

A Lipid Raft Environment Enhances Lyn Kinase Activity by Protecting the Active Site Tyrosine from Dephosphorylation*

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The plasma membrane contains ordered lipid domains, commonly called lipid rafts, enriched in cholesterol, sphingolipids, and certain signaling proteins. Lipid rafts play a structural role in signal initiation by the high affinity receptor for IgE. Cross-linking of IgE-receptor complexes by antigen causes their coalescence with lipid rafts, where they are phosphorylated by the Src family tyrosine kinase, Lyn. To understand how lipid rafts participate in functional coupling between Lyn and FcεRI, we investigated whether the lipid raft environment influences the specific activity of Lyn. We used differential detergent solubility and sucrose gradient fractionation to isolate Lyn from raft and nonraft regions of the plasma membrane in the presence or absence of tyrosine phosphatase inhibitors. We show that Lyn recovered from lipid rafts has a substantially higher specific activity than Lyn from nonraft environments. Furthermore, this higher specific activity correlates with increased tyrosine phosphorylation at the active site loop of the kinase domain. Based on these results, we propose that lipid rafts exclude a phosphatase that negatively regulates Lyn kinase activity by constitutive dephosphorylation of the kinase domain tyrosine residue of Lyn. In this model, cross-linking of FcεRI promotes its proximity to active Lyn in a lipid raft environment.

Cross-linking of IgE-FcεRI complexes on mast cells by multivalent antigens initiates a series of signaling events that culminate in the exocytosis of mediators of the allergic response. The earliest detectable biochemical event following receptor aggregation is phosphorylation of the ITAM sequences on FcεRI β and γ subunits by Src family tyrosine kinases, primarily Lyn (1, 2). The mechanism by which cross-linking promotes this initial phosphorylation remains unclear.

One current hypothesis focuses on the interaction of Lyn kinase with FcεRI. Metzger and colleagues (3, 4) described evidence that a small percentage of Lyn is constitutively associated with FcεRI on RBL-2H3 mast cells but is unable to phosphorylate these receptors in the absence of aggregation. In the transphosphorylation model proposed in these studies, Lyn can only phosphorylate an adjacent FcεRI following aggregation of two or more receptors. Subsequent studies from the Metzger laboratory showed that the β subunit of FcεRI is

capable of weak interactions with Lyn in the absence of phosphorylation (5, 6). However, other studies showed that signaling occurs via human FcεRI in the absence of the β subunit (7, 8) and that β plays an amplifying role in FcεRI signaling (9, 10).

An alternative model focuses on the ordered lipid environment of lipid rafts that are proposed to facilitate the productive interaction between aggregated FcεRI and Lyn (11, 12). Lipid rafts are enriched in cholesterol, sphingolipids, and glycerophospholipids with saturated acyl chains and can be isolated due to their insolubility in nonionic detergents at 4 °C (13, 14). A large percentage of cellular Lyn fractionates with lipid rafts following sucrose gradient analysis of Triton X-100-lysed RBL mast cells (11, 15). Cholesterol depletion by methyl-β-cyclodextrin reversibly inhibits antigen-stimulated tyrosine phosphorylation of FcεRI and in parallel causes reversible loss of both Lyn and cross-linked FcεRI from lipid rafts (16). Thus, either the association of Lyn or FcεRI or both with rafts is important for initiating this process.

Previous studies that focused on the relatively low abundance of proteins in isolated lipid rafts suggested that localization of signaling proteins in rafts serves to concentrate them and thereby promote signaling (17, 18). However, it is now clear that these ordered regions of the plasma membrane constitute a large percentage of its lipid and surface area (19, 20). To gain more insight about the mechanism by which lipid rafts facilitate functional coupling between cross-linked FcεRI and Lyn, we investigated the role of membrane environment on the kinase activity of Lyn. We find that Lyn solubilized from RBL mast cells by Triton X-100 represents a subset of Lyn with reduced kinase activity, and this subset largely fractionates with nonraft proteins in sucrose gradients. Lyn isolated with lipid rafts has a substantially higher kinase activity than Lyn from nonraft fractions, and this increase in activity correlates with greater tyrosine phosphorylation of raft-associated Lyn in its kinase domain. Cross-linking of FcεRI does not increase the overall kinase activity of Lyn in these cells but rather promotes functional coupling by causing co-compartmentalization of these receptors with the active form of Lyn in the raft environment.

EXPERIMENTAL PROCEDURES

Differential Detergent Solubility—RBL-2H3 cells (21) were suspended at $8\text{--}10 \times 10^6$ cells/ml in bovine serum albumin containing buffered saline solution (BSS (16)) and lysed by mixing 1:1 (v/v) with a detergent solution for a final concentration of either 0.5% Triton X-100 or RIPA¹ detergents (0.5% Triton X-100, 0.5% deoxycholate, and 0.05% SDS) in lysis buffer (11). Cells were lysed for 10 min on ice and then clarified by microcentrifugation ($14,800 \times g$) for 20 min at 4 °C to yield a postnuclear supernatant (PNS). In some experiments, half of the Triton X-100-lysed cells were loaded directly on a sucrose gradient, the

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¹ The abbreviations used are: RIPA, radioimmune precipitation assay; PNS, postnuclear supernatant; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

other half were microcentrifuged for 20 min, and the PNS was loaded. The amount of Lyn solubilized by RIPA was determined by quantitative anti-Lyn Western blot data (see below) through comparisons of RIPA-lysed samples before and after clarification. The relative amount of Lyn solubilized in the Triton X-100 lysate was determined by comparing Lyn immunoprecipitation yields from RIPA PNS with Triton X-100 PNS.

Sucrose