

Observing FcεRI Signaling from the Inside of the Mast Cell Membrane

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Abstract. We have determined the membrane topography of the high-affinity IgE receptor, FcεRI, and its associated tyrosine kinases, Lyn and Syk, by immunogold labeling and transmission electron microscopic (TEM) analysis of membrane sheets prepared from RBL-2H3 mast cells. The method of Sanan and Anderson (Sanan, D.A., and R.G.W. Anderson. 1991. *J. Histochem. Cytochem.* 39:1017–1024) was modified to generate membrane sheets from the dorsal surface of RBL-2H3 cells. Signaling molecules were localized on the cytoplasmic face of these native membranes by immunogold labeling and high-resolution TEM analysis. In unstimulated cells, the majority of gold particles marking both FcεRI and Lyn are distributed as small clusters (2–9 gold particles) that do not associate with clathrin-coated membrane. Approximately 25% of FcεRI clusters contain Lyn. In contrast, there is essentially no FcεRI-Syk colo-

calization in resting cells. 2 min after FcεRI cross-linking, ~10% of Lyn colocalizes with small and medium-sized FcεRI clusters (up to 20 gold particles), whereas ~16% of Lyn is found in distinctive strings and clusters at the periphery of large receptor clusters (20–100 gold particles) that form on characteristically osmiophilic membrane patches. While Lyn is excluded, Syk is dramatically recruited into these larger aggregates. The clathrin-coated pits that internalize cross-linked receptors bud from membrane adjacent to the Syk-containing receptor complexes. The sequential association of FcεRI with Lyn, Syk, and coated pits in topographically distinct membrane domains implicates membrane segregation in the regulation of FcεRI signaling.

Key words: microdomains • Lyn • Syk • rafts

Introduction

The discovery that different membrane proteins partition away from or into membrane that is internalized during phagocytosis provided early evidence that proteins can segregate nonrandomly in the plasma membrane and implicated cytoskeletal proteins in the segregation (Tsan and Berlin, 1971; Oliver et al., 1974; Oliver and Berlin, 1979). Subsequent studies raised the possibility that membrane proteins can be confined to small membrane domains or rafts characterized by unique lipid compositions (Edidin, 1997; Simons and Ikonen, 1997; Brown and London, 1998; Jacobson and Dietrich, 1999). The hypothesis that membrane-associated signaling complexes can be topographically restricted was introduced by Anderson and colleagues based on evidence that growth factor receptors and receptor-associated signaling molecules are concentrated with glycosyl-phosphatidylinositol (GPI)¹-anchored

proteins, glycosphingolipids, gangliosides, and cholesterol in smooth membrane invaginations called caveolae in fibroblasts and epithelial cells (reviewed in Anderson, 1998). Immune cells lack morphologically distinct caveolae. Nevertheless, it has been proposed that they contain membrane microdomains that are similarly enriched for GPI-anchored proteins, glycosphingolipids, gangliosides, and cholesterol and may serve as “hot spots” for signal transduction by accumulating lipid-anchored signaling molecules such as Src (Stulnig et al., 1998) and Ras family members (Mineo et al., 1997). Evidence for noncaveolar signaling domains has come largely from biochemical experiments in which signaling proteins coisolated with detergent-resistant membrane fractions during sucrose gradient centrifugation. Whether such domains exist in vivo remains controversial because detergent extraction and membrane fractionation might force interactions between previously independent membrane components and because parallel microscopic experiments have generally

enriched membranes; GPI, glycosyl-phosphatidylinositol; ITAMs, immunoreceptor tyrosine-based activation motifs; MIRRs, multi-chain immune recognition receptors; TCR, T cell receptor; TEM, transmission electron microscopy.

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¹Abbreviations used in this paper: BCR, B cell receptor; DIGs, detergent-insoluble glycolipid-enriched domains; DIMs, detergent-insoluble membranes; DRMs, detergent-resistant membranes; GEMs, glycolipid-

failed to detect microdomains of similar apparent size and stability. There are also differences in the interpretation of microdomain function. Some investigators propose that the putative microdomains serve to promote signaling, while others suggest that they may instead segregate signaling molecules from each other (Edidin, 1997; Simons and Ikonen, 1997; Kenworthy and Edidin, 1998; Jacobson and Dietrich, 1999). Recent studies suggest that such microdomains, variously called GEMs (glycolipid-enriched membranes), DIGs (detergent-insoluble glycolipid-enriched domains), DRMs (detergent-resistant membranes), DIMs (detergent-insoluble membranes), and lipid rafts, may be far smaller and more dynamic than originally proposed (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998).

The high-affinity IgE receptor, FcεRI, is a tetrameric complex consisting of an IgE-binding α subunit, a tetraspan β subunit and two disulfide-linked γ subunits (Blank et al., 1989). It is a member of a larger family of multi-chain immune recognition receptors (MIRRs), which also includes the T cell receptor (TCR), the B cell receptor (BCR), and two IgG receptors, FcγRI and FcγRIII (Cambier, 1995). These receptors have no intrinsic catalytic activity. Instead, signaling is initiated when Src-related kinases phosphorylate ITAMs (immunoreceptor tyrosine-based activation motifs) within the cytoplasmic tails of receptor subunits, creating phospho-ITAM-binding sites for the binding of Syk-related kinases. These interactions result in the activation and phosphorylation of Syk, that in turn recruit and phosphorylate SH2 domain-containing downstream signaling molecules (reviewed in Tamir and Cambier, 1998). Previous biochemical studies identified Lyn and Syk as the principal FcεRI-associated kinases of RBL-2H3 mast cells, showed that Lyn's primary substrates are the FcεRIβ and γ ITAMs, and linked Syk activation to FcεRI signal propagation (Eiseman and Bolen, 1992; Hutchcroft et al., 1992; Oliver et al., 1994; Yamashita et al., 1994; Rivera and Brugge, 1995; Zhang et al., 1996; Kinet, 1999).

It has been suggested that FcεRI signaling may occur in microdomains. Field et al. (1995, 1997, 1999) reported that the myristoylated src family member, Lyn, associates in RBL-2H3 cells with large detergent-resistant membrane domains characterized by the presence of the GPI-linked protein, Thy-1, glycosphingolipids, gangliosides, and cholesterol. Cross-linked, but not monomeric, FcεRI were also detected in these membranes, leading to the hypothesis that aggregated FcεRI might move into Lyn-containing ordered lipid microdomains and thereby initiate FcεRI signaling. However, contrasting biochemical evidence suggests that the association of FcεRI with Lyn relies primarily upon defined protein-protein interactions. In particu-

lar, Vonakis et al. (1997) demonstrated that a portion of Lyn binds to monomeric FcεRI via interactions of its unique NH₂-terminal SH4 domain with non-ITAM regions in the FcεRI β subunit COOH terminus. Furthermore, fluorescence microscopic experiments by Stauffer and Meyer (1997) suggested that aggregated FcεRI may colocalize with Syk rather than Lyn in ordered lipid microdomains. These investigators showed that FcεRI redistribute after cross-linking to ganglioside GM1-enriched membrane domains that also recruit Syk and PLC-γ by SH2 domain interactions. Peptide inhibitors of Lyn that blocked FcεRI phosphorylation also blocked Syk recruitment to the membrane (Stauffer et al., 1997).

In this report, signaling molecules were localized on native membrane sheets from the dorsal surface of RBL-2H3 mast cells by immunogold labeling and high-resolution TEM analysis. Our results show that a portion of monomeric FcεRI and Lyn are colocalized in small clusters in unstimulated cells and that Lyn is dramatically excluded from the large receptor clusters that form after FcεRI cross-linking. Moreover, cross-linked FcεRI encounter Syk and coated pits in topographically distinct membrane domains.

