Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9201–9205, September 1995
Immunology

FcεRI-mediated recruitment of p53/56^{lyn} to detergent-resistant membrane domains accompanies cellular signaling

(mast cells/high-affinity IgE receptor/caveolae/tyrosine kinases)

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Communicated by Leon A. Heppel, Cornell University, Ithaca, NY, July 3, 1995

ABSTRACT **Detergent-resistant plasma membrane structures, such as caveolae, have been implicated in signaling, transport, and vesicle trafficking functions. Using sucrose gradient ultracentrifugation, we have isolated low-density, Triton X-100-insoluble membrane domains from RBL-2H3 mucosal mast cells that contain several markers common to caveolae, including a src-family tyrosine kinase, p53/56^{lyn}. Aggregation of FcεRI, the high-affinity IgE receptor, causes a significant increase in the amount of p53/56^{lyn} associated with these low-density membrane domains. Under our standard conditions for lysis, IgE-FcεRI fractionates with the majority of the solubilized proteins, whereas aggregated receptor complexes are found at a higher density in the gradient. Stimulated translocation of p53/56^{lyn} is accompanied by increased tyrosine phosphorylation of several proteins in the low-density membrane domains as well as enhanced *in vitro* tyrosine kinase activity toward these proteins and an exogenous substrate. With a lower detergent-to-cell ratio during lysis, significant FcεRI remains associated with these membrane domains, consistent with the ability to coimmunoprecipitate tyrosine kinase activity with FcεRI under similar lysis conditions [Pribluda, V. S., Pribluda, C. & Metzger, H. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11246–11250]. These results indicate that specialized membrane domains may be directly involved in the coupling of receptor aggregation to the activation of signaling events.**

The src-family protein-tyrosine kinase (PTK) p53/56^{lyn} is known to associate with and participate in the signaling mediated by aggregation of FcεRI on RBL-2H3 cells (1–3). FcεRI, predominantly found on mast cells and basophils, has structural and functional features in common with the T-cell receptor for antigen and the surface immunoglobulin-containing B-cell receptor (4). Aggregation of these three types of receptors stimulates similar signaling pathways, including activation of src-family and ZAP-70/syk-family PTKs, stimulation of phosphatidylinositol hydrolysis, elevation of cytosolic Ca^{2+}, and activation of protein kinase C and other serine/threonine kinases (5, 6). In mast cells, including RBL-2H3 cells, activation of these signaling pathways leads to exocytosis of various inflammatory mediators, including histamine, as well as synthesis and secretion of leukotrienes and certain cytokines (5, 7). The stoichiometry and affinity of p53/56^{lyn} association with FcεRI are low in lysates from unstimulated cells, but antigen-mediated aggregation of IgE–receptor complexes causes an increased association of active p53/56^{lyn} (1–3). In a recent study, Pribluda *et al.* (8) reported that coimmunoprecipitation of tyrosine kinase activity with FcεRI depends critically on the ratio of detergent to cell lipid in the lysate, for both antigen-stimulated and unstimulated cells. This kinase activity, which appears to be due to p53/56^{lyn}, increases 3- to 10-fold upon aggregation of the receptor.

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In RBL-2H3 cells, p53/56^{lyn} has been shown to coimmunoprecipitate with a glycosylphosphatidylinositol (GPI)-linked protein, Thy-1 (9), and a GD_{1b} ganglioside derivative (10, 11), both of which are attached to the outer leaflet of the plasma membrane via lipid tails. These complexes, which lack FcεRI, contain 39- to 42-kDa and 70- to 80-kDa tyrosine kinase substrates (9, 11) and appear to exist as very large structures ($>10^6$ apparent molecular weight) as determined by gel permeation chromatography of postnuclear supernatants from Nonidet P-40-lysed cells (9). Using fluorescence microscopy we recently showed that the aggregation of FcεRI on RBL-2H3 cells causes coredistribution of membrane domains that are labeled with the fluorescent lipid analog, 3,3′-dihexadecylindocarbocyanine (12). These studies showed that large-scale aggregation of ganglioside GD_{1b} derivatives by the monoclonal antibody AA4 (10, 13) also causes redistribution of these domains, suggesting that they are related to the detergent-resistant complexes that are immunoprecipitated by AA4 and contain p53/56^{lyn} (11). In several other cell types, the src-family PTKs lck, fyn, and c-yes have been found to associate with GPI-linked proteins in large, detergent-resistant complexes (14, 15). Related membrane structures, known as caveolae or plasmalemmal vesicles, are found on a variety of cell types and have been found to be enriched in glycosphingolipids, GPI-linked proteins, certain src-family PTKs, GTP-binding proteins, and other signaling molecules as well as the v-src substrate, caveolin (16–18). Simple purification methods employing sucrose density ultracentrifugation of Triton X-100 (TX-100)-lysed cells yield a low-density fraction that is highly enriched in these membrane domains (15, 18–20).

