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Detergent-resistant Microdomains Offer No Refuge for Proteins Phosphorylated by the IgE Receptor*

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When the high affinity receptor for IgE and related receptors become aggregated, they emigrate to specialized microdomains of the plasma membrane that are enriched in certain lipids and lipid-anchored proteins. Among the latter are the kinases that initiate signaling cascade(s) by phosphorylating the receptors. In studying the IgE receptor, we explored whether, in addition to their potential role in enhancing the initiation of signaling by the kinase(s), the microdomains might augment the stimulation by excluding phosphatases. *In vitro* assessment of phosphatase activity, using either a relevant or irrelevant substrate, suggested that the microdomains were deficient in phosphatase activity, but, *in vivo*, proteins confined to the microdomains were found to be no less vulnerable to dephosphorylation than those outside such domains. In the course of our experiments, we observed that the procedures routinely used to isolate the detergent-resistant domains dissociated the receptor for IgE, thereby artificially accentuating the observed preferential distribution of phosphorylated subunits in the microdomains.

Binding of multivalent antigen by the IgE bound to the high affinity receptors for IgE (FcεRI)[1] aggregates them and leads to the rapid phosphorylation of specific tyrosines on the β and γ subunits of the receptors. The results from experiments using inhibitors of enzymes (1–3), and from other studies in which the clustered receptors were dissociated under the influence of excess monovalent antigen (hapten) (4, 5), demonstrate that the number of phosphorylated FcεRI and their lifetime is influenced both by protein kinases and protein phosphatases. In RBL-2H3 cells, a widely used line of rat mast cells, the aggregation-induced phosphorylation of FcεRI is mediated by p53/56^{lyn} (6), a member of the Src family of tyrosine kinases; the identity of the protein-tyrosine phosphatase(s) (PTP) responsible for dephosphorylating the IgE receptors remains uncertain.

Alternative, although not mutually exclusive, mechanisms, by which the formation of receptor aggregates promotes phosphorylation of FcεRI, have been proposed. One model posits that aggregates that include at least one IgE receptor constitutively associated with a molecule of the kinase enable a "transphosphorylation" of the juxtaposed receptors (7). It proposes that the constitutive protein:protein interaction between the kinase and the receptor are essential regardless of where in the membrane they occur. A second model proposes that aggregation drives receptors into specialized regions of the membrane, variously called lipid rafts or detergent-resistant membranes (DRM), which are enriched in the initiating kinase (8–10). Exposure to the high local concentrations of Lyn is thought sufficient to account for the phosphorylation of the immigrant receptors.

How PTP fit into these models remains unclear. It has been suggested that in T-cells some PTP, notably the transmembrane PTP, CD45, are excluded from DRM (11). In the latter instance, however, this was thought to lead to decreased activity of Src family kinases within the DRM, rather than to prolong the phosphorylation of resident proteins. In another instance, evidence was presented that phosphoproteins corralled in DRM by co-aggregation of CD3 and CD28 on T cells remained phosphorylated longer than those not so confined (12). Prior studies by our group showed that FcεRI, presumptively located within DRM, were nevertheless rapidly dephosphorylated when the kinase was inhibited (5). That result argued against protection from PTP within these domains, but alternative interpretations of those data are possible.

Our laboratory has presented evidence that a "kinetic proofreading" regimen (13, 14) governs the capacity of aggregates of FcεRI of varying stability to initiate downstream signals (15). In such a regimen, the likelihood that downstream signals are propagated depends not only on the concentration but also on the lifetime of the "activated" receptor. Therefore, the notion that rafts would protect phosphoproteins from deactivation by phosphatases raises the possibility that the domains not only increase the intensity of the signals but also their potential to stimulate later, "downstream," events.

We have now explored this possibility in greater detail. Specifically, we examined the distribution of PTP activity in cellular compartments fractionated on sucrose gradients to assess the level of PTP activity in DRM directly. In addition, we compared the distribution of the initiating kinase Lyn and of SHP-1, a PTP mooted as a potential negative regulator of the IgE receptor (16). We also examined a protein constitutively associated with the rafts, the linker for activation of T cells (LAT) (17–19), and compared its susceptibility to dephosphorylation with that of FcεRI, which resided in the microdomains only transiently. The *in vivo* data suggest that phosphoproteins in the DRM of RBL-2H3 cells are not protected from dephosphorylation.

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[1] The abbreviations used are: FcεRI, receptor with high affinity for IgE; DNP, dinitrophenyl; DRM, detergent-resistant membrane; HRP, horseradish peroxidase; ITAM, immunoreceptor tyrosine activation motif; PNS, post-nuclear supernatant; PTP, protein-tyrosine phosphatase; LAT, linker for activation of T cells; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