Materials and Methods

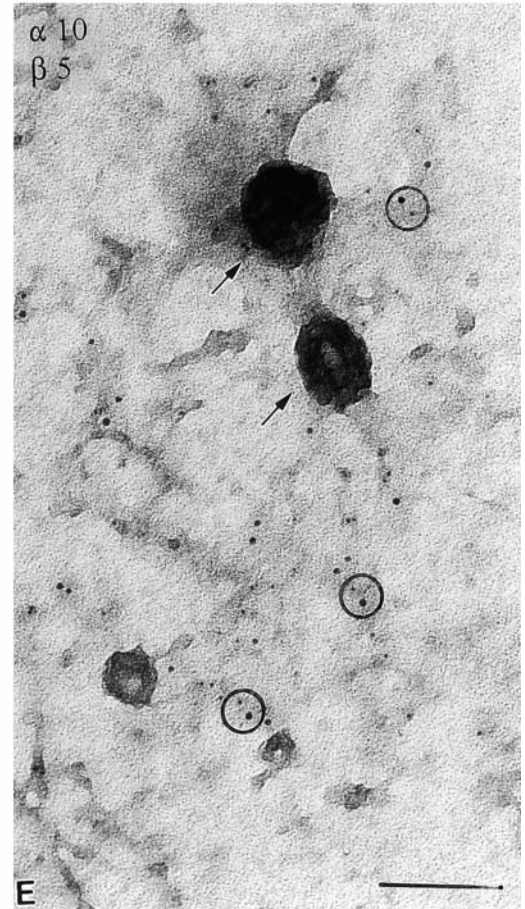
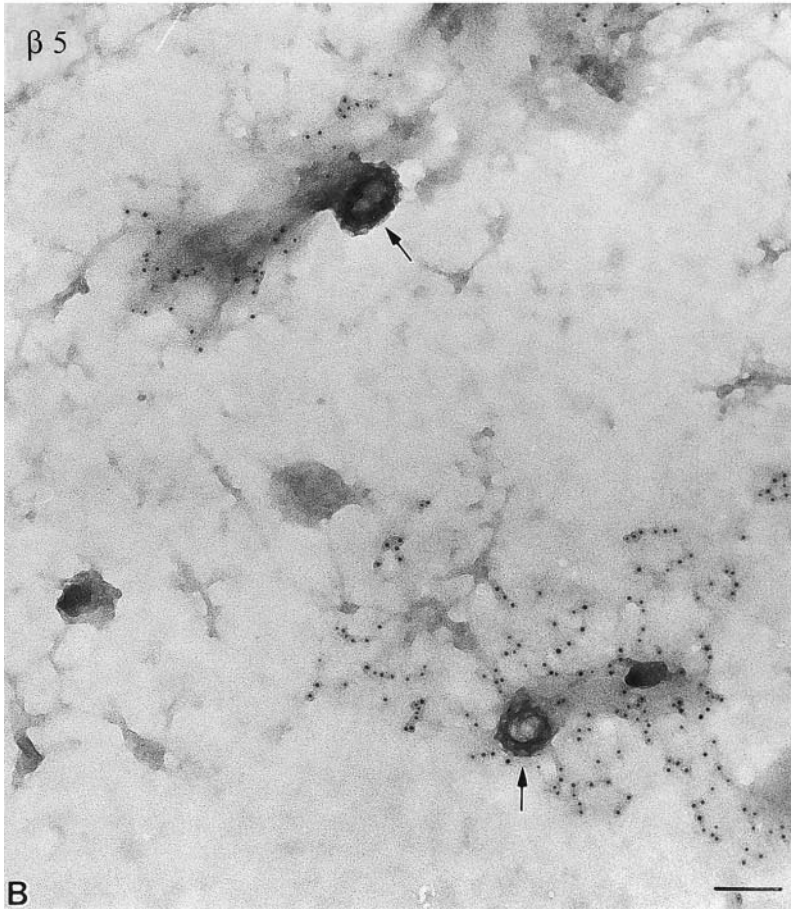
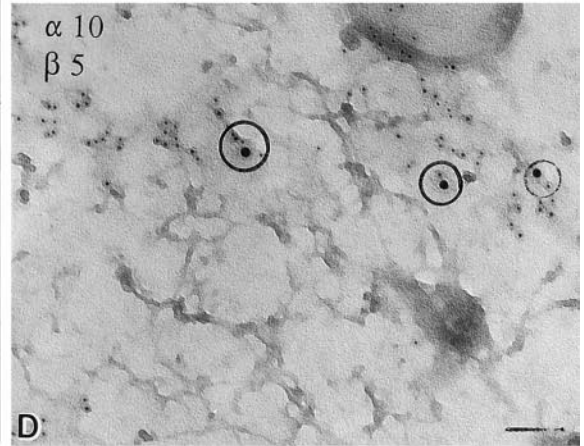
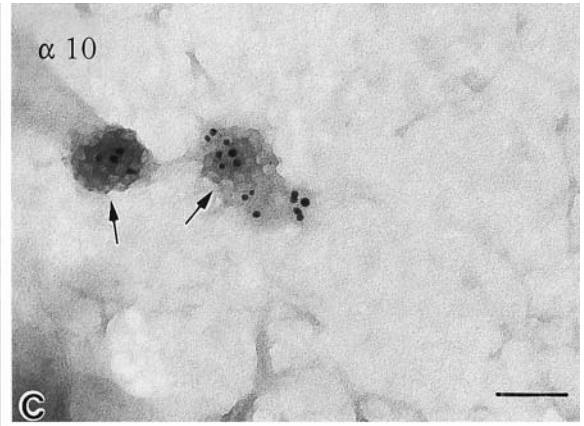
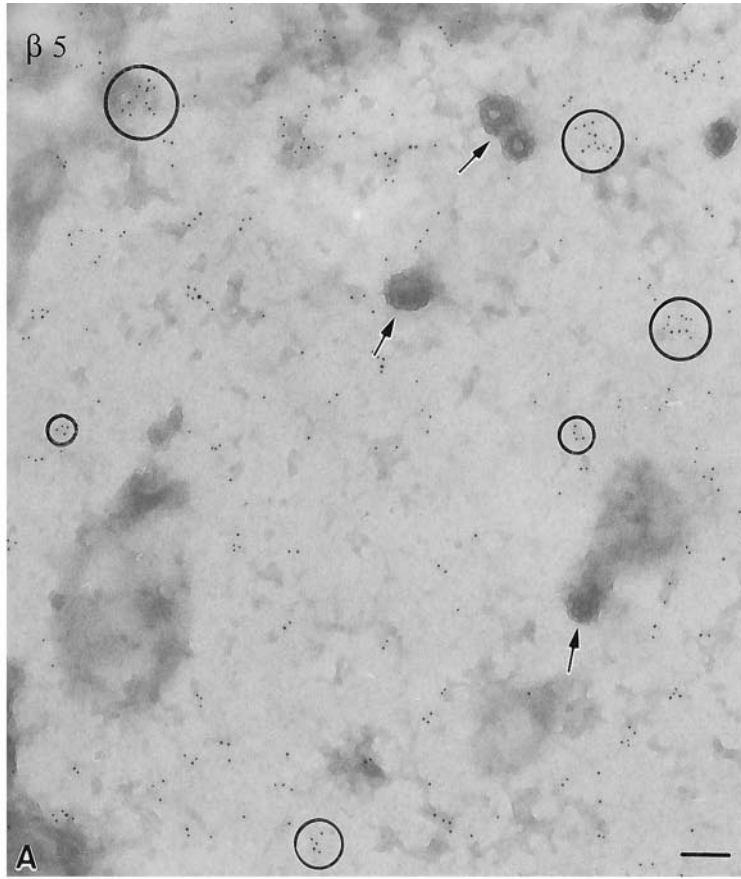
Reagents and Cell Culture

RBL-2H3 cells were grown in MEM (GIBCO BRL) supplemented with 15% fetal calf serum, penicillin-streptomycin, and L-glutamine. Rabbit anti-Lyn (44) and anti-Syk (N-19) Abs were from Santa Cruz Biotechnology, Inc. Mouse anti-FcεRI β mAb was a generous gift from Dr. Juan Rivera (NIH, Bethesda, MD). Affinity-purified mouse anti-DNP IgE and rabbit anti-mouse IgE Abs were prepared as described (Liu et al., 1980; Seagrave et al., 1991). Colloidal gold particles (3–10 nm in diameter) conjugated with anti-rabbit IgG, anti-mouse IgG, and streptavidin were from Nanoprobe and Amersham Pharmacia Biotech. Biotin-phalloidin and DNP-BSA were from Molecular Probes.

