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# Compartmentalized Activation of the High Affinity Immunoglobulin E Receptor within Membrane Domains*

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**The earliest known step in the activation of the high affinity IgE receptor, FcεRI, is the tyrosine phosphorylation of its β and γ subunits by the Src family tyrosine kinase, Lyn. We report here that aggregation-dependent association of FcεRI with specialized regions of the plasma membrane precedes its tyrosine phosphorylation and appears necessary for this event. Tyrosine phosphorylation of β and γ occurs in intact cells only for FcεRI that associate with these detergent-resistant membrane domains, which are enriched in active Lyn. Furthermore, efficient *in vitro* tyrosine phosphorylation of FcεRI subunits occurs only for those associated with isolated domains. This association and *in vitro* phosphorylation are highly sensitive to low concentrations of detergent, suggesting that lipid-mediated interactions with Lyn are important in FcεRI activation. Participation of membrane domains accounts for previously unexplained aspects of FcεRI-mediated signaling and may be relevant to signaling by other multichain immune receptors.**

The plasma membrane contains specialized regions that have distinct compositions and can serve unique functions in the regulation of cell surface receptor activation. For example, caveolae have been shown to associate with certain signaling proteins (1, 2) and have been implicated in receptor activation (3–6), vesicular transport (7, 8), and the uptake of small molecules (9). Compositionally related membrane domains, which lack the invaginated morphology of caveolae as well as the membrane protein caveolin, have also been identified and biochemically separated from caveolae (10). These membrane domains, like caveolae, are resistant to solubilization in nonionic detergents such as Triton X-100, are enriched in sphingolipids and glycosylphosphatidylinositol-linked proteins, and are associated with palmitoyl-anchored signaling molecules including Src family tyrosine kinases (10–14). Detergent-resistant membrane domains isolated from rat basophilic leukemia (RBL)[1] cells, a mast cell line, contain at least 30% of the cellular Lyn, a Src family tyrosine kinase, and no detectable caveolin (15).

Aggregation of FcεRI on mast cells and basophils by multivalent antigens leads to phosphorylation of immunoreceptor tyrosine-based activation motifs within the β and γ receptor subunits by Lyn (16–19). This initiates a signaling cascade culminating in secretion of inflammatory mediators and cytokines that play an important role in the allergic response (20, 21). The molecular mechanism by which aggregation of FcεRI initiates its phosphorylation by Lyn is incompletely understood. Selective binding of Lyn directly to unphosphorylated FcεRI β (22) has been proposed to mediate an initial *trans*-phosphorylation of aggregated FcεRI (23), but this does not account for the capacity of FcεRI lacking the β subunit (24, 25) or chimeric receptors containing only the γ cytoplasmic tail (26–28) to become tyrosine-phosphorylated upon aggregation. The involvement of detergent-resistant membrane domains in FcεRI signaling was recently suggested by the observation that aggregation of FcεRI on RBL cells significantly increased the amount of active Lyn associated with these structures (15). Furthermore, fluorescence microscopy studies showed that aggregation of FcεRI at the surface of intact cells co-redistributes ganglioside-enriched membrane patches that are related to the isolated membrane domains (29, 30). The aggregation-dependent association of FcεRI with these less fluid regions of the membrane (30, 31) is also consistent with decreased lateral and rotational mobility of aggregated FcεRI (reviewed in Ref. 32). In the present study, we establish conditions for preserving the interaction of aggregated FcεRI with these membrane domains following cell lysis, and we demonstrate the importance of this interaction to the initial step in signaling, the tyrosine phosphorylation of FcεRI.