We find that RBL-2H3 cells contain detergent-resistant membrane structures that have properties common to caveolae that have been characterized on other cells. In particular, we find the PTK p53/56^{lyn} associated with these caveolae-like domains, and aggregation of FcεRI recruits additional p53/56^{lyn} to this location with a concomitant increase in tyrosine kinase activity. Our results point to the possibility that receptor-mediated redistribution of important signaling proteins to specialized regions of the plasma membrane is integrally involved in the process of FcεRI-mediated signal transduction.

METHODS

Sucrose Density Ultracentrifugation of RBL-2H3 Cell Lysates. Murine monoclonal anti-dinitrophenyl IgE (21), anti-rat Thy-1 (clone OX7; PharMingen), and anti-GD_{1b} ganglioside [clone AA4 (10, 13)] were iodinated with chloramine T. ^{125}I-labeled IgE (^{125}I-IgE) was biotinylated with a 25-fold molar excess of 6-({6-[(biotinoyl)amino]hexanoyl}amino)-hexanoic acid, succinimidyl ester (Molecular Probes) for 2 hr

Abbreviations: TX-100, Triton X-100; PTK, protein-tyrosine kinase; GPI, glycosylphosphatidylinositol.

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at 22°C and then dialyzed extensively. RBL-2H3 cells (22) were harvested in 1.5 mM EDTA, washed, and then resuspended in buffered salt solution [BSS (12)] with 1 mg of bovine serum albumin per ml. After stimulation, as indicated, cells were centrifuged and lysed by resuspension in lysis buffer [25 mM Mes, pH 6.5/50 mM NaCl/1 mM phenylmethylsulfonyl fluoride or 4-(2-aminoethyl)benzenesulfonylfluoride (Calbiochem)/0.02 unit of aprotinin per ml/0.01% (wt/vol) NaN_3/1 mM Na_4VO_3/30 mM pyrophosphate/10 mM glycerophosphate] containing TX-100 at indicated concentrations and then incubated on ice for 10 min. Except where indicated, the lysates were gently homogenized with 10 strokes in a 0.0010–0.0030 inch (1 inch = 2.54 cm) clearance Dounce homogenizer and adjusted to 40% (wt/vol) sucrose by diluting with an equal volume of 80% (wt/vol) sucrose. For solubilization in 0.05% or 0.2% (vol/vol) TX-100, the appropriate concentrations of TX-100 were maintained when the lysate was diluted with sucrose. Sucrose step gradients contained 25 mM Mes (pH 6.5) and 0.15 M NaCl and were formed by the successive addition of 80%, 60%, 40% (containing the cell lysate), 30%, 20%, and 10% (wt/vol) sucrose to ultraclear centrifuge tubes (Beckman). Gradients were spun in a SW60.1 rotor (Beckman) at 250,000 × *g* for 12–18 hr at 4°C. Gradients were fractionated into 0.2-ml aliquots withdrawn from the top and the ^{125}I present in each fraction was determined with a Beckman model 4000 γ counter. The sucrose concentration (% wt/vol) in each fraction was estimated based on the known concentration of the stock solutions and the volume of each step in the gradient prior to centrifugation.