Cell Activation and Membrane Labeling

RBL-2H3 cells were allowed to settle overnight onto 15-mm round, clean glass coverslips in the presence of anti-DNP IgE (1 μg/ml) to prime cell surface FcεRI. After washing to remove excess IgE, FcεRI was cross-linked by incubation with DNP-BSA (0.1–1 μg/ml), with rabbit polyclonal anti-IgE (1 μg/ml) or with gold conjugates of these reagents (prepared as in Seagrave et al., 1991). Plasma membrane sheets were prepared by a modification of a procedure described by Sanan and Anderson (1991). The coverslips were rapidly chilled by immersion in ice-cold Hepes buffer (25 mM Hepes, pH 7, 25 mM KCl, and 2.5 mM MgAcetate) and inverted onto nickel EM grids that had been coated with formvar and carbon and, on the day of the experiment, glow-discharged and floated on poly-L-lysine (0.8 mg/ml for 30 min, followed by 10 s dH₂O rinse and air drying). Pressure was applied to the coverslip for 20 s by bearing down with a cork. The coverslips were lifted, leaving sections of the upper cell surface adher-

Figure 1. FcεRI is distributed nonrandomly on resting and activated mast cells. Membrane sheets were prepared from RBL-2H3 cells before (A and D) or after (B, C, and E) cross-linking the FcεRI with DNP-BSA or anti-IgE. The FcεRI α subunit was labeled from the outside of the membrane using 10-nm gold particles conjugated to DNP-BSA (C) or rabbit anti-IgE Ab (D and E). The FcεRI β subunit was labeled from the inside of the membrane using 5-nm gold particles conjugated to anti-FcεRI β mAb (A, B, D, and E). 5-nm gold particles marking FcεRI β are distributed in small dispersed clusters and strings (circled) in the membranes of resting cells (A) and as larger clusters in the membranes of IgE-primed cells that were activated for 2 min at 37°C with DNP-BSA (B). The endocytosis of cross-linked receptors through coated pits (arrows) is illustrated in (C), taken from an experiment where IgE-primed cells were activated for 2 min with 10-nm gold-conjugated DNP-BSA. In D, fixation (10 min at 37°C with 2% paraformaldehyde) preceded outside labeling with anti-IgE-gold particles. In E, cells were activated by incubation for 2 min at 37°C with anti-IgE-gold particles. In both micrographs, 10-nm gold particles marking FcεRI α colocalize with 5-nm particles marking FcεRI β (circles). Bars, 0.1 μm.



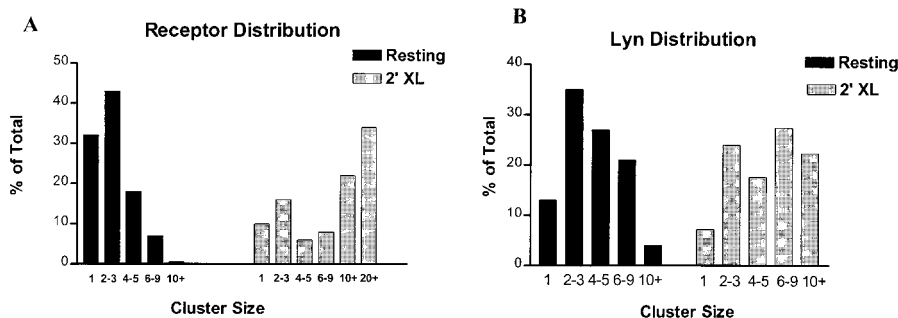


Figure 2. FcεRI and Lyn cluster sizes increase after receptor cross-linking. Gold particles marking either FcεRI (A) or Lyn (B) were scored for cluster size in membrane sheets prepared from resting cells or cells 2 min after FcεRI cross-linking. A minimum of 3,000 gold particles were counted for each experimental condition.

ent to the poly-L-lysine-coated grid. Membranes were rinsed in 4°C HEPES buffer and fixed in 2% paraformaldehyde for 10 min. For experiments using anti-IgE-gold to label FcεRI in the resting state, cells were fixed with 0.5% paraformaldehyde for 5 min and then incubated with anti-IgE-gold before inversion onto EM grids. All membranes were labeled from the inside by inverting the grids onto droplets containing primary antibodies or biotin-phalloidin (5 units/ml) followed by gold-conjugated secondary reagents. Incubations were for 30 min. Intermediate washes in PBS were performed by inverting the grids onto droplets. Primary antibodies were diluted in PBS, 0.1% BSA at the following concentrations: Syk, 10 μg/ml; FcεRI β, 28 μg/ml; Lyn, 2 μg/ml. Gold-conjugated secondary reagents were diluted 1:20 from commercial stocks in PBS-BSA. The samples were post-fixed in 2% glutaraldehyde in PBS and held overnight in PBS. Next, samples were stained for 10 min with 1% OsO₄ prepared in 0.1 M cacodylate buffer and washed 5 min with cacodylate buffer and twice for 5 min in dH₂O. Samples were then processed for 10 min in 1% aqueous tannic acid, followed by two 5-min rinses with dH₂O, 10 min with 1% aqueous uranyl acetate and two 1-min rinses with dH₂O. Grids were air-dried and examined using an Hitachi 600 transmission electron microscope.

Quantifying Gold Particle Distributions

Micrographs from up to four separate experiments were sorted into six groups of at least 25 according to the following treatment and labeling conditions: labeled only with 5- or 10-nm gold particles marking FcεRI β; labeled with 5-nm gold particles marking Lyn plus 10-nm particles marking FcεRI β; labeled with 5-nm gold particles marking Syk plus 10-nm particles marking FcεRI β; resting and activated. A minimum of 3,000 gold particles per set of micrographs were counted for (a) numbers of gold particles marking FcεRI β distributed as singlets or as clusters (with cluster size), (b) numbers of gold particles marking Lyn distributed as singlets or as clusters (with cluster size), and (c) numbers of gold particles marking Lyn that were or were not colocalized with FcεRI β clusters (distinguishing, in activated cells, between gold particles marking Lyn that occurred within FcεRI clusters versus gold particles marking Lyn that instead surrounded these clusters). Our procedure simply involved highlighting singlets and clusters and recording their composition until no unmarked particle or group remained on the micrograph. Numbers of gold particles marking Syk were counted over 55 μm² of membrane and scored for colocalization or not with FcεRI β.

Results

The Distribution of FcεRI on Resting and Activated Mast Cells

The distribution of 5-nm gold particles marking FcεRI β in resting cells is illustrated in Fig. 1 A and quantified in Fig. 2 A. The micrograph shows that gold particles marking FcεRI β are distributed as a mixture of singlets and small dispersed clusters that rarely exceed a diameter of 100 nm (the length of the scale bar in Fig. 1 A). No gold particles associate with clathrin-coated vesicles that are major features of the cytoplasmic face of the plasma membrane.

Particle counting (Fig. 2 A) established that ~33% of gold particles marking FcεRI β are distributed as singlets and that the rest are in clusters that most commonly contain 2–3 gold particles and only rarely exceed 9 gold particles.