## EXPERIMENTAL PROCEDURES

*Sucrose Gradient Ultracentrifugation*—RBL-2H3 cells were lysed in 10 mM Tris, pH 8.0, 50 mM NaCl, 10 mM EDTA, 1 mM $Na_3VO_4$, 30 mM pyrophosphate, 10 mM glycerophosphate, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem, San Diego, CA), 0.02 units/ml aprotinin, 0.01% (w/v) $NaN_3$, and 0.05% (v/v) Triton X-100. The lysates were then diluted 1:1 in 80% sucrose and analyzed by ultracentrifugation as described (15). In some experiments, the lysis buffer contained 0.025% Triton X-100, and 0.025% Triton X-100 was also present in the 80% sucrose solution used to dilute the lysate. The two lysis procedures yielded identical results.[2] Sucrose solutions contained 25 mM Tris, pH 7.5, 125 mM NaCl, and 2 mM EDTA.

*Immunoblotting*—Electrophoresis of samples was carried out on 12.5% acrylamide SDS gels under nonreducing conditions, and semidry transfer to Immobilon P (Millipore, Bedford, MA) was performed as described (15). Anti-phosphotyrosine immunoblots were performed using 0.1 μg/ml monoclonal antibody 4G10 conjugated to horseradish peroxidase (UBI, Lake Placid, NY) and Supersignal ECL substrate (Pierce, Rockford, IL). For the results in Fig. 2, tyrosine phosphorylation of the FcεRI β subunit was quantified from anti-phosphotyrosine immunoblots of post-nuclear supernatants of $10^6$ RBL cells lysed in 0.2% Triton X-100. The prominent 34-kDa band detected in these blots after FcεRI stimulation was identified as β based on selective immunodepletion by IgE-specific agarose beads.[2] This band was quantified with a 256 gray-scale scanner (Umax Vista-S6E) and NIH Image software.

*Immunoprecipitations*—After adjusting the sucrose fractions to 0.2% Triton X-100 to extract FcεRI from the membrane domains, FcεRI was immunoprecipitated for 90 min with trinitrophenyl-conjugated Sepharose 4B (which efficiently binds anti-DNP IgE). The immunoprecipitates were washed twice with 0.2% Triton X-100 and once in lysis buffer

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[1] The abbreviations used are: RBL, rat basophilic leukemia; DNP, dinitrophenyl.

[2] K. A. Field, D. Holowka, and B. Baird, unpublished observations.

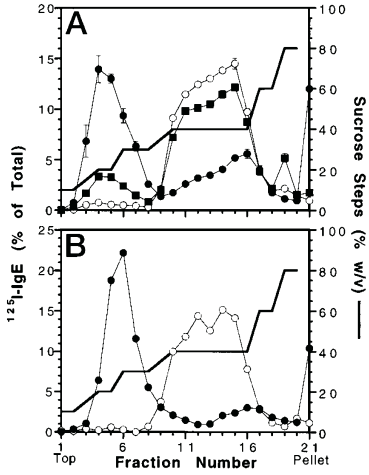


FIG. 1. **Aggregation-dependent association of FcεRI with membrane domains.** *A*, RBL cells sensitized with biotinylated $^{125}$I-anti-DNP IgE and suspended in buffered salt solution (15) were stimulated for 5 min at 37 °C with 500 ng/ml antigen (■, DNP-bovine serum albumin), 10 nM streptavidin (●), or left unstimulated (○) prior to lysis in 0.025% Triton X-100 at $4 \times 10^6$ cells/ml. Lysates were then diluted 1:1 with 80% sucrose containing 0.025% Triton X-100 and loaded into sucrose step gradients (*right axis*) followed by ultracentrifugation at 250,000 × *g* overnight at 4 °C. After fractionating the gradients in 0.2-ml aliquots, the distribution of $^{125}$I-IgE-FcεRI was determined and is expressed as the fraction of total $^{125}$I present in the gradient, including the pellet. *Error bars* show the range of duplicate gradients run on the same day. *B*, RBL cells sensitized as above and suspended in 20 mM phosphate, pH 7.5, 150 mM NaCl, and 5 mM EDTA were permeabilized with 0.4 units/ml of Streptolysin O (Burroughs-Wellcome, Research Triangle Park, NC) for 15 min at 37 °C. The permeabilized cells were then stimulated with streptavidin (●) or not (○) followed by lysis and ultracentrifugation as in *A*. Staining with Trypan blue confirmed that nearly 100% of the cells were permeabilized by Streptolysin O, and immunoblotting showed no stimulated phosphorylation of FcεRI β under these conditions.[2]

lacking detergent prior to elution by boiling in nonreducing SDS sample buffer.