EXPERIMENTAL PROCEDURES

Materials

Goat anti-mouse IgE was purified on an affinity column of mouse IgE; the mouse anti-DNP IgE (20) was purified as described (21); biotinylated anti-phosphotyrosine (anti-PY) 4G10, and rabbit anti-LAT, anti-human Lyn, anti-SHP-1 and anti-SHP-2 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY); the antibody to the β subunit was JRK (22); OX-7 monoclonal mouse anti-rat CD90.1 (Thy-1.1) was from Pharmagen (San Diego, CA); the mouse monoclonal anti-Syk used for Western blotting was from clone Syk01/Pr (23). Horseradish peroxidase (HRP) coupled to avidin (Extravidin®) and to sheep anti-mouse IgG and DNP_{25-30} HSA were from Sigma; Protein A and reagents for enhanced chemiluminescence were from Amersham Pharmacia Biotech. Two substrates were used in PTP assays; a nonspecific control substrate supplied with the kit (below) was *N*-biotinylated residues 1–17 of human gastrin phosphorylated on tyrosine 12. The test substrate, synthesized by Quality Controlled Biochemicals, Inc. (Hopkinton, MA) had a sequence corresponding to residues 42–65 of the γ chain from the rat FcεRI (24). A biotin moiety was attached to the N-terminal lysine, and phosphotyrosines were substituted for the canonical tyrosines at positions 47 and 58. Covalent oligomers of mouse IgE were prepared as described (25); the RBL cells used were from clone RBL-2H3 (26).

Separation of Cellular Compartments

Crude Cell Fractions—Performing all procedures at 0–4 °C, 6–8 × 10^7 RBL cells were suspended at 2 × 10^7/ml in buffer A described by Smart *et al.* (27) (0.25 M sucrose, 20 mM Tricine, pH 7.8, 1 mM EDTA) with protease inhibitors (aprotinin, leupeptin, pepstatin, all at 10 μg/ml), and 1 mM 4-(2-aminoethyl)benzenesulfonyl- fluoride and sonicated with a microtip probe (Ultrasonic Processor XL; Heat Systems, Farmingdale, NY) for 5 s, at maximum power. This sonicate was centrifuged at 800 × *g*, for 10 min, to get a post-nuclear supernatant (PNS). A portion of the latter was then centrifuged in a 70Ti fixed angle rotor (Beckman Instruments, Fullerton, CA) at 100,000 × *g*, for 60 min. The supernatant of the latter centrifugation is referred to as "cytosol"; the pellet as "crude membrane."

Plasma Membranes—Our method followed closely that of Smart *et al.* (27). A PNS was prepared as above from 0.5–1 × 10^8 RBL cells, except that a second sonication of the initial pellet was performed and the supernatant added to the initial PNS. Two-ml aliquots of this pooled supernatant containing approximately 3–4 × 10^7 cell eq were layered on top of a 23-ml cushion of 30% Percoll and centrifuged for 30 min, at 84,000 × *g*. The fractions containing a visible band (fractions 8–13) were pooled. They typically contained 70–90% of the radioactive IgE, but only 20–30% of the total protein. After addition of some diluent, the plasma membrane fractions were concentrated by centrifugation at 100,000 × *g* for 1 h. The loose pellet was aspirated with a Pasteur pipette in approximately 0.5 ml, 0.8 ml TNE buffer (see below) was added, and the solution was made 0.1% in Triton X-100. After 40 min, the solution was centrifuged on a step gradient of sucrose as described below with the omission of orthovanadate and iodoacetate from all buffers. An aliquot of the unfractionated Triton X-100 extract and of the PNS was retained for use in the PTP assay and protein analysis.

Fractionation of Detergent Extracts

We prepared lipid rafts using the method described by Rodgers and Rose (11) with some modifications. In short, 1–2 × 10^7 adherent RBL cells were incubated for 30 min at 4 °C in 1 ml of a buffer containing 0.1% (≈1.6 mM) Triton X-100 in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 1 mM sodium iodoacetate and protease inhibitors as described above (TNEV). (In experiments to assess PTP activity, the vanadate and iodoacetate were omitted (TNE buffer).) At these concentrations, the value of ρ (= ([detergent] − cmc_{eff})/[phospholipid]) is ≈2–3 (28). The suspension was then transferred to a Dounce homogenizer and subjected to 15 strokes. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C and the supernatant mixed in an Ultra-clear™ centrifuge tube (Beckman) with an equal volume of 85% sucrose made up in water. This mixture was successively overlaid with 6 ml of 30% sucrose and 3.5 ml of 5% sucrose, neither of which contained Triton X-100. The tubes were then centrifuged at 200,000 × *g* at 4 °C, in a Beckman SW40Ti rotor for either 4 or 16 h. Sequential 1-ml fractions were harvested from the top of the gradient. An opaque band at the interface between the 5% and 30% layers was routinely harvested in fraction 4 and contained the lipid rafts. All fractions were counted in a γ counter, and aliquots were analyzed by the bicinchoninic acid method for total protein (29) with reagents from Pierce, and by Western blotting for specific proteins that had been separated by electrophoresis on polyacrylamide gels in SDS, as described previously (30).

Precipitation of FcεRI from Sucrose Fractions

Sucrose fractions were diluted with TNEV so that the final concentration of sucrose was approximately 15% in all cases. Triton X-100 was added to give a final concentration of 0.2% in all fractions. Anti-IgE (2 μg/ml) and a 50% suspension of protein A-Sepharose beads (20 μl/ml) were added to each tube and incubated overnight or for 4 h. In a few instances, the time for immunoprecipitations was reduced by incubating the protein A-Sepharose beads with goat anti-mouse IgE for 4 h, at 4 °C, washing them, and then adding them to the fractions from the sucrose gradient.