Immunoblotting of Sucrose Gradient Fractions. Aliquots from sucrose gradient fractions were pooled as indicated and then boiled in sample buffer containing 1.0% (wt/vol) SDS, 40 mM Tris (pH 6.8), and 2.0% (vol/vol) 2-mercaptoethanol. Samples were electrophoresed on 12.5% acrylamide/SDS gels and then transferred to Immobilon poly(vinylidene difluoride) (Millipore) using a semidry transfer apparatus (Integrated Separation Systems, Hyde Park, MA). Membranes were blocked with 4% (wt/vol) bovine serum albumin and probed with either 1:25,000 rabbit anti-lyn (Upstate Biotechnology, Lake Placid, NY) or 1:5000 mouse anti-src [clone 327 (23)]. Membranes were then washed and probed with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham) at 1:5000 dilution. Anti-phosphotyrosine blots were performed using 1:2500 horseradish peroxidase-conjugated PY20 antibody (Transduction Laboratories, Lexington, KY). Blots were developed using ECL chemiluminescence and Hyperfilm-ECL (Amersham). Densitometry was performed with a Bio-Rad model 620 video densitometer in transmittance mode. Peak intensities were integrated after subtracting the appropriate background. Averages are reported as the mean of the 53- and 56-kDa forms of lyn; errors represent the standard error of the mean.

***In Vitro* Tyrosine Kinase Assays.** Twenty percent sucrose gradient fractions were pooled (20 μl each of fractions 4, 5, and 6) and diluted to 250 μl in kinase buffer (20 mM Tris, pH 7.4/150 mM NaCl/10 mM $MgCl_2$/0.5 mM dithiothreitol/25 μM ATP). Eight micrograms of acid-denatured enolase was added and the samples were incubated for 15 min at 37°C, followed by the addition of 5× SDS sample buffer with 2-mercaptoethanol and boiling. Control samples were boiled in SDS before kinase buffer was added. Samples were electrophoresed, transferred, and immunoblotted with anti-phosphotyrosine as described above.

RESULTS AND DISCUSSION

To investigate whether detergent-resistant membrane domains are involved in the association of $p53/56^{lyn}$ with GD_{1b} ganglioside or Thy-1, and to determine the relevance of these interactions to FcεRI-mediated activation of RBL-2H3 cells, we used sucrose density gradient ultracentrifugation to characterize the subcellular location of $p53/56^{lyn}$. In initial experiments, RBL-2H3 cells, sensitized with biotinylated ^{125}I-IgE, were lysed in 1.0% TX-100 lysis buffer at a concentration of 4 × 10^7 cells per ml either before or after treatment with 100 nM streptavidin for 30 min at 4°C. Under these conditions, we observed that streptavidin treatment causes a large increase in tyrosine phosphorylation of cellular proteins, similar to that seen for antigen-triggered cells at 4°C and at 37°C (ref. 24; unpublished data). Cell lysates were adjusted to 40% sucrose and included within sucrose step gradients for fractionation by equilibrium ultracentrifugation. As shown in Fig. 1*A*, crosslinking by streptavidin caused a significant shift of receptor-bound ^{125}I-IgE from the 40% sucrose lysate fraction, which contains most of the cellular proteins, to a higher-density (60–80% sucrose) fraction. No change in the location of ^{125}I-IgE in the gradient was observed if streptavidin was added after cell lysis (data not shown). When similar experiments were performed on cells pretreated with ^{125}I-labeled anti-Thy-1, most of this GPI-linked protein was located in the 20% sucrose fraction, which has the density of lipid-containing structures (Fig. 1*B*).