*In Vitro Kinase Assays*—Kinase assays were performed by adding kinase buffer (20 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, and 1 mM $Na_3VO_4$) to the sucrose fractions and incubating at 37 °C for 15 min. The reaction was quenched either with 5 × nonreducing SDS sample buffer or by adding 50 mM EDTA followed by immunoprecipitating FcεRI.

## RESULTS AND DISCUSSION

In order to determine if the interaction of FcεRI with membrane domains is involved in the activation of this immunoreceptor, we developed conditions that preserve this association during the isolation of these complexes by equilibrium sucrose density ultracentrifugation. As shown in Fig. 1*A*, limiting amounts of Triton X-100 used for cell lysis preserve the association of aggregated FcεRI (●, ■) with the detergent-resistant membrane domains which migrate as low density vesicles (fractions 3–7). In 29 separate experiments, 54 ± 7% of biotin-IgE FcεRI complexes aggregated with streptavidin associate with the membrane domains (●). Significant but lesser amounts of antigen-aggregated receptors associate (■; 11 ± 1%, $n = 6$), most likely reduced by the partial reversal of IgE-antigen binding during the overnight ultracentrifugation. In contrast, monomeric FcεRI (○) is nearly absent from the membrane domains (3 ± 1%, $n = 26$) and found almost entirely in the 40% sucrose fractions containing solubilized proteins (fractions 10–16). The association of FcεRI with isolated membrane domains depends on its aggregation at the cell surface, as less than 5% association is seen for FcεRI aggregated after cell lysis or for FcεRI aggregated with antigen on cells and then dissociated with monovalent hapten after lysis.[2] The interaction between aggregated FcεRI and the membrane domains is very sensitive to the detergent:cell lipid ratio during solubilization and ultracentrifugation, as indicated by its disruption when concentrations of Triton X-100 greater than 0.05% are used (15). This sensitivity is similar to that observed by Pribluda *et al.* (23) for FcεRI coupling to Lyn in cell lysates, and it contrasts with cytoskeleton-mediated detergent insolubility of aggregated FcεRI (33–35), which is not disrupted by high Triton X-100 concentrations.

Although this reduction in Triton X-100 used for cell lysis dramatically increases the amount of aggregated FcεRI that remains associated with detergent-resistant membrane domains, these domains are otherwise very similar to those isolated after lysis in high Triton X-100 (≥0.2%). When directly compared, domains from low and high detergent lysis conditions contain the same fraction of cellular Lyn, and neither has detectable amounts of Src.[2] In addition, both preparations contain a similar spectrum of tyrosine kinase substrates as revealed in *in vitro* tyrosine kinase assays (Ref. 15 and as described below), and both contain similar amounts of cellular protein (<2% of the total).[2] By these criteria, the domains obtained using 0.05% Triton X-100 for cell lysis appear to be identical to other membrane domains previously described that do not contain caveolin (10, 13–15). Furthermore, the aggregation-dependent association of FcεRI with membrane domains shows selectivity among transmembrane cell surface receptors, as FcεRI but not Type I interleukin-1 receptors, both expressed on Chinese hamster ovary cells, associate with membrane domains following aggregation.[3]

Association of FcεRI with membrane domains does not require tyrosine phosphorylation of the receptor subunits. As shown in Fig. 1*B*, RBL cells permeabilized with Streptolysin O in the presence of excess EDTA to inhibit kinase activity show a similar amount of aggregation-dependent association of FcεRI with domains as intact cells (Fig. 1*A*). As previously shown with broken cells (36), stimulated tyrosine phosphorylation of FcεRI β and other substrates is prevented by EDTA in these permeabilized cells.[2]