Assay for PTP

Aliquots (0.6 ml) of diluted cell extracts or of fractions from sucrose gradients were adjusted to contain 2 mM dithiothreitol and 0.5% Triton X-100. They were warmed to 37 °C, and either the nonspecific or specific biotinylated phosphopeptide substrate (above) was added to a final concentration of 294 nM. Duplicate samples were removed after 1 min to 18 h., and assayed for residual phosphotyrosine with a tyrosine phosphatase assay kit (Roche Molecular Biochemicals; catalog no. 1 534 153) using the instructions provided. Briefly, 34-μl aliquots (10 pmol of substrate) were added to the wells of a streptavidin-coated, 96-well microtiter plate containing 16 μl of 0.1 M orthovanadate to quench the reaction. For any given sample, at least four time points were taken. Duplicate standards, prepared in the same buffer used for the test samples and comprising sequential 2-fold dilutions containing between 10 and 0.01 pmol of the substrate peptide, were added to the same microtiter plate. We observed no effect on peptide binding to the plate even at concentrations of up to 40% sucrose, a concentration higher than any present in the assays on the gradient fractions.

After the biotinylated substrate had been allowed to bind for at least 1 h at room temperature, the wells were washed and HRP-conjugated monoclonal anti-phosphotyrosine antibody (4G10-HRP) was added. After 1 h at 37 °C, the wells were washed and a chromogenic substrate for HRP (ABTS®) was added. Ten minutes later, the absorbance at 405 nm was read on a MR5000 plate reader (Dynatech). Our standard curve data consistently showed a linear relationship between absorbance and the amount of substrate added between 0.01 and 0.3 pmol/well and was convincingly able to detect 2-fold differences in PTP activity as assessed by sequential dilutions of whole cell extracts.

Activation of Cells

Adherent cells (≈2 × 10^6/well) sensitized with anti-DNP IgE were rinsed three times with activation buffer and then incubated at 37 °C with or without antigen (0.3 μg/ml DNP_{25-30}-HSA). After 4 min, the cells were either solubilized immediately in 0.5% Triton X-100 or first reacted with 100 μM DNP-caproate for 10–80 s. Lysates from four replicate wells were pooled and duplicate aliquots prepared. Octylglucoside (final concentration, 60 mM) was added to one set of lysates. After solubilization (30 min at 4 °C), lysates were centrifuged for 2 min at 10,000 × *g* at 4 °C, and aliquots removed for later analysis. LAT was immunoprecipitated from the lysates containing octylglucoside; IgE-bound FcεRI were immunoprecipitated from corresponding lysates prepared in the absence of octylglucoside, using an affinity-purified goat anti-mouse IgE. After overnight incubations, the beads were washed three times with ice-cold solubilization buffer and reacted with an approximately equal volume of 2× SDS sample buffer and placed in a boiling water bath for a few minutes. Likewise, whole cell lysates were mixed with an equal volume of 2× SDS sample buffer and heated. All samples were separated by electrophoresis on 12% Tris-glycine gels. The separated proteins were transferred to nitrocellulose membranes and the latter incubated overnight in a Tris buffer containing 4% bovine serum albumin and 0.02% Tween detergent. The membranes were blotted with biotin-conjugated anti-phosphotyrosine and then with avidin-conjugated HRP. The bound enzyme was quantitated using the enhanced chemiluminescence method (Amersham Pharmacia Biotech), and autophotographs scanned on a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Distribution of FcεRI and Other Proteins Before and After Aggregation of FcεRI—Previous studies of the RBL-2H3 mast cell line demonstrated that when detergent lysates are subjected to centrifugation on sucrose density gradients, unaggre-

TABLE I
Translocation of aggregated FcεRI to Triton X-100-resistant domains

Stimulus	Concentration of Triton X-100								
	0.1%				0.2%				
	Ag[a]	Dimer	Trimer	Oligomer	None	Ag	Dimer	Trimer	Oligomer
μg/ml	0.3	0.5	0.75	1.0		0.3	0.5	0.75	1.0
Time (min)	4	30	30	30	4	4	30	30	30
	% cpm								
DRM	18	25	32	30	2.4	27	5.3	3.6	7.8
Soluble	66	59	50	52	95	59	92	95	91

[a] Ag, antigen.

gated FcεRI localize to the lower (denser) fractions containing Triton X-soluble proteins from the cytoplasm and membranes, whereas aggregates of the receptors accumulated in the more buoyant fractions containing the Triton X-100 DRM or "lipid rafts" (8, 9). We were readily able to reproduce these findings. Adherent RBL-2H3 cells were passively sensitized with ^{125}I-labeled mouse anti-DNP IgE (or covalent oligomers of IgE), and then challenged or not with DNP_{25-30}-HSA. In resting cells only a small fraction of the recovered radioactivity, generally 2–4%, was associated with the Triton X-100-resistant lipid rafts. Following challenge with antigen, 10–50% of the counts were recovered in the fractions containing the lipid rafts. In three successive experiments in which antigen increased the percent of receptors in the DRM by an average of 29%, the increase was reversed to just over 2% by subsequent addition of hapten. This demonstrates that the ligand-induced aggregation of the receptors must be maintained in order for the association with the rafts to persist.

Although smaller aggregates of receptors translocated into the DRM, their association was weaker than that exhibited by larger aggregates. Thus as shown in the results of a comparative study in Table I, dimers of IgE remained largely in the denser gradient fractions when the Triton X-100 concentration was increased to 0.2%, whereas the larger covalent aggregates or aggregates induced by the multivalent antigen continued to float in the fractions containing the DRM.