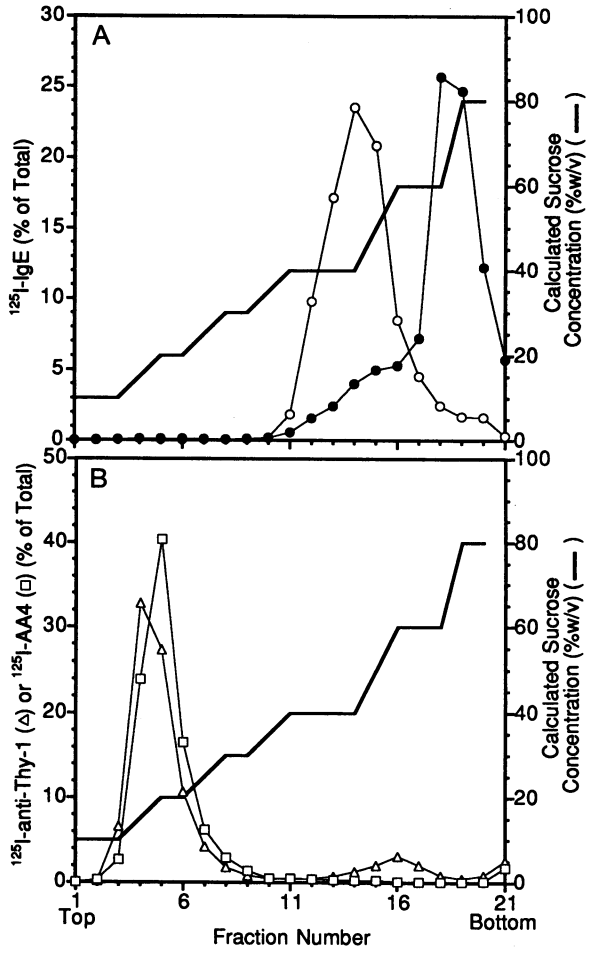

FIG. 1. Sucrose gradient ultracentrifugation of RBL-2H3 cell lysates. (*A*) RBL-2H3 cells sensitized with biotinylated ^{125}I-IgE were treated (●) or not treated (○) with 100 nM streptavidin, lysed in 1.0% TX-100, loaded within the 40% sucrose fraction of sucrose step gradients, and fractionated by ultracentrifugation. (*B*) RBL-2H3 cells were labeled with either ^{125}I-OX7 anti-Thy-1 antibody (△) or ^{125}I-AA4 anti-ganglioside antibody (□), washed twice in BSS, lysed in 1.0% TX-100, and fractionated by sucrose gradient ultracentrifugation. Data are representative of 12 experiments with ^{125}I-IgE and 2 each with ^{125}I-anti-Thy-1 and ^{125}I-AA4. Points represent the percent of the total cpm present in the gradient (on the left axis); fraction 21 contains the pellet. The calculated sucrose concentration (heavy line) is shown on the right axis.

If cells were lysed in 1.0% TX-100 prior to addition of the antibody, a similar amount of Thy-1 was detected in this low-density fraction (data not shown), indicating that prior crosslinking of this protein by the antibody is not required for its association with these membrane structures. In addition, ^{125}I-labeled anti-ganglioside GD_{1b} monoclonal antibody AA4 was found almost entirely in the low-density fraction when this antibody was added before (Fig. 1*B*) or after (not shown) TX-100 lysis of the cells. The density and opaque appearance of this fraction are identical to that previously described for GPI protein-rich membrane domains (19). Furthermore, transmission electron microscopy of uranyl acetate-stained 20% sucrose fractions revealed many 200- to 700-nm vesicular structures similar in appearance to detergent-resistant structures previously isolated (ref. 15; K.A.F. and D. J. Reczek, unpublished data).

When these sucrose gradients were analyzed by immunoblotting with anti-lyn we observed that a significant fraction of cellular p53/56^{lyn} was present in this low-density (≈20% sucrose) region of the gradient (Fig. 2*A*, lanes 1–5). Interestingly, the amount of p53/56^{lyn} in this region increased substantially upon FcεRI aggregation (Fig. 2*A*, lanes 6–10). In 11 separate experiments analyzed with densitometry, the percentage of total cellular p53/56^{lyn} associated with this fraction was found to be 31% ± 4% in the absence of stimulation. Upon FcεRI aggregation, an additional 17% ± 3% of the total cellular p53/56^{lyn} was translocated from the 40% sucrose fraction, which contains solubilized protein, to this low-density fraction containing caveolae-like membrane structures. The ratio of p53/56^{lyn} in the 20% fraction to that in the 40% fraction increased 2-fold (from 0.64 to 1.27) upon receptor aggregation. This translocation is statistically significant ($P < 0.001$) based on Student's *t* test.