The presence of Lyn and aggregated FcεRI within the same subregions of the plasma membrane suggests that domain-associated Lyn could be responsible for the initial phosphorylation of the immunoreceptor tyrosine-based activation motifs. FcεRI associates with membrane domains very rapidly at 37 °C (○, Fig. 2*A*) and is more than 50% complete within 30 s, whereas substantially less than 50% of the maximal tyrosine phosphorylation of FcεRI β occurs during this time (●, Fig. 2*A*). The amount of β tyrosine phosphorylation declines after 2 min at 37 °C, and domain-associated receptor also decreases between 5 and 30 min in parallel with its internalization.[2] At 4 °C, the association of FcεRI with domains occurs more slowly (○, Fig. 2*B*), but is clearly more rapid than the β tyrosine phosphorylation during the first 5 min (●, Fig. 2*B*). FcεRI internalization and downstream signaling such as $Ca^{2+}$ mobilization and phosphatidylinositol hydrolysis do not occur at 4 °C (37), indicating that they are not required for domain association. These results demonstrate that association of FcεRI with membrane domains on cells is an early, aggregation-dependent event that is sufficiently rapid to mediate receptor tyrosine phosphorylation.

Evidence for FcεRI tyrosine phosphorylation occurring within membrane domains of intact cells is shown in Fig. 3. Stimulation of biotin-IgE-sensitized RBL cells with streptavidin dramatically increases the tyrosine phosphorylation of many proteins. When lysates of these cells are analyzed by sucrose gradient ultracentrifugation, most of the proteins with

[3] K. A. Field, D. Holowka, and B. Baird, manuscript in preparation.

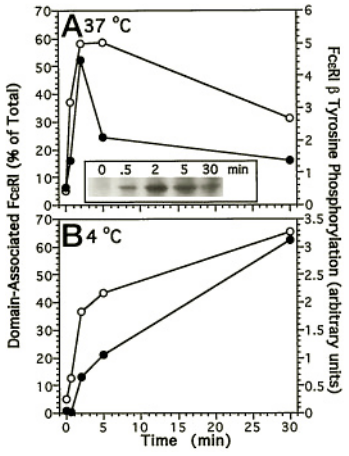


FIG. 2. **Comparison of the kinetics of FcεRI association with domains with its tyrosine phosphorylation.** RBL cells were stimulated for various times at 37 °C (*A*) or at 4 °C (*B*) with 10 nM streptavidin, then lysed with 0.05% Triton X-100 in the presence of 1 μM biotin to prevent cross-linking subsequent to lysis and analyzed as for Fig. 1. The percentage of $^{125}$I-IgE-FcεRI present in the membrane domain fractions (○, gradient fractions 3–7) at different times is compared to the relative tyrosine phosphorylation of the FcεRI β subunit (●) determined as described under "Experimental Procedures." The *inset* displays the tyrosine phosphorylation of the β subunit used to obtain the results presented in *A*. The relative intensities of tyrosine phosphorylation are represented on the same scale in *A* and *B*.