In addition to FcεRI, we examined the distribution of several plasma membrane proteins previously reported to reside within lipid rafts (Fig. 1). The Src family kinase, Lyn, the glycosylphosphoinositide-tethered protein, Thy 1, and LAT (17) each resided predominantly and constitutively within Triton X-100-resistant lipid rafts as had been described (18). Syk kinase and SHP-1 were virtually absent from the fractions containing the lipid rafts as expected, given their predominantly cytosolic location. Unlike the IgE receptor (Table I), neither those two enzymes nor any other proteins we observed migrated to the DRM in response to antigen-induced aggregation of FcεRI as judged by this assay (Fig. 1).

Distribution of Phosphorylated FcεRI after Aggregation of FcεRI—It has been proposed that the preferential localization of the initiating Lyn kinase within lipid rafts makes these domains favored sites at which the β and γ chains of FcεRI become phosphorylated after the receptors are aggregated (9). To examine this possibility further, we measured the phosphotyrosine content of antigen-aggregated FcεRI recovered from the different portions of the gradient. Appropriately pooled fractions were incubated with anti-IgE, and the protein-bound phosphotyrosine on the immunoprecipitated FcεRI was quantitated by Western blotting with anti-phosphotyrosine. Using this methodology, it appeared that only those receptors in the lipid rafts were detectably tyrosine-phosphorylated, as has been reported (9) (Fig. 2, *upper blot* (with anti-PY)).

Notably, when the same immunoprecipitates were re-probed

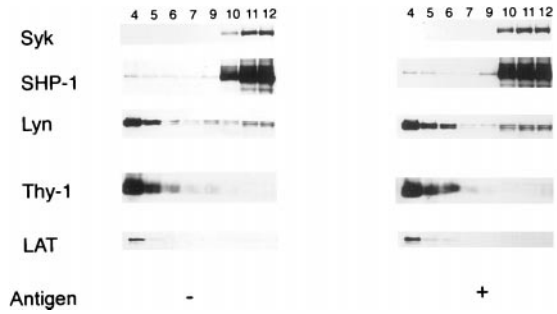

FIG. 1. **Distribution of selected cellular proteins in detergent-resistant domains.** Resting cells (*left*) or cells stimulated for 4 min with 300 ng/ml DNP-HSA (*right*) were lysed with 0.1% Triton X-100 lysates and then centrifuged on sucrose gradients. Aliquots of individual fractions were subjected to gel electrophoresis in SDS prior to Western blotting.

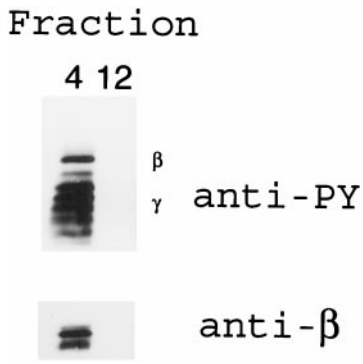

FIG. 2. **Analysis of receptors for PY in gradient fractions.** The figure shows Western blots of fractions 4 and 12 from a sucrose gradient on which a 0.1% Triton X-100 lysate of cells first incubated with ^{125}I-labeled IgE and then stimulated as in Fig. 1, was analyzed. The fractions were immunoprecipitated with anti-IgE under conditions that precipitated ≥60% of the cell bound IgE. The SDS extract of the immunoprecipitates from fractions 4 and 12, which were subjected to gel electrophoresis prior to Western blotting, contained 9,000 and 10,000 cpm of labeled IgE, respectively.

with an antibody to the β chain of FcεRI, the subunit was virtually undetectable in the fractions from the denser part of the gradient which contain the Triton X-100-soluble proteins, even though the IgE in those fractions had been efficiently precipitated (Fig. 2, *lower blot* (with anti-β)). When unstimulated cells were similarly analyzed, we observed that, likewise, over 75% of the receptors had dissociated during the gradient centrifugation of the detergent extracts (data not shown).

Whereas in mild detergent IgE remains stably bound to the α chain, the β and γ subunits of FcεRI dissociate from the α chain unless adequate lipids are present (28, 31, 32). Under the conditions we used for solubilization, ρ, which is a function of the detergent:lipid concentrations (see "Experimental Procedures") was in the range where the FcεRI is relatively stable.

Therefore, it appeared that the centrifugation was depleting the denser part of the gradient of lipid and thereby inducing dissociation of the receptors in those fractions. Addition of ethanol to these fractions precipitated most of the β and γ subunits that had dissociated from the α-IgE complexes retained in those fractions (data not shown).

Because the disruption of the receptors is gradual (32), we sought to minimize the time of centrifugation, and found that 4 h were sufficient to separate the low density, Triton X-100-resistant fraction (Fig. 3). The profile of total proteins from similar fractions of a gradient prepared in parallel, but centrifuged for 16 or 4 h alternatively, were also virtually indistinguishable (data not shown). Even with the more rapid isolation procedure, the amount of β chain associated with the IgE-bound α chain was on average 5-fold less (range, 2–10-fold) in anti-IgE immunoprecipitates from the denser fractions than in immunoprecipitates from the fractions containing the DRM (Table II, 6th column of data). Shortening the time of the immunoprecipitation did not substantially further improve the relative yield (data not shown).