After cross-linking for 2 min at 37°C with DNP-BSA (0.1 μg/ml), most FcεRI β is found in clusters that are moderately or substantially larger than the clusters on resting cells (Fig. 1 B). These clusters are particularly associated in activated cells with distinct membrane regions that stain darkly with the mixture of osmium, tannic acid, and uranyl acetate used to provide contrast to these samples. Clathrin-coated pits typically occur at the periphery of these osmiophilic membrane regions. Further particle counting, also reported in Fig. 2 A, showed that ~25% of 5-nm gold particles marking FcεRI β in activated cells are in clusters of 10–20 (intermediate clusters) and that ~40% are in clusters of greater than 20 (and sometimes more than 100; large clusters). The internalization of cross-linked receptors by coated pit-mediated endocytosis was confirmed by activating cells with DNP-BSA 10-nm colloidal gold and localizing the gold particles within clathrin-caged vesicles (Fig. 1 C, arrow).

To establish the reliability of the membrane sheet technique for demonstrating topographical relationships between membrane-associated proteins, we labeled FcεRI from the extracellular as well as the cytoplasmic faces of the membrane and looked for colocalization of gold particles. In Fig. 1 D, IgE-primed cells were fixed lightly, then labeled from the outside with 10-nm rabbit anti-IgE-gold particles and subsequently, from the inside, with 5-nm anti-FcεRI β particles. 5-nm gold particles marking FcεRI β are again distributed in the singlets and small dispersed clusters that characterize unstimulated cells. The small clusters now also contain 10-nm gold particles marking FcεRI α. In Fig. 1 E, cells were activated for 2 min at 37°C with 10-nm rabbit anti-IgE-gold particles before labeling from the inside with 5-nm anti-FcεRI β particles. This time both sizes of gold colocalize in the larger receptor clusters surrounding coated pits (arrows) that characterize activated cells. We interpret the very regular distance between the 5-nm particles marking FcεRI β and the 10-nm gold particles marking FcεRI α as strong evidence that the same receptor is being labeled, although on different subunits and from different sides of the membrane.

Previous studies using the lower resolution techniques of fluorescence and scanning electron microscopy had not revealed FcεRI clusters in resting cells. Therefore, we performed further control experiments to ensure that the

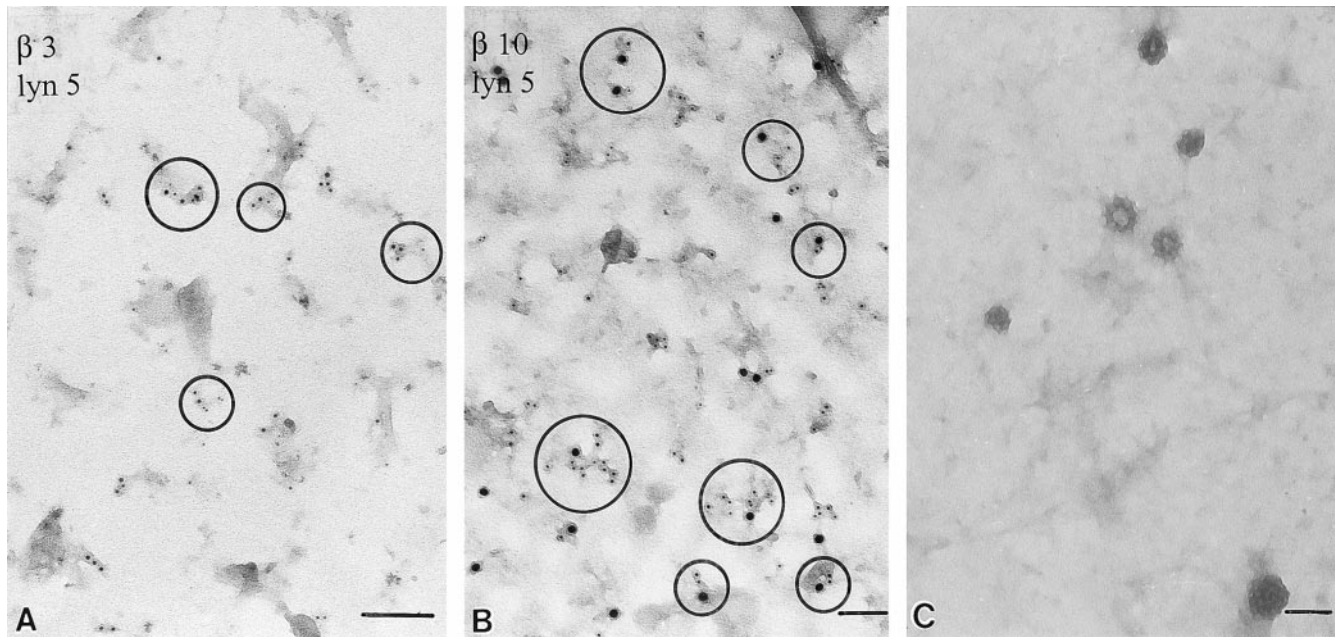


Figure 3. Lyn associates with FcεRI in resting mast cells. Membrane sheets were prepared from untreated RBL-2H3 cells and labeled from the inside with 5-nm gold particles specific for Lyn and with either 3- (A) or 10-nm (B) gold particles specific for FcεRI β. In both micrographs, a substantial portion of 5-nm gold particles marking Lyn are colocalized with FcεRI β (circles). (C) Demonstrates the absence of background binding when both sizes of gold particles are incubated with membrane sheets in the absence of specific antibodies. Bars, 0.1 μm.

small clusters were not induced in the fixed samples by the labeling Abs. In one control experiment, membrane sheets were fixed with 2% paraformaldehyde for either the standard 10 min or for only 5 min, then labeled for 30 min with monoclonal anti-FcεRI β and polyclonal anti-Lyn followed by gold secondary antibodies for 30 min. In another, membrane sheets were fixed for 10 minutes with 0.5, 2, and 4% paraformaldehyde and labeled as above. As an additional control, sheets were fixed for 10 min with 2% paraformaldehyde and labeled with anti-FcεRI β, anti-Lyn, and gold secondary antibodies and held for up to 12 h before fixation with glutaraldehyde. Gold particle labeling densities and distributions were not altered by reducing the fixation time or fixative concentration. They also were not altered by increasing the holding time before the glutaraldehyde post-fixation step, that could conceivably promote reagent-induced clusters to form in lightly fixed samples.

The Topography of Lyn and Its Association with FcεRI

Fig. 3 demonstrates the relative distributions of FcεRI and Lyn, the kinase that phosphorylates the β and γ subunits of the cross-linked FcεRI (Oliver et al., 1994; Yamashita et al., 1994), in resting cells. In these images, either 3- (Fig. 3 A) or 10-nm (Fig. 3 B) gold particles mark the location of FcεRI β, while Lyn is labeled with 5-nm gold particles. Two inferences can be drawn from these micrographs. First, gold particles marking Lyn are frequently in clusters. Second, Lyn clusters frequently, but not always, colocalize with FcεRI β in resting mast cells. Fig. 3 C is included to show there is essentially no background binding of either 5- or 10-nm anti-mouse IgG gold particles to membrane

sheets; backgrounds are similarly low using all of the colloidal gold reagents in this study (not shown).

Counting gold particle numbers and distributions on replicate micrographs confirmed that indeed the majority (at least 85%) of gold particles marking Lyn are in clusters (Fig. 2 B). Clusters of 2–3 particles are most common and clusters of >9 particles are rare. We found that ~20% of total Lyn gold particles were in close proximity to gold particle(s) marking FcεRI β. If counted from the complementary perspective of total FcεRI gold particles, the percentage of these in close proximity to Lyn was ~25%. These quantitative data confirm the visual observation that a significant percentage of Lyn and FcεRI are colocalized in resting cells.

The redistribution of Lyn that follows FcεRI cross-linking with multivalent antigen or anti-IgE is demonstrated by the series of micrographs in Fig. 4. Fig. 4 A is a relatively low-power field to show multiple associations of Lyn, marked by 5-nm gold, and FcεRI β, marked by 3-nm gold, in membrane sheets. Lyn has three principal distributions in this micrograph. First, there are multiple Lyn particles and clusters that do not associate with receptor. Second, a portion of Lyn and FcεRI β are colocalized within receptor clusters of small to intermediate size (circles). Finally Lyn surrounds, but is very rarely intercalated within, the large clusters of cross-linked FcεRI β that form on osmiophilic membrane regions in activated cells (arrows). Fig. 4, B–D, provide additional examples of the receptor-rich osmiophilic membrane regions (arrows) of activated cells to demonstrate both the absence of intercalated Lyn and the presence of Lyn in strings and clusters surrounding the receptor clusters (rectangular boxes).