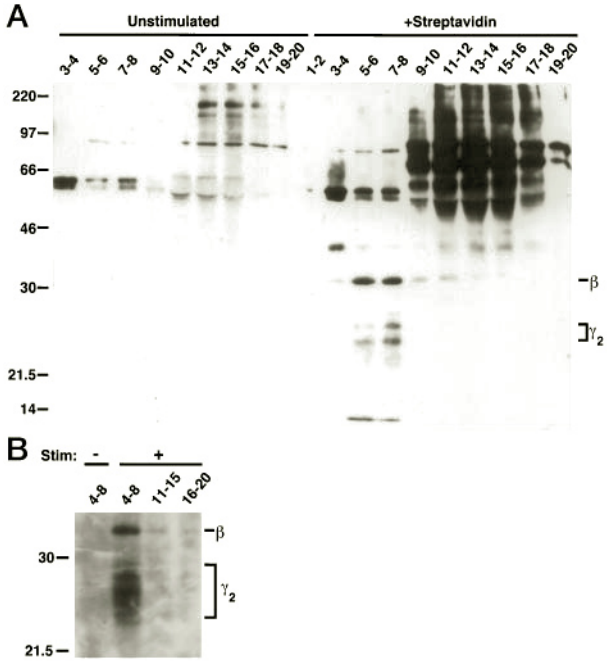


FIG. 3. **Tyrosine phosphorylation in intact cells of domain-associated FcεRI.** *A*, receptor-stimulated tyrosine phosphorylation in sucrose gradient fractions. RBL cells either unstimulated or stimulated with 10 nM streptavidin for 2 min at 37 °C were lysed with 0.05% Triton X-100 and analyzed as for Fig. 1. Sucrose fractions containing 1 mM $Na_3VO_4$ were pooled as indicated and electrophoresed and analyzed with anti-phosphotyrosine immunoblotting. Apparent molecular masses are indicated on the *left* in kDa. In this experiment, 43% of the $^{125}$I-IgE-FcεRI was present in fractions 5–8 after stimulation. *B*, tyrosine phosphorylation of domain-associated FcεRI. IgE-FcεRI was immunoprecipitated in the presence of 0.2% Triton X-100 from the indicated sucrose fractions from the streptavidin-stimulated (+) or unstimulated (−) gradients shown in *A*. The relative amount of FcεRI in each lane is (*left* to *right*) 0.10, 1.00, 0.33, and 0.46 as determined by the associated $^{125}$I-IgE.

enhanced tyrosine phosphorylation are found with the solubilized proteins at 40% sucrose (fractions 11–16), as expected (15). Associated with the membrane domains (fractions 3–8) after stimulation are tyrosine-phosphorylated proteins of approximately 90, 53/56, 45, 34, and 25–30 kDa. The 53/56-kDa doublet was identified as Lyn by reprobing the blot with rabbit anti-Lyn (UBI).[2] Significantly, the 45-, 34-, and 25–30-kDa bands appear only with stimulation and are markedly enriched in membrane domains relative to the other fractions. The domain-associated proteins of 34 and 25–30 kDa correspond to phosphorylated β and $\gamma_2$ FcεRI subunits, respectively, as identified by immunoprecipitating FcεRI from the sucrose gradient fractions (Fig. 3*B*). Fig. 3 clearly shows that the *tyrosine-phosphorylated* β and γ subunits are almost entirely associated with membrane domains. The majority of other tyrosine kinase substrates phosphorylated as the result of FcεRI aggregation are located in the solubilized protein fractions, presumably because they are cytosolic or associated with membranes that are solubilized in 0.05% Triton X-100. Syk, the ZAP-70-related tyrosine kinase responsible for phosphorylating the majority of substrates downstream of FcεRI (19, 38, 39), is also found exclusively in these soluble fractions,[2] as expected because activated Syk is localized primarily in the cytosol after receptor stimulation (40, 41).[2] Thus, following FcεRI aggregation on cells, Lyn phosphorylates the β and γ subunits of domain-associated receptors. This apparently leads to a transient association and the activation of Syk, followed by Syk-mediated phosphorylation of downstream substrates, most of which are not stably associated with membrane domains.

Additional support for the involvement of these domains in initiating FcεRI activation comes from *in vitro* tyrosine kinase assays performed on sucrose fractions, followed by immunoprecipitation of FcεRI in the presence of 0.2% Triton X-100 (which releases FcεRI from membrane domains). Fig. 4*A* shows that aggregated FcεRI associated with membrane domains isolated after cell lysis in 0.05% Triton X-100 (*MD*+) are efficiently tyrosine-phosphorylated *in vitro*, whereas receptors in the sucrose fractions containing solubilized proteins (40+ and 40−) are not phosphorylated, and membrane domains from unstimulated cells (*MD*−) also show no phosphorylated FcεRI. Streptavidin-aggregated FcεRI from cells lysed in 0.2% Triton X-100 migrate at a high density (50–70% sucrose) in these gradients (15). When *in vitro* tyrosine kinase assays are performed on these high density sucrose fractions (*HD*+), a relatively small amount of β subunit phosphorylation is seen. This fraction does contain a small amount of Lyn that may be responsible for the phosphorylation detected,[2] but it is not known whether this represents Lyn directly associated with FcεRI, Lyn contaminating this fraction from the 40% sucrose fraction, or fragments of membrane domains which remain receptor-associated in 0.2% Triton X-100. Consistent with the last possibility, the *in vitro* phosphorylation in the HD+ fraction shows a Triton X-100 sensitivity similar to that of the MD+ fraction (see below).