With the more rapid fractionation, we recovered sufficient intact receptors from the critical fractions to determine their content of phosphotyrosine reliably. The receptors located in the denser fraction (fraction 12) contained substantial tyrosine-phosphorylated FcεRI. However, in the nine experiments summarized in Table II, the receptors that had translocated to the lipid rafts after aggregation contained on average 5-fold more phosphotyrosine per β subunit (range, 2–13-fold), (Table II, 5th column of data).

Distribution of Phosphatases: in Vitro Studies—We investigated the distribution of PTP activity within RBL-2H3 cells. We were particularly interested in gauging the localization of PTP activity capable of dephosphorylating the subunits of the IgE receptor itself. To this, end we prepared a bis-phosphorylated peptide corresponding to the ITAM sequence of the γ chain of FcεRI (33, 34) as a surrogate for the intact receptor, and assayed the PTP activity in PNS from sonicated cells, cytosol, crude membrane, and plasma membranes (Table III). A tyrosine-phosphorylated peptide derived from human gastrin served as a control, nonspecific substrate in parallel assays.

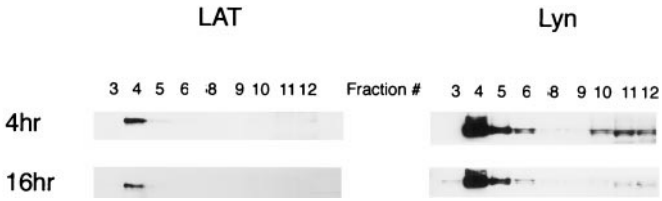

FIG. 3. **Separation of membrane domains after brief or prolonged gradient centrifugation.** Triton X-100 lysates of unstimulated cells were analyzed on sucrose density gradients with centrifugation for 4 or 16 h alternatively. Aliquots of the individual fractions were subjected to gel electrophoresis in SDS prior to Western blotting for either LAT (*left panel*) or Lyn (*right panel*).

TABLE II
Distribution of intact and phosphorylated receptors in gradients

	Fraction 4 (Triton X-resistant)		Fraction 12 (Triton X-soluble)		Fraction 4/fraction 12	
	PY/β	β/IgE	PY/β	β/IgE	PY/β	β/IgE
Average	"1.00"	"1.00"	0.16	0.23	5.17	5.42
S.E.	0.57	0.24	0.042	0.067	1.05	1.20

The sum of the activities measured in the crude membrane and cytosol agreed well with the activity measured in the unfractionated post-nuclear supernatant. As documented in Table III, PTP activity toward the ITAM substrate was 10–20-fold lower than that detected in the same fractions toward the nonspecific peptide. A significant majority of the PTP activity against both substrates was located in the crude membrane fraction of RBL cells consistent with previously reported data (5). Table III also shows that the activity toward the ITAM relative to that toward nonspecific peptide was highest in the plasma membrane and lowest in the cytosolic fraction.

It was of interest to examine the presence of PTP activity within detergent-resistant lipid rafts, specifically. We prepared sucrose gradients of whole cell extracts identical to those used in the analyses above. Protein-tyrosine phosphatase activity, as well as the content of total protein and Lyn kinase, was then assessed for each gradient fraction and compared with the unfractionated whole cell extract. The sum of PTP activity, total protein (data not shown), and Lyn kinase detected in individual gradient fractions accounted almost exactly for the values measured in the unseparated cell extract. The fractions containing the lipid rafts were not devoid of PTP activity, although the great majority of PTP activity was found in the Triton-soluble fractions of the gradient, and per unit of protein, the PTP activity was substantially less in the fractions containing the DRM (Table IV).

Because the lipid rafts contained a major portion of the total Lyn (above), it was apparent that the ratios of Lyn kinase protein to PTP activity were strikingly different in the DRM and the Triton X-100-soluble fractions. We made a similar, potentially more meaningful comparison between rafts and the bulk plasma membranes. The latter were prepared as described under "Experimental Procedures" and then solubilized in 0.1% Triton X-100. The preparations were then fractionated on the same sucrose gradients used in the experiments with whole cell extract described above. The data reproduced in Table IV are typical of those observed in the three experiments that used these conditions. The distribution of PTP activity and Lyn protein accurately mirrored the situation in similar analyses of whole cell lysates. Thus, in each experiment, a small but detectable fraction ($1.4 \pm 1\%$, $n = 3$) of the total recovered PTP activity was present in the DRM fractions. As depicted in Fig. 1, these fractions contained most of the Lyn kinase (at least 55%) and almost all the recovered LAT. The Triton X-100-soluble fraction of the plasma membrane by contrast contained the vast majority of the PTP activity but only approximately 20% of the total Lyn kinase. The total PTP activity recovered in the sucrose fractions again reflected almost exactly the activity

TABLE III
Distribution of specific and nonspecific phosphatase activity in discrete cellular compartments

Fraction	Substrate peptide								Average (ITAM/nonspecific)
	ITAM				Nonspecific				
	n	Average	S.E.	PNS	*n*	Average	S.E.	PNS	
				%				%	
Post-nuclear supernatant	7	8.42	2.52	100	7	80.8	20.32	100	0.104
Cytosol	9	1.24	0.27	15	9	28.9	5.42	36	0.043
Total membrane	9	7.23	1.89	86	9	57.6	9.95	71	0.126
Plasma membrane	6	2.98	0.46	35	6	26.1	4.49	32	0.1

TABLE IV
Distribution of PTP activity and Lyn on sucrose gradients

Results from one of three experiments with closely similar results. In this particular comparison, phosphotyrosine phosphatase activity was assessed using the nonspecific substrate; the distribution of Lyn and SHP-1 (plasma membrane only) was estimated by Western blotting.