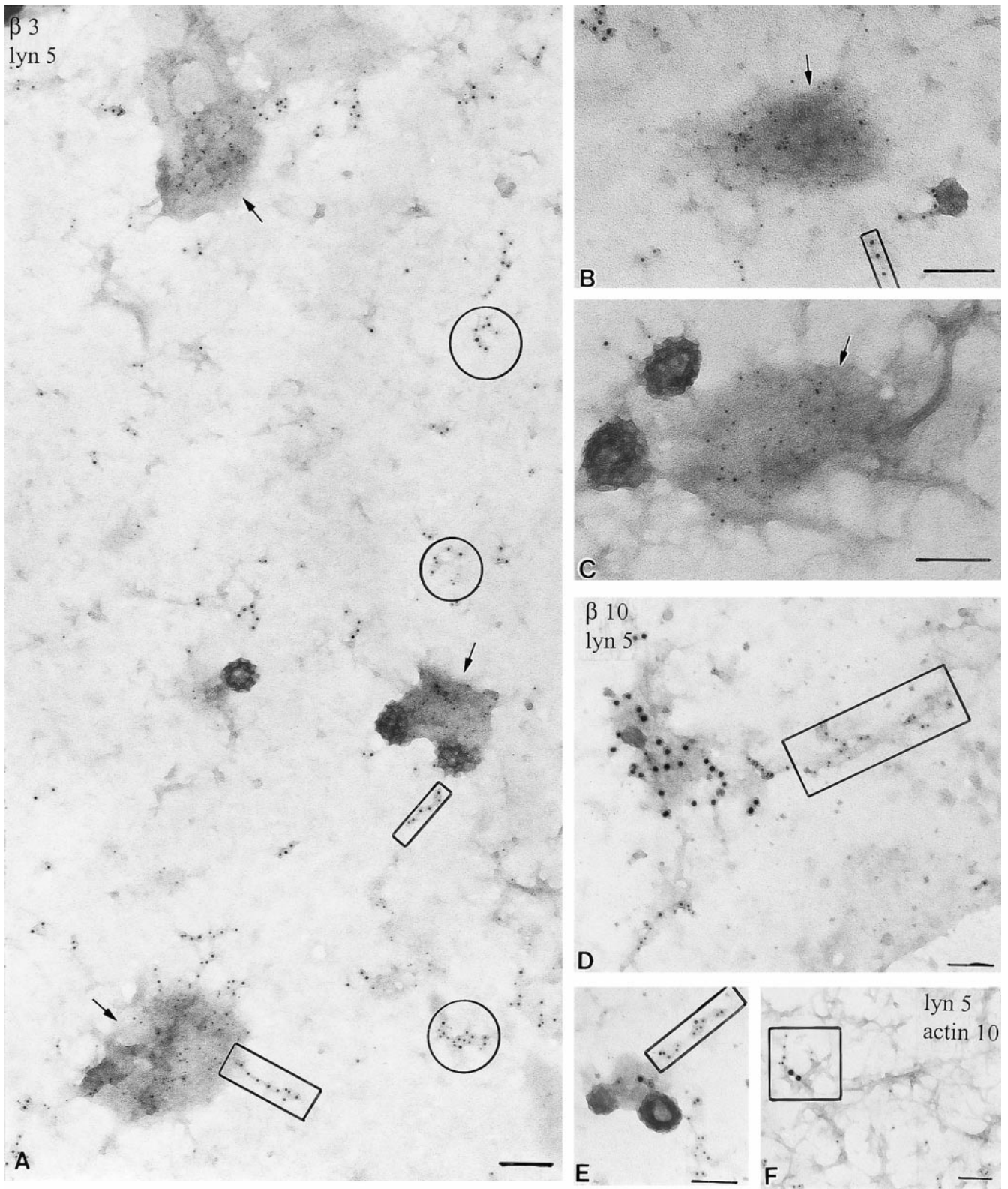


Figure 4. Lyn segregates from FcεRI in activated mast cells. Membrane sheets were prepared from IgE-primed RBL-2H3 cells that were previously activated for 2 min with anti-IgE Ab. In each micrograph, the sheets were labeled from the inside with 5-nm gold particles specific for Lyn. Sheets were double labeled with: (A–C) 3-nm gold particles specific for FcεRI β; (D and E) 10-nm gold specific for FcεRI β; and (F) 15-nm phalloidin-conjugated particles specific for F-actin. In A, a portion of Lyn is associated with FcεRI β in membrane clusters of intermediate size (circles). The large clusters of cross-linked FcεRI β that are striking features of A–C (arrows) are almost completely lacking in colocalized Lyn. Lyn is found characteristically in strings and clusters at the periphery of the aggregates (rectangles in A, B, D, and E). In F, 5-nm gold particles marking Lyn strings are continuous with 15-nm gold-conjugated biotinylated-phalloidin marking F-actin. Bars, 0.1 μm.

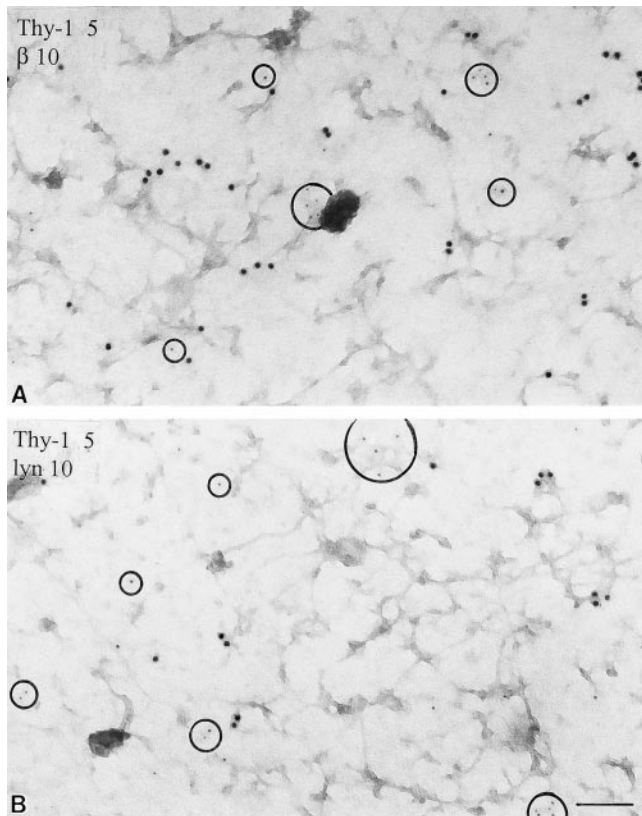


Figure 5. Thy-1 is distributed in singlets and small clusters over the RBL cell surface but does not colocalize with Lyn or FcεRI. Membrane sheets were prepared from resting, prefixed RBL-2H3 cells that were labeled from the outside with 5-nm gold particles conjugated to anti-Thy-1 antibodies. The sheets were double labeled from the inside with 10-nm gold particles specific for either FcεRI β (A) or Lyn (B). Bar, 0.1 μm.

Counting gold particle numbers and distributions emphasized the topographical differences in Lyn distribution between resting and activated cells. First, Lyn clusters increase in size in response to 2 min of FcεRI cross-linking so that >50% of clusters now contain >6 gold particles (Fig. 2 B). This observation implies that the population of Lyn as a whole has altered properties in activated cells. Second, 74% of gold particles marking Lyn were in clusters that showed no apparent association with receptor. Importantly, 10% of total Lyn was distributed within small or medium clusters of FcεRI β gold particles, whereas 16% of total Lyn was localized at the periphery of the large clusters of FcεRI β on osmiophilic membrane.