When *in vitro* tyrosine kinase assays are performed on membrane domains, stimulated FcεRI phosphorylation is highly sensitive to the concentration of Triton X-100 present. As shown in Fig. 4*B*, addition of submicellar (0.01%) Triton X-100 to the membrane domains causes a slight enhancement of FcεRI β and γ tyrosine phosphorylation, but higher Triton X-100 concentrations dramatically reduce this phosphorylation. The association of Lyn with membrane domains, as well as its activity toward the exogenous substrate, enolase, is not significantly affected by Triton X-100,[2] and neither is phosphorylation of Lyn itself or the 45-kDa substrate (Fig. 4*B*). The exquisite sensitivity of FcεRI *in vitro* phosphorylation to Triton X-100 indicates that lipid-mediated association of these receptors with the membrane domains is required for this activation

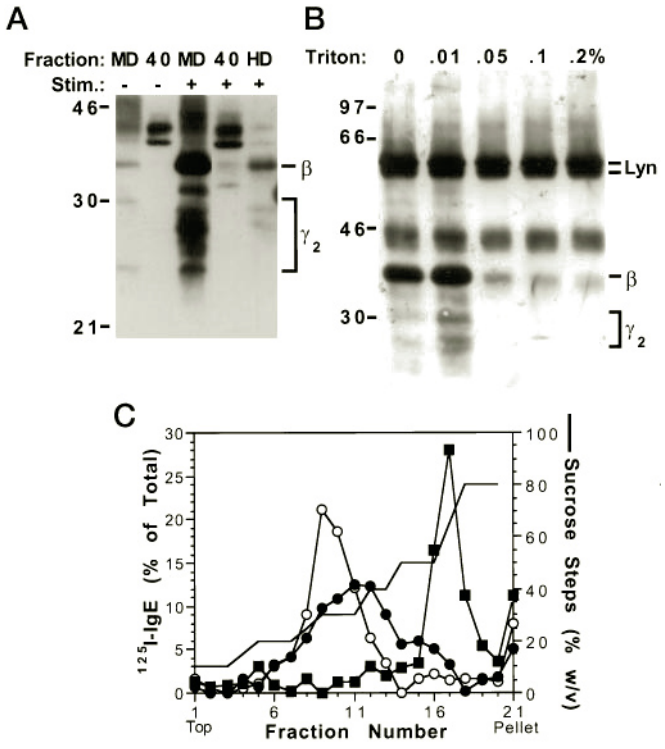


FIG. 4. ***In vitro* tyrosine phosphorylation of FcεRI associated with membrane domains.** *A*, preferential phosphorylation of FcεRI associated with membrane domains from stimulated cells. Kinase assays were performed on fractions from sucrose gradients without $Na_3VO_4$ containing either detergent-resistant membrane domains (*MD*), the 40% sucrose fraction (*40*), or immune complexes in high density sucrose (*HD*), from either unstimulated RBL cells (−) or cells stimulated with 10 nM streptavidin for 5 min at 37 °C (+). FcεRI was then immunoprecipitated and subjected to anti-phosphotyrosine immunoblotting as in Fig. 3*B*. The relative amount of $^{125}$I-IgE loaded in each lane is (*left* to *right*) 0.04, 1.00, 0.76, 0.34, and 0.42. Experiments where ATP and $Mg^{2+}$ were omitted from the kinase assay, or where cells without IgE were used, showed no detectable phosphorylation of β and γ under these conditions.[2] *B*, detergent sensitivity of *in vitro* FcεRI phosphorylation. Membrane domains isolated as in Fig. 1 from streptavidin-stimulated cells were incubated with the indicated concentration of Triton X-100 prior to performing kinase assays on the sucrose fractions. The samples were then boiled with SDS, electrophoresed, and immunoblotted with anti-phosphotyrosine. *C*, extraction of FcεRI from membrane domains with Triton X-100. Membrane domain fractions isolated from streptavidin-stimulated cells as for *B* were treated with no Triton X-100 (○), with 0.01% Triton X-100 (●), or with 0.05% Triton X-100 (■), readjusted to 40% sucrose, and ultracentrifuged overnight within a sucrose gradient (*right axis*) as in Fig. 1.