Fraction	Starting material				
	PNS		Plasma membrane		
	PTP activity	Lyn	PTP activity	Lyn	SHP-1
	pmol/min	*% total*	*pmol/min*	*% total*	*% total*
4–6 (DRM)	1.3	86	0.14	65	"0"
7–9	5.3	5.1	0.06	4.9	9.2
10–12	30	8.1	4.1	31	91

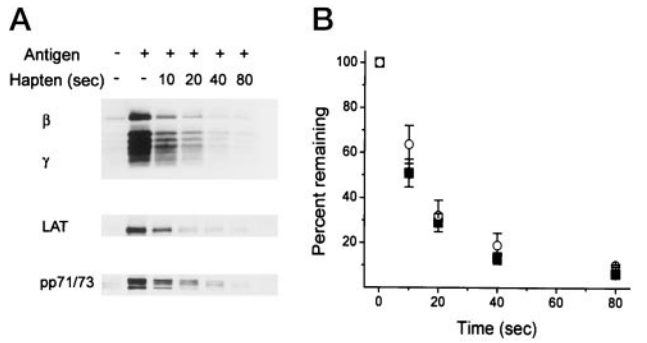

FIG. 4. **Rates of protein dephosphorylation after addition of hapten.** Cells sensitized with anti-DNP IgE were stimulated (or not) with 300 ng/ml antigen at time 0 and then reacted (or not) with 100 μM DNP-ϵNH_2-caproate at 240 s. *A*, autophotograph of Western blot from a single representative experiment. Reading from left to right, the cells were lysed at 240, 240, 250, 260, 280, and 320 s, respectively. The amount of PY associated with several proteins was determined by Western blotting of immunoprecipitates (FcεRI, LAT) or lysate (pp71/73), alternatively. *B*, composite data for dephosphorylation of FcεRI and LAT from six such experiments. The data from successive samples were normalized relative to the signal measured in the stimulated cells not exposed to hapten. The *error bars* are for the standard errors. *Filled squares*, FcεRI; *open circles*, LAT.

present in the unfractionated plasma membrane.

Similarly, Western analyses demonstrated that the upper, DRM-containing fractions generated from whole cell lysates or plasma membrane-enriched preparations were depleted or devoid respectively of the Src homology 2 domain containing PTP, SHP-1, and SHP-2, whereas the lower, denser fractions contained large amounts of both PTP (data not shown). Together these data appeared to support the possibility that the lipid rafts could serve as sites of refuge from PTP.

Distribution of Phosphatases: in Vivo Studies—The *in vitro* procedures could have led to an artifactual loss or redistribution of PTP activity. Therefore, we also assessed PTP activity in the lipid rafts *in vivo*. If the lipid rafts were in fact deficient in PTP activity, we reasoned that substrates constitutively associated with these domains, such as LAT, would be more resistant to dephosphorylation than substrates more fleetingly associated with the rafts, *e.g.* the hapten-dissociated aggregates of FcεRI (see above). To investigate this possibility directly, we assessed the level of phosphorylation of FcεRI and LAT in samples of the same cells at various time points following the addition of monovalent hapten and the consequent decrease in aggregated receptors. Initial experiments demonstrated that, under the conditions of stimulation we used, phosphorylation of LAT reached a maximum at 4 min after addition of antigen (data not shown). Monovalent hapten was added, and after various periods the reactions were stopped by the addition of ice-cold solubilization buffer. Data from one such experiment

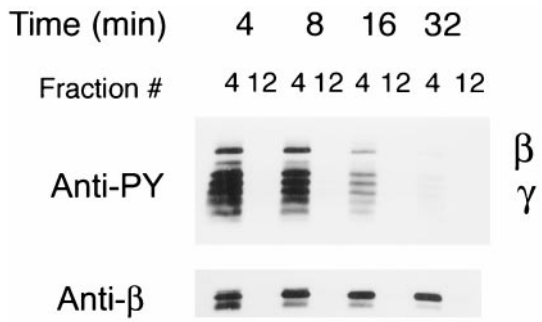

FIG. 5. **Dephosphorylation of aggregated receptors.** Cells sensitized with ^{125}I-labeled IgE were reacted with 300 ng/ml DNP-HSA for the times indicated. Lysates were prepared after successive intervals from equal numbers of cells, and individually fractionated on sucrose gradients. At each time point, the receptors found in the fractions containing the lipid rafts (fraction 4) were assessed by immunoprecipitation with anti-IgE and Western blotting with antibodies to phosphotyrosine (*upper set*) or to the receptors' β chain (*lower set*), alternatively.

are illustrated in Fig. 4 (*left panel*). It is apparent that the subunits of the receptor, the LAT protein, and the pp71/73 proteins of uncertain identity were each rapidly dephosphorylated. The composite data from all (six) such experiments in which the dephosphorylation of FcεRI and LAT were compared are plotted in Fig. 4 (*right panel*). The data show that the DRM-confined LAT protein is dephosphorylated no less rapidly than the disaggregated receptors, which have abandoned those domains. Likewise, and consistent with previously reported data (5), the rate of dephosphorylation of other phosphoproteins detected in whole cell lysates was similar to those observed for both LAT and the receptor with half-times between 13 s and 16 s (data not shown).