Table I. Syk Distribution on Membrane Sheets

	Resting cells	Activated cells
Total Syk-gold particles	978	1,218
% Increase in membrane Syk	—	24.5%
Syk-gold particles colocalized with FcεRI-gold particles	86 (8.7%)	861 (70.6%)

Membrane sheets were prepared from IgE-primed resting or activated (2 min with 0.1 mg/ml DNP-BSA) cells and labeled for Syk and RceRI β. Numbers of gold particles marking Syk and their proximity to particles marking RceRI β were counted for 55 μm² of membrane. Micrographs were from four independent experiments.

The segregation of Lyn from highly clustered receptors was further demonstrated by comparing the topography of FcεRI and Lyn with the topography of clathrin and actin. As already noted, cross-linked FcεRI can be internalized through clathrin-coated pits that often bud from the edges of these clusters. These pits consistently label with gold particles marking the clathrin adaptin, AP-2, but they almost never label with gold particles marking Lyn (data not shown). Conversely, strings of Lyn are seen on fibrous structures that also label with phalloidin, a marker for F-actin (Fig. 4 F, boxed). Phalloidin-gold particles do not associate with FcεRI β clusters (not shown).

Based principally on sucrose density fractionation of detergent-solubilized membranes, previous investigators have proposed that Lyn associates in resting cells with cholesterol and ganglioside GM-1-enriched microdomains that also accumulate GPI-linked proteins including Thy-1 (Surviladze et al., 1998). However, the membrane distribution of Thy-1, labeled from the outside of lightly fixed resting cells, is rarely coincident with either Lyn (Fig. 5 A) or FcεRI β (Fig. 5 B) labeled from the inside of the same cells.

The Topography of Syk and Its Association with FcεRI

Previous biochemical studies have shown that a second tyrosine kinase, Syk, couples the cross-linked FcεRI to downstream responses (Hutchcroft et al., 1992; Oliver et al., 1994; Zhang et al., 1996). Lyn-mediated tyrosine phosphorylation of paired ITAMs within the cytoplasmic tail of the cross-linked FcεRI γ subunit creates sites for the binding and activation of Syk (Shiue et al., 1995).

The distribution of Syk was determined in membrane sheets prepared from resting (Fig. 6) and antigen-stimulated (Fig. 7) RBL-2H3 cells. In resting cells, gold particles marking Syk occurred in singlets and small clusters with essentially no interaction with FcεRI (Fig. 6, circles). In contrast, the majority of Syk label is found intercalated into both small and large receptor clusters in activated cells (Fig. 7, arrows). A few clusters of isolated Syk persist in the membrane sheets of activated cells (Fig. 7, circles).

Results of counting gold particles specific for Syk are shown in Table I. The total number of gold particles marking Syk over 55 μm² of resting plasma membrane was 978, compared with 1,218 gold particles over the same area of activated plasma membrane. Thus, at 2 min of continuous receptor cross-linking, there was a 24% increase in Syk labeling. Although FcεRI cross-linking induced only a modest increase in total Syk labeling at the membrane, the topography of Syk was dramatically altered. Specifically, in membranes from resting cells, <10% of Syk label is found in close proximity to gold particles marking FcεRI β. In contrast, >70% of gold particles marking Syk are found in receptor clusters after activation.

Discussion

Results of previous biochemical studies have suggested a model for FcεRI signaling in which receptor cross-linking leads to the Lyn-mediated tyrosine phosphorylation of ITAMs within the cytoplasmic tails of the β and γ subunits of the FcεRI. FcεRI γ phosphorylation in turn provides

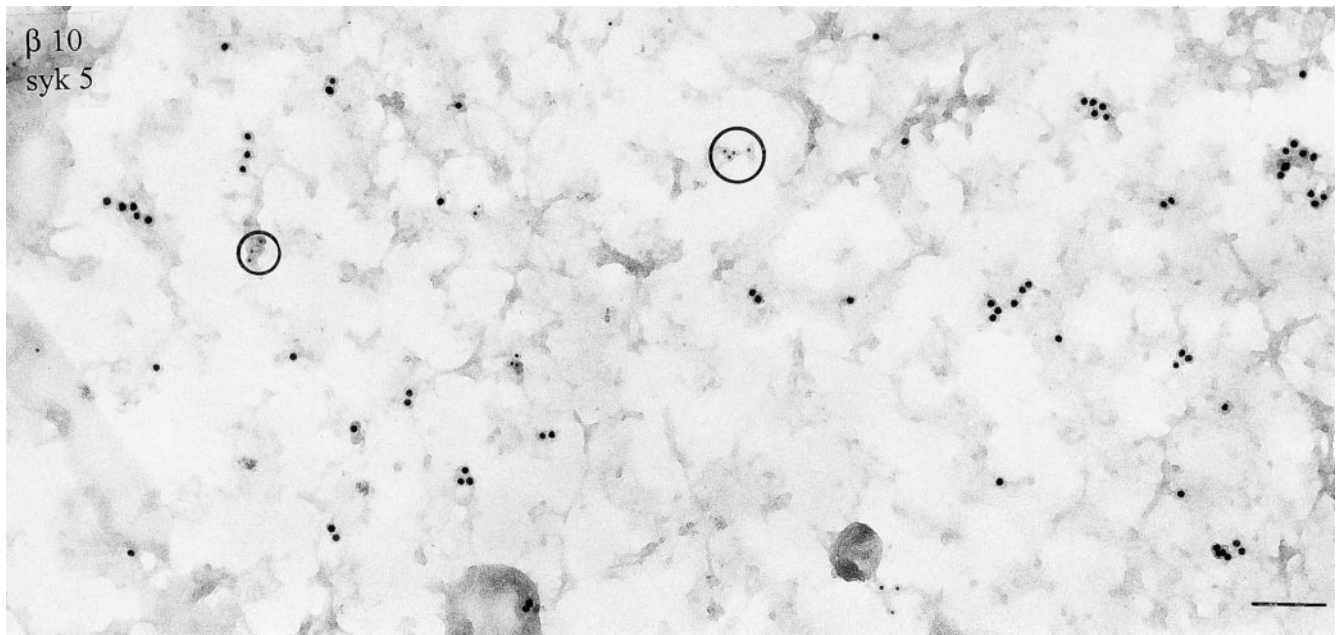


Figure 6. Syk and FcεRI do not colocalize in the membranes of resting cells. Membrane sheets were prepared from untreated RBL-2H3 cells and labeled from the inside with 5-nm gold particles specific for Syk and with 10-nm gold particles specific for FcεRI β. Examples of isolated Syk clusters are marked with circles. Bar, 0.1 μm.

binding sites for the binding and activation of Syk. Our goal was to determine the membrane topography of the high-affinity IgE receptor, FcεRI, and of its associated tyrosine kinases, Lyn and Syk, during this biochemical cascade. We used a modification of the method of Sanan and Anderson (1991) to generate membrane sheets from the dorsal surface of RBL-2H3 mast cells. Signaling molecules were localized on these native membranes by immunogold labeling and high-resolution TEM analysis.

We found that the majority of gold particles marking FcεRI (>65%) and Lyn (>85%) in resting cells occur as clusters and that a significant proportion (20–25%) of these are mixed clusters, containing both FcεRI and Lyn. Neither FcεRI nor Lyn associated with clathrin-coated membrane in resting cells. The colocalization of Lyn and monomeric receptor is consistent with previous evidence from the Metzger laboratory (Vonakis et al., 1997; Metzger, 1999) showing that a portion of Lyn associates with the resting FcεRI via interactions of its SH4 domain with determinants on the FcεRI β subunit. In contrast, cell fractionation studies by Field et al. (1995, 1997, 1999) identified Lyn in detergent-insoluble, glycosphingolipid-enriched membrane microdomains that were also enriched in cholesterol, sphingomyelin, and glycosylphosphatidylinositol (GPI)-anchored proteins, including Thy-1, but specifically did not include monomeric receptor. To explain this discrepancy, we suggest that the interaction of monomeric FcεRI with myristoylated Lyn may be disrupted when cells are treated with Triton X-100 and subjected to sucrose gradient centrifugation. In short, our results support the concept that Lyn occurs in mast cells in microdomains but show clearly that these microdomains do not exclude unactivated FcεRI.