Under the lysis conditions of Fig. 1, the low-density fraction contained <1% of receptor-bound ^{125}I-IgE before and after stimulation. Also, <5% of p53/56^{lyn} was in the high-density fraction (60–80% sucrose) that contains most of the crosslinked ^{125}I-IgE under these conditions. Thus, maintenance of p53/56^{lyn} in these fractionated detergent-resistant domains does not require physical interaction with IgE–receptor complexes. Qualitatively similar redistribution of p53/56^{lyn} was observed in separate experiments in which cells were stimulated for 5 min at 37°C instead of 30 min at 4°C or with multivalent antigen instead of streptavidin (unpublished data). Since aggregation of FcεRI at 4°C does not trigger downstream signaling events such as phosphatidylinositol hydrolysis, Ca^{2+} mobilization, and degranulation (25), and because recruitment of p53/56^{lyn} to these domains is seen as rapidly as 1 min after stimulation at 37°C (unpublished data), this recruitment appears to represent an early signal transduction event directly linked to PTK activation.

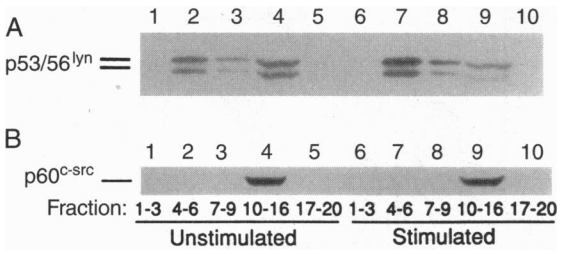

FIG. 2. Location of p53/56^{lyn} and p60$^{c\text{-}src}$ in sucrose gradients. Immunoblots from SDS gels were performed with anti-lyn (*A*) or anti-src (*B*) on sucrose gradient fractions from either unstimulated (lanes 1–5) or 100 nM streptavidin-stimulated (lanes 6–10) cell lysates. Equal aliquots from each fraction were pooled from sucrose gradient fractions 1–3 (≈10% sucrose, lanes 1 and 6), fractions 4–6 (≈20% sucrose, lanes 2 and 7), fractions 7–9 (≈30% sucrose, lanes 3 and 8), fractions 10–16 (≈40% sucrose, lanes 4 and 9), and fractions 17–20 (≈60–80% sucrose, lanes 5 and 10). The observed position of each protein is indicated on the left, as determined by appropriate molecular mass and reproducible blotting with two different antibodies specific for p53/56^{lyn}. Densitometry of the fractions in *A* showed that 37% and 63% of total p53/56^{lyn} were present in the 20% sucrose fraction from unstimulated and stimulated cells, respectively.

Anti-src immunoblots of sucrose gradient fractions show that this PTK was not significantly associated with the membrane domains in the 20% sucrose fraction either before or after stimulation (Fig. 2*B*). This is consistent with the suggestion that palmitoylation on a cysteine residue near the N terminus may be involved in the association of some PTKs with these membrane structures (26), since p53/56^{lyn} but not p60$^{c\text{-}src}$ contains a site for this modification (27). Aggregation of FcεRI may cause an increase in the palmitoylation of p53/56^{lyn} and other proteins, thereby mediating their association with the membrane domains. This type of regulation has recently been observed for the activated β-adrenergic receptor, which increases the palmitoylation turnover on the α-subunit of the G_s heterotrimeric GTP-binding protein (28).

Caveolin is a 22-kDa protein that is considered to be a marker for caveolae on endothelial and epithelial cells (17). RBL-2H3 cells contain a 22-kDa protein that is recognized by anti-caveolin antibodies in immunoblots, but this protein does not appear to associate with the same low-density structures that contain p53/56^{lyn} (unpublished data). Low-density membrane domains containing Thy-1 and GM_1 ganglioside have recently been isolated from T- and B-cell lines that do not express caveolin (29). It is likely that the structures we have isolated from RBL-2H3 mast cells are analogous to these latter domains.