We further reasoned that if, *in vivo*, the lipid rafts were deficient in PTP activity, then FcεRI resident in these domains might be spared from the spontaneous dephosphorylation prominently observed in suspended RBL cells following aggregation of the receptors. FcεRI were immunoprecipitated from gradient fractions corresponding to lipid rafts isolated from cells challenged for various periods with antigen. As documented in Table I, FcεRI are readily detectable in lipid rafts after 4 min of stimulation with antigen. In the experiment illustrated in Fig. 5, cells previously incubated with ^{125}I-labeled IgE were washed and reacted with antigen and sampled periodically. At each time point the number of receptors found in the lipid rafts was assessed on sucrose gradients and anti-IgE immunoprecipitates from the DRM-containing fractions were assessed by Western blotting with antibodies to the receptors' β chain and to phosphotyrosine, alternatively. The number of receptors recovered in the DRM-containing fraction decreased about 35% over 28 min after the addition of antigen (data not shown), but the level of phosphotyrosine present in the same receptor immunoprecipitates decreased to almost undetectable levels over the same period. Relative to the recovered β chain, the phosphotyrosine in the β and γ subunits together, fell from its starting value at 4 min, to 0.88, 0.35, and 0.05, at 4, 12, and 28 min respectively, thereafter. The amount of Lyn kinase in the same fractions did not change appreciably over the same period (data not shown).

DISCUSSION

The goal of these studies was to define further certain factors that significantly affect the concentration and persistence of tyrosine-phosphorylated FcεRI. In particular, we wished to determine whether aggregated receptors were shielded from phosphatases by recruitment to the distinctive lipid microdomains.

Shift of Receptors after Aggregation—A shift of a fraction of

receptors to rafts is reproducibly observed after aggregation (8), and both the size of the aggregates and the concentration of detergent influence the magnitude of the shift observed (Table I). Possibly, the less stable interaction of small relative to large receptor aggregates with DRM is related to the reduced efficacy of smaller aggregates to stimulate cellular responses (25).

It might be supposed that, once conveyed into the microdomains, the multivalent interaction of aggregates of the receptors with the special lipids or with co-localized cytoskeletal structures (35) would stabilize the aggregates. If so, the effect is too small to prevent the disappearance from the DRM of those dissociated IgE·receptor complexes whose rebinding to antigen was prevented by addition of excess hapten.

Role of Shift in Phosphorylation—Whereas the shift of aggregated receptors to DRM is readily demonstrable, what is less clear is whether the interaction of the receptors with Lyn and their consequent phosphorylation occurs only in those regions. Reports of studies in which the DRM were disrupted have come to virtually contradictory conclusions (36).[2] Other studies, employing genetically engineered constructs of either the Lyn kinase[3,4] or of the subunits of the receptor thought to interact with the enzyme,[4] suggest that those interactions can occur quite normally outside those domains.

In part, certain technical aspects complicate the interpretation of the biochemical analyses. We found that, in the fractions containing Triton X-100-soluble proteins, a significant proportion of the α chains of the receptors had dissociated from the β and γ chains. This dissociation was exaggerated by prolonging the centrifugation step in the protocol used to separate the microdomains. However, the dissociation was not appreciably accompanied by fractionation of the receptor such as occurs in Triton X-114 (37). In the latter studies, IgE·receptor complexes were solubilized in cold Triton X-114 and the temperature raised to the "cloud point" where aggregates ("coacervates") of the detergent micelles occur. The β and γ chains of the receptor became selectively partitioned into the coacervate phase whereas IgE·α chain complexes were concentrated in the aqueous phase. In the gradients, it appears that by floating out the lipids from the bulk solution, the centrifugation exposes the receptors in the higher density fractions of the gradient to a high ρ (28) promoting their dissociation, whereas those receptors that co-migrate with the DRM remain protected. Therefore, it is important to correct for the yield of β and γ chains in the anti-IgE precipitates. Possibly, other multichain receptors sensitive to the detergent:lipid environment also dissociate during such analyses, so that the same precaution is required.

Under conditions that reduced the dissociation of receptors, we recovered sufficient intact receptors from the critical fractions to evaluate their relative phosphorylation. We found that, on average, the receptors translocated by the multivalent antigen to the fractions in which the rafts were located, constituted approximately three-fourths, but clearly not all, of the receptor-incorporated phosphotyrosine (Table III). Field *et al.* (9) found that phosphorylated β and γ chains were located exclusively in the DRM but appear not to have assessed whether any β and γ chains were precipitated when the IgE·α remaining in the lipid-depleted fractions was immunoprecipitated with anti-IgE.

Exclusion of Phosphatases from Microdomains—There is evidence that the transmembrane phosphatase CD45 is excluded from DRM (11), and other data have suggested a more general depletion of phosphatase activity at least from aggregated DRM (12). Those results suggest a model in which tyrosine phosphorylation of substrates located in DRM is favored both by the enrichment of kinases *and* the paucity of phosphatases. We examined this possibility by several approaches.