While the unique composition of DRMs has provided strong evidence for the concept of membrane microdomains,

previous investigators have also described discrepancies between the protein compositions of low-density sucrose fractions and the protein composition of microdomains in the native plasma membrane. In particular, Chang et al. (1994) demonstrated that isolated caveolae are enriched in a complex profile of proteins and that Triton X-100 selectively extracts a subset of these proteins, including acylated G proteins. Adding to the complexity, the recovery of membrane proteins in the light fractions of density gradients is highly dependent on the choice of detergent (Surviladze et al., 1998) and on the temperature during preparative steps (Harder et al., 1998). This variability emphasizes the need to complement cell fractionation studies with direct analyses of microdomains in native membranes, made possible here by high-resolution electron microscopy.

Previous studies by fluorescence, transmission, and scanning electron microscopy (Menon et al., 1984; Pfeiffer et al., 1985; Stump et al., 1988; Seagrave et al., 1991; Mao et al., 1993; Santini and Keen, 1996; Stauffer and Meyer, 1997) have all failed to reveal the inherently clustered distribution of FcεRI that we now readily demonstrate using 3- and 5-nm gold particles to mark receptors. We speculate that these small clusters are below the resolution of the light microscope (~250 nm; Jacobson and Dietrich, 1999) and depend on the substantially higher resolution achieved here by TEM, which readily resolves gold particles as small as 3 nm. Interestingly, our own previous studies of FcεRI topography by immunogold labeling with 15-nm gold probes and SEM analysis initially suggested an inherently random distribution of the FcεRI (Seagrave et al., 1991). However, subsequent quantitative analysis of gold particle distributions on digitized negatives from these SEM experiments showed a nonrandom distribution of the 15-nm particles on resting cells (Anderson, 2000). Thus, the more

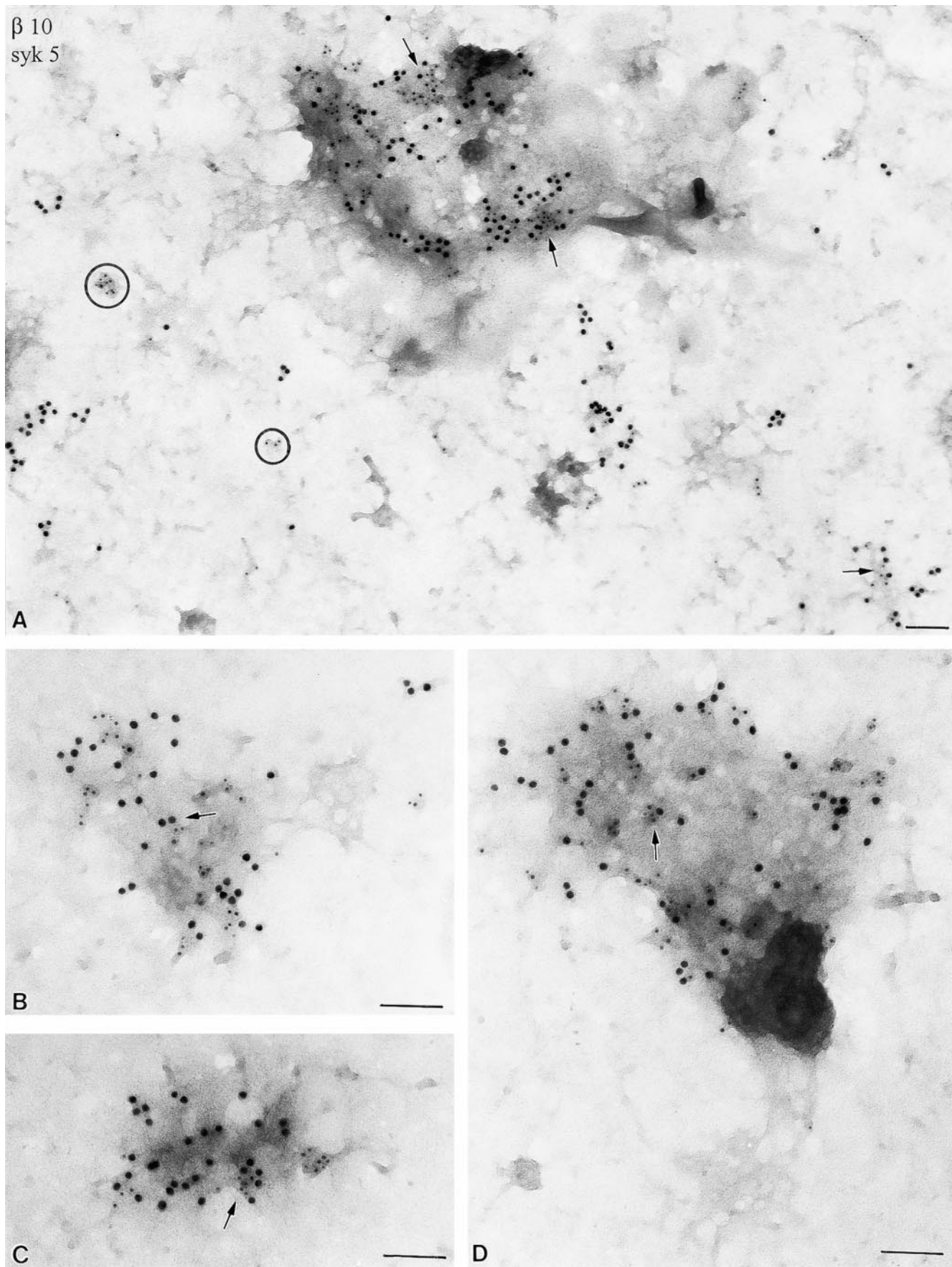


Figure 7. Syk is recruited to Fc ϵ RI-rich osmiophilic membrane domains. Membrane sheets were prepared from IgE-primed RBL-2H3 cells that were previously activated for 2 min with anti-IgE Ab. The sheets were labeled from the inside with 5-nm gold particles specific for Syk and with 10-nm gold particles specific for Fc ϵ RI β . Arrows point to gold particles marking Syk that are strikingly colocalized with large clusters of gold particles marking Fc ϵ RI β in activated cells. Circles in A indicate isolated Syk clusters. Bars, 0.1 μ m.

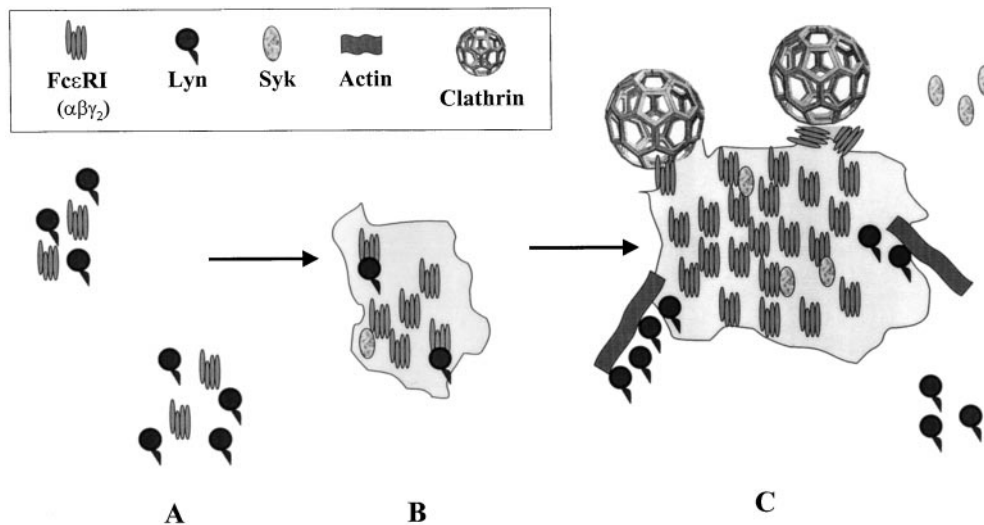


Figure 8. The FcεRI encounters Lyn, Syk, and coated pits in topographically distinct membrane domains. In this model, FcεRI and Lyn are loosely colocalized in resting cells in dispersed clusters that do not associate with clathrin-coated membrane (A). Syk does not colocalize with FcεRI in resting cells. FcεRI cross-linking (B) induces Lyn-mediated phosphorylation needed for Syk recruitment. Larger FcεRI aggregates (C) exclude Lyn by an actin-dependent process and include Syk to form functional signaling domains. The clathrin-coated vesicles that internalize cross-linked receptors bud from membrane adjacent to the Syk-FcεRI complexes.