By including ATP and vanadate in the sucrose gradients, we could examine the phosphorylation of the proteins fractionated from stimulated and unstimulated cells. Anti-phosphotyrosine immunoblots of the 20% sucrose fraction revealed a heavily phosphorylated doublet of 53/56 kDa and another broad band at 75–95 kDa, both of which were significantly enhanced in stimulated cells (Fig. 3, lanes 1 and 2). A longer exposure revealed additional bands at 28–32, 41, and 50 kDa

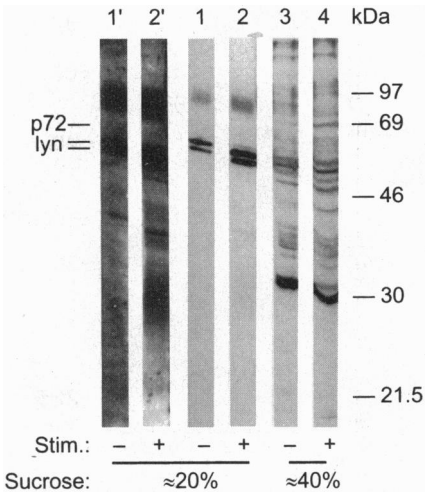

FIG. 3. Stimulation of tyrosine phosphorylation in 20% and 40% sucrose fractions. Shown are immunoblots from SDS gels loaded with aliquots (100 μl) from the 20% sucrose (lanes 1 and 2) or 40% sucrose (lanes 3 and 4) gradient fractions of unstimulated (lanes 1 and 3) or 100 nM streptavidin-stimulated (lanes 2 and 4) cell lysates. Lanes 1′ and 2′ represent longer exposures of lanes 1 and 2. The migration of molecular mass standards is indicated on the right, and the approximate positions of p53/56^{lyn} and p72^{syk} are indicated on the left. This figure is representative of six separate experiments. Samples were prepared as described for Fig. 2 except that 1 mM vanadate and 1 mM ATP were included throughout the sucrose gradient.

that are also enhanced upon stimulation (Fig. 3, lanes 1′ and 2′). This pattern of phosphoproteins is similar to those seen for AA4 or anti-Thy-1 immunoprecipitates from RBL-2H3 cell lysates (9, 11), suggesting that these components are all part of a large, low-density complex. In the 40% sucrose fraction we observed stimulated phosphorylation of several proteins (Fig. 3, lanes 3 and 4), most notably p72, which probably includes the PTK $p72^{syk}$ (30, 31). This phosphoprotein was not seen in the 20% fraction.

To determine if $p53/56^{lyn}$ kinase activity was retained during our fractionation procedure, *in vitro* tyrosine kinase assays were performed. The assays were done on 20% sucrose fractions from either unstimulated or antigen-stimulated cells by diluting the sucrose fractions into kinase buffer containing acid-denatured enolase, which is commonly used as a substrate for src-family PTKs (32). As shown in Fig. 4, lanes 1 and 2, the 20% sucrose fractions contained abundant tyrosine kinase activity that reproducibly phosphorylated enolase as well as endogenous proteins of 75–95, 53/56, and 35–42 kDa. Substrates at 30 and 22 kDa were also seen in Fig. 4, lanes 1 and 2, although these were less frequently observed in these experiments. The apparent molecular masses of these various protein substrates are very similar to those seen in anti-phosphotyrosine immunoblots of 20% sucrose fractions (Fig. 3 and data not shown). They are also similar to the tyrosine phosphorylation substrates previously identified in anti-Thy-1 (9) and anti-GD_{1b} (11) immunoprecipitates of postnuclear Microfuge supernatants of Nonidet P-40- or Brij-96-extracted RBL-2H3 cells.

Antigen stimulation caused a substantial increase in the amount of tyrosine kinase activity found in these low-density fractions (Fig. 4, compare lanes 1 and 2) that paralleled the amount of $p53/56^{lyn}$ recruitment seen in anti-lyn immunoblots from the same experiment (data not shown). Although it seems likely that the additional tyrosine phosphorylation detected in the low-density fractions following stimulation is due to the increase in $p53/56^{lyn}$, it is possible that stimulation also causes recruitment of these kinase substrates (Figs. 3 and 4). Comparison of the tyrosine phosphorylation in these samples to the amount present in these fractions boiled in SDS prior to the kinase assay (Fig. 4, lanes 3 and 4) confirms that the observed tyrosine phosphorylation is due to *in vitro* activity. Centrifugation of the diluted 20% sucrose fractions at 100,000 × *g* for 5 min prior to performing the kinase assay sedimented virtually all the tyrosine kinase activity in these samples (not shown), further supporting the conclusion that the kinase activity is associated with a particulate structure. At lower centrifugation speeds typically used to pellet nuclei and for immunoprecipitations (≤12,000 × *g* for 5 min), most of the $p53/56^{lyn}$ and the tyrosine kinase activity in these fractions remained in the supernatant (data not shown). The anti-lyn antibody used to detect $p53/56^{lyn}$ by immunoblotting in these fractions failed to immunoprecipitate $p53/56^{lyn}$ in these structures (unpublished data), suggesting that the sequence recognized by this polyclonal antibody (residues 39–58; Upstate Biotechnology) is inaccessible in the intact domains. Correspondingly, we are unable to immunodeplete the *in vitro* kinase activity in these fractions. It is likely that a large fraction of the kinase activity detected in Fig. 4 is due to $p53/56^{lyn}$, although it remains possible that other tyrosine kinases contribute to the phosphorylations detected, possibly under regulation by $p53/56^{lyn}$.