We quantitated PTP activity in different cellular compartments, using two peptide substrates: one containing the tyrosine-phosphorylated ITAM sequence of the γ chain, and the other an irrelevant tyrosine-phosphopeptide (Table IV). PTP activity against both peptides was predominantly associated with the membrane fraction of cell sonicates, and approximately half of this activity was found in partially purified plasma membranes. These results agree with previous data indicating that at least half the PTP activity toward intact "*ex vivo*" receptors was associated with crude membranes (5). The plasma membranes appeared to be slightly enriched in PTP that act on the ITAM peptide, but PTP activity in each fraction toward the ITAM peptide was 10–20-fold lower than that in the same fractions against the nonspecific peptide substrate. Whether the ITAM peptide is a relatively poor substrate for most or all PTP or whether only a small fraction of the total PTP population can efficiently dephosphorylate the ITAM peptide is unknown.[5] We did check one PTP that has been specifically implicated-SHP-1 (16), but it showed a preference for the nonspecific phosphopeptide similar to that of the extracts as a whole. To pursue this further, the approach used by Walchli *et al.* (38) could be used. They recently showed that "substrate trapping" mutants, when applied in a "brute force" approach, can be used to "short-list" candidate PTP for the insulin receptor kinase (39). Nevertheless, much further work is required to determine whether dephosphorylation of that receptor is principally controlled by a specific phosphatase.

PTP activity was abundant in Triton X-100-soluble fractions of either whole cell extracts or partially purified plasma membranes, but the fractions containing the DRM from the same gradients were largely depleted but not devoid of detectable PTP activity. Similarly, Western analyses demonstrated that DRM fractions generated from whole cell lysates or plasma membrane-enriched preparations were depleted or devoid, respectively, of the Src homology 2 domain containing PTP, SHP-1, and SHP-2, whereas the Triton X-100-soluble fractions contained large amounts of both PTP (*cf.* Ref. 5).

In isolation, these findings would support the notion that rafts act as sites of refuge from PTP. However, we found that, following exposure of the cells to hapten, the rate of dephosphorylation of the raft-confined substrate, LAT, was no less rapid than that of a substrate located outside DRM, the disaggregated IgE receptor. In addition, receptor aggregates stably resident within DRM were nevertheless subject to "spontaneous" dephosphorylation. These results are consistent with our previous results, which suggested rapid dephosphorylation of receptor aggregates *presumptively* located in rafts, following the attenuation of kinase activity (5). In the latter study, however, it remained possible that the aggregates of IgE receptors were moving rapidly in and out of the lipid rafts, and could in fact have been dephosphorylated while briefly located outside the rafts. In the experiments presented here, we obviated this uncertainty by studying a phosphoprotein known to associate

[2] T. Yamashita, T. Yamaguchi, K. Murakami, and S. Nagasawa, submitted for publication.

[3] Vonakis, B., Haleem-Smith, H., Benjamin, P., and Metzger, H. (2000) *J. Biol. Chem.*, in press.

[4] M. Kovarova, L. Draberova, J. Rivera, and P. Draber, submitted for publication.

[5] We estimate that, in our *in vitro* assays, where the bis-phosphorylated ITAM peptide was saturating, the detergent extract of 10^6 cell eq was hydrolyzing about 17 pmol of phosphotyrosine/min. Mao *et al.* (5) estimated that in cells treated with hapten after activation, the rate of dephosphorylation of receptor ITAMs was equivalent to 1.3 pmol/10^6 cell eq/min. The same paper reported that, *in vitro*, using immunoprecipitated receptors, the extract of cells hydrolyzed only 0.4 pmol/10^6 cell eq/min but the substrate was not saturating under the conditions used.

stably and constitutively with DRM, LAT. That LAT and the receptor, as well as two other phosphoproteins in whole cell lysate identified only outside the DRM, are dephosphorylated at almost identical rates, argues strongly against the idea that rafts provide a privileged site at which the phosphorylated state of proteins is protected. Parallel findings have been described in Jurkat T cells; using PP1, a cell-permeant inhibitor of Src family kinases, raft-associated substrates such as CD 59 were observed to be rapidly dephosphorylated (35).

The discrepancy between the rapid dephosphorylation of substrates confined in DRM in intact cells, and the apparent deficiency of PTP in isolated DRM, could be explained in two ways. Possibly, PTP are abundant in lipid rafts but are lost during the isolation procedure. Presumably, this is the case with phosphorylated Syk, which we were unable to recover with the isolated DRM despite its likely association with the phosphorylated receptors there (40). Making reasonable assumptions,[6] it can be readily calculated that, whereas Syk's avidity for the bis-phosphorylated ITAM (K_D = 80 nM; Ref. 42) is sufficient to bind it to the phosphorylated receptors *in situ*, it is insufficient to maintain that association at the high dilution accompanying the disruption of cells and subsequent procedures.

Alternatively, whereas the PTP may be literally excluded from the interior of the domains, they may be able to attack whenever the normal diffusive path of the phosphorylated proteins brings them close enough to the perimeter. Recent estimates of the size of the DRM (43) suggest they are sufficiently small to make such a scenario plausible.

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[6] Assuming that the cells contain roughly equivalent numbers of receptors and molecules of Syk, that the two tyrosines in all of the receptor ITAMs were phosphorylated (41), and that no other proteins competed for Syk, then 85% of the Syk and 67% of the receptors would be in Syk·phosphorylated receptor complexes. If only 10% of the receptors were fully phosphorylated, the corresponding values would be 16% and 96%, respectively. In the gradient, the dilution is such that, even if all the receptors were fully phosphorylated, ≤1% of the receptors and Syk would be expected to be associated at equilibrium.