptor complexes have substantially different signaling characteristics and that the relative proportion of each Fc $\epsilon$ RI complex existing on mast cells would determine the net signal intensity of the cells. To control the aggregation of individual receptor complexes could be the potential target of pharmacological intervention in Fc $\epsilon$ RI-mediated allergic reactions. Further studies are necessary to provide a clearer picture of how proximal Fc $\epsilon$ RI signaling is initiated in relation to subunit dynamics.

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