Pribluda *et al.* (8) showed recently that a large amount of kinase activity can be coimmunoprecipitated with FcεRI if the cells are lysed with TX-100 at a micellar detergent/cell lipid ratio significantly lower than that used for our standard lysis conditions (e.g., Fig. 1). When we tested solubilization conditions identical to theirs (4×10^6 cells per ml, 0.05% TX-100), we found that 4–6% of IgE-FcεRI remained associated with the low-density membrane domains in the sucrose gradient (Fig. 5, circles). As expected from our previous results, this FcεRI association was disrupted at a higher TX-100/lipid ratio (4×10^6 cells per ml, 0.2% TX-100; Fig. 5, squares). The cell lysate was diluted 2-fold with 80% sucrose containing the same concentration of TX-100 as used to lyse the cells, and this

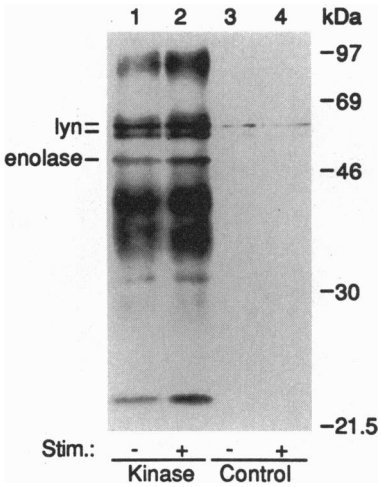

FIG. 4. *In vitro* tyrosine kinase activity in 20% sucrose gradient fractions. Anti-phosphotyrosine immunoblotting of SDS gels was used to detect the *in vitro* tyrosine kinase activity of the 20% sucrose fraction from unstimulated cells (lane 1) and from cells stimulated with 0.5 μg of dinitrophenyl-bovine serum albumin (antigen) per ml for 5 min at 37°C (lane 2). This is compared to the amount of phosphotyrosine present in a corresponding sample that was boiled in 1% SDS before the kinase assay (lanes 3 and 4, respectively). Kinase activity was measured for 15 min at 37°C in kinase buffer with acid-denatured enolase as added substrate (32). The positions of $p53/56^{lyn}$ and enolase are indicated on the left and molecular mass standards are indicated on the right. This figure is representative of four separate experiments.