obviously nonrandom distributions of monomeric FcεRI observed here on membrane sheets is probably the result of the higher resolution achieved with smaller (3–10 nm) gold particles and is not an artifact of the method of observation.

FcεRI cross-linking causes the formation of large receptor clusters seen previously by fluorescence, transmission, and scanning electron microscopy (Menon et al., 1984; Pfeiffer et al., 1985; Stump et al., 1988; Seagrave et al., 1991; Mao et al., 1993; Santini and Keen, 1996; Stauffer and Meyer, 1997). Remarkably, Lyn, the kinase that phosphorylates ITAMs within the β and γ subunits of the cross-linked FcεRI (Yamashita et al., 1994) colocalizes with FcεRI in clusters of small and intermediate size but is dramatically segregated to the periphery of the large clusters. This segregation may be regulated in part by interactions of Lyn with components of the actin cytoskeleton in activated cells. Supporting this suggestion, early biochemical and flow cytometric studies showed that increased F-actin assembly is an immediate response of RBL-2H3 mast cells to FcεRI cross-linking (Pfeiffer et al., 1985), whereas the current work shows that Lyn associates with fibrous structures that also label with phalloidin, a marker for F-actin. One important unanswered question is where Lyn-mediated FcεRI tyrosine phosphorylation actually occurs. We suspect the clusters of intermediate size of serving this function.

Syk, the cytoplasmic kinase that couples the cross-linked FcεRI to downstream responses (Hutchcroft et al., 1992; Oliver et al., 1994; Rivera and Brugge, 1995; Zhang et al., 1996), is not colocalized with the receptor in resting cells. Nevertheless some Syk is resident at the membrane of resting cells. Unlike Lyn, Syk has no membrane localization domain. However, it may well interact in resting mast cells with other receptor tyrosine kinases, for example c-Kit, or with binding proteins like tubulin and Cbl that may in turn interact with membranes (Ota and Samelson, 1997; Fernandez et al., 1999).

Syk is dramatically recruited into the large receptor clusters that form on osmiophilic membrane patches within minutes after cross-linking. This recruitment is likely to occur both by the translocation of Syk from the cytosol to the membrane and by the recruitment of Syk from other sites of membrane association. We propose the Syk-enriched, Lyn-negative FcεRI clusters as the probable sites of downstream signaling. The identities of other components within these putative signaling domains are not yet known. However, their distinct appearance as darkened membrane patches suggests that they are not merely the products of the aggregation of smaller clusters but may instead contain a unique subset of membrane lipids and proteins that stain differently from bulk membrane with the combination of osmium, tannic acid, and uranyl acetate used here to provide contrast to the membrane sheets.

Because the Syk-enriched clusters are sufficiently large to be observed readily by fluorescence microscopy, they are very likely to correspond to the microdomains seen previously by Stauffer and Meyer (1997) that labeled with fluorescent cholera toxin B (a ligand for the GM1 ganglioside) and accumulated cross-linked FcεRI and GFP-conjugated SH2 domains of both Syk and PLC-γ1. Although the clusters seen by TEM exclude Lyn, they are typically surrounded by Lyn in an F-actin-associated (presumably detergent-insoluble) form. Thus the detergent-insoluble, FcεRI- and Lyn-containing membranes isolated by Field et al. (1995, 1997, 1999) from activated mast cells could possibly represent cross-linked receptors surrounded by, rather than intercalated with, Lyn.

We observed that clathrin-coated vesicles internalize cross-linked receptors from membrane adjacent to the Syk-FcεRI complexes. Previous thin section TEM studies established that cross-linked FcεRI are internalized through coated pits that often have long necks and can support several clathrin-coated buds (Pfeiffer et al., 1985). These structures are major features of the membrane sheets described here. In membrane sheets from resting cells, their

distribution seems unrelated to particular topographical features of the membrane. In membrane sheets from activated cells, they appear particularly at the edges of the darkened patches proposed to represent signaling domains. Gold particles marking Syk were seen at the necks of coated pits but not in clathrin cages, suggesting that Syk dissociates before cross-linked Fc ϵ RI are internalized. Lyn appears to be actively excluded from membrane adjacent to coated pits in both resting and activated cells.

Previous thin section TEM studies in mast cells have not so far demonstrated clear increases in coated pit density induced by Fc ϵ RI cross-linking (Mao et al., 1993; Santini and Keen, 1996). However, we previously localized the majority of coated pits to smooth membrane near the bases of microvilli or lamellae in both resting and activated mast cells (Pfeiffer et al., 1985; Mao et al., 1993). Additionally, we established by statistical analyses of thin section TEM micrographs that coated pits are distributed in dispersed clusters, and not randomly, on a range of immune cells and that events that polarize cell shape, for example capping or chemotaxis, further polarize coated pits (Pfeiffer et al., 1980; Oliver and Berlin, 1983). Recently, Gaidarov et al. (1999) presented further evidence that coated pits may assemble as clusters in specific locations on membranes. These indications of the nonrandom distribution of coated pits raises the possibility that their assembly at the periphery of signaling domains is regulated.

There is substantial evidence that efficient TCR signaling occurs by the segregation of the TCR and coreceptors like CD4 and CD28 to detergent-resistant membrane domains that accumulate GPI-linked proteins, Src kinases, Zap70, scaffolding proteins like LAT and molecules implicated in signal propagation such as PLC- γ (Montixi et al., 1998; Xavier et al., 1998; Zhang et al., 1998; Ilangumaran et al., 1999). Once signaling is initiated there is evidence that the signaling domains can additionally recruit cytoskeletal and adhesive proteins that may stabilize an "immunological synapse" between T cells and APCs (Monks et al., 1998; Grakoui et al., 1999; Penninger and Crabtree, 1999). Because TCR and Fc ϵ RI signaling are similar, it is possible that closer microscopic analysis in T cells will show that the TCR actually encounters Src and Syk kinases in distinct domains. Indeed, Monks et al. (1998) used fluorescence techniques to identify discrete organization of the TCR and accessory molecules at the sites of T cell and APC contact. They placed the TCR in the center of an activation cluster, which they termed the central supramolecular activation cluster (c-SMAC), surrounded by proteins such as LFA-1 and talin in a more peripheral ring (p-SMAC). Further studies in both systems will be required to localize at high resolution the other constituents involved in signal propagation via the TCR and Fc ϵ RI, including important scaffolding proteins, such as LAT and SLP76.

In summary, we have discovered that Fc ϵ RI encounters Lyn, Syk, and coated pits in topographically distinct membrane domains. The sequence of events revealed to date is shown schematically in Fig. 8. Studies are in progress to determine where Fc ϵ RI β and γ subunit phosphorylation occurs, to identify other components of the small and large receptor clusters, to determine how Fc ϵ RI cross-linking induces the segregation of Lyn from Fc ϵ RI and to explore

mechanisms regulating the topography of coated pit formation.

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