step. Fig. 4*C* supports this conclusion, demonstrating that treatment of isolated membrane domains with 0.05% Triton X-100 (■) dissociates the aggregated receptors from these membrane domains as indicated by reanalysis on a second sucrose gradient. Treatment of the same domains with 0.01% Triton X-100 (●) does not dissociate the receptor, although it does cause a slight change in the distribution of the receptor within the gradient relative to untreated membrane domains (○). Thus, FcεRI associated with membrane domains is functionally coupled to Lyn, and this association is easily disrupted by detergent.

The involvement of membrane domains in this early step of FcεRI activation provides a new model in which the initial phosphorylation of the β and γ subunits by Lyn is mediated by lipid-protein interactions. Although previous results have explained how the phosphorylation of FcεRI β and γ and subsequent events proceed after the association of active Lyn with a receptor cluster (23, 25, 38, 39), the structural basis for the initial interaction between Lyn and FcεRI in the activation process has remained poorly defined. Several studies that detected association of Lyn with unstimulated FcεRI utilized methods that could stabilize the association of these receptors with membrane domains, including chemical cross-linking (18) or low detergent:cell lipid ratios (23). We find that unstimulated receptors do not co-isolate with the Lyn-containing domains to a large extent (Fig. 1), although weak and/or transient interactions could occur on intact cells. The size and stability of the domains on the surface of intact, unstimulated cells are unknown. These domains are likely to be small and dynamic in composition, but appear to coalesce together with aggregated FcεRI (29, 30). Thus, localization of Lyn within membrane domains could serve to sequester this kinase away from FcεRI prior to receptor aggregation and, in turn, provide a pool of active or readily stimulated Lyn for aggregated FcεRI that stably associate with the domains. Support for this aspect of the model comes from our observation that isolated membrane domains contain abundant tyrosine kinase activity even in the absence of FcεRI activation (15), as well as from other studies on Src family members that associate with detergent-resistant membrane domains. These other investigations have found that Fyn, Lck, and Fgr associated with isolated membrane domains show higher specific activity *in vitro* than soluble forms of these kinases (42, 43), possibly because of the capability for kinases concentrated within domains to *trans*-autophosphorylate readily.[4]

Our results demonstrate that aggregation of FcεRI causes its rapid and efficient association with specialized domains in the plasma membrane that are enriched in the tyrosine kinase, Lyn. FcεRI associated with membrane domains are rapidly tyrosine-phosphorylated in intact cells, and this phosphorylation is also observed *in vitro* preferentially for receptors associated with membrane domains. The interaction of FcεRI with these specialized membrane domains does not depend on the β subunit,[3] and thus can account for the initiation of receptor signaling independent of specific protein-protein interactions between the β subunit and Lyn (24–28). In addition, this receptor-membrane domain association may facilitate coupling to processes such as $Ca^{2+}$ mobilization, lipid metabolism, and exocytic vesicle fusion. Recent evidence indicates that specialized membrane domains, including caveolae, are involved in the signaling of other cell surface receptors such as certain growth factor receptors (3, 5) and glycosylphosphatidylinositol-linked mitogenic receptors (12, 44, 45). Receptor-domain interactions also may be important for other multichain immune recognition receptors that utilize Src family kinases during their initial signaling steps (46, 47).

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