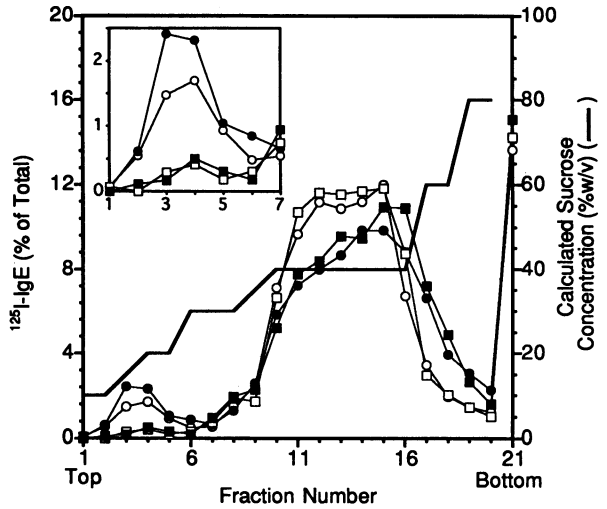

FIG. 5. Association of FcεRI with low-density membrane domains. ^{125}I-IgE-sensitized RBL-2H3 cells, either unstimulated (open symbols) or stimulated with 0.5 μg of antigen per ml (filled symbols), were solubilized with either 0.05% (circles) or 0.2% (squares) TX-100 at 4×10^6 cells per ml. The lysates were diluted with sucrose containing TX-100, loaded onto sucrose gradients without homogenization, and then centrifuged and analyzed. This figure is representative of four separate experiments. (*Inset*) Expanded scale of the low-density fractions. The calculated concentration of sucrose (heavy line) is shown on the right axis. In this figure, 4.6% and 6.4% of the ^{125}I-IgE are located in fractions 2–5 for unstimulated and stimulated samples, respectively, isolated with 0.05% TX-100; ≈1.0% of the ^{125}I-IgE is found in these fractions in both samples isolated with 0.2% TX-100.

increase in the ratio of micellar detergent to cell equivalents could actually reduce the amount of receptors that remain associated with the domains. Activation of cells with antigen for 5 min at 37°C caused some increase in the amount of the FcεRI associated with the membrane domains following lysis with 0.05% TX-100 but not with 0.2% TX-100 (Fig. 5 *Inset*). For both concentrations of TX-100, activation by antigen caused a significant shift to higher densities for the IgE-FcεRI located elsewhere in the gradient. These results show that some IgE-FcεRI associates with the low-density membrane domains, and this association can be maintained under sufficiently mild lysis conditions. In comparison with the study of Pribluda *et al.* (8), our results raise the possibility that the kinase activity that can be coimmunoprecipitated with unstimulated IgE-FcεRI may be due to $p53/56^{lyn}$ associated with the membrane domains that we have characterized. Correspondingly, the additional kinase activity observed after antigen stimulation may be due, in some part, to the recruitment of additional $p53/56^{lyn}$ to these domains.

In summary, our results indicate that a large fraction of cellular $p53/56^{lyn}$ is associated with TX-100-insoluble plasma membrane domains and that aggregation of IgE-FcεRI causes an increase in the amount of associated $p53/56^{lyn}$ and a concomitant increase in tyrosine phosphorylation and *in vitro* tyrosine kinase activity in these domains. Our previous results (12) suggest that aggregation of FcεRI causes the coaggregation of some of these membrane domains, and it is possible that the recruitment of $p53/56^{lyn}$ that we observe occurs only to the subset of membrane domains that is associated with aggregated FcεRI. In this scenario, the local enhancement of $p53/56^{lyn}$ within domains adjacent to aggregated FcεRI may be very substantial. Since the $p53/56^{lyn}$ associated with the domains appears to be highly active, this $p53/56^{lyn}$ may be responsible for the initial phosphorylation of the β and/or γ subunits of FcεRI that follows aggregation (33). Furthermore, we propose that $p53/56^{lyn}$ recruitment may initiate a cascade of events by phosphorylating proteins localized to this structure that regulate other signaling molecules. G_S, which has been found to be substantially enriched in caveolae-like domains (15, 18) and has been implicated in FcεRI-mediated Ca^{2+} mobilization and secretion (34, 35), may be involved in this cascade. The localization of certain Ca^{2+} channels and Ca^{2+}-ATPases to caveolae (36–38) also suggests a functional role for these structures in Ca^{2+}-dependent signaling. Similar membrane domains have been shown to be involved in the polarized delivery of GPI-linked proteins and certain viral coat proteins to the apical surface of epithelial cells (39), and it is possible that such structures participate in the stimulated exocytosis of intracellular granules. In this regard, caveolae-like domains contain the Ca^{2+}-sensitive, phospholipid-binding protein annexin II (15), which has been implicated in the fusion of membrane bilayers (40). Taken together, these studies suggest possible roles for caveolae-like membrane domains as centers for the generation of second messengers and as target sites for the terminal steps in stimulation–secretion coupling.

We thank W. W. Webb for inspiring this investigation, R. Siraganian for generously providing the AA4 antibody, J. Rivera for advice on immunoblotting and supplying reagents, D. Shalloway for providing anti-src antibody, and D. Shalloway, R. Cerione, and H. Metzger for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants AI22449, AI18306, and GM07273 (K.A.F.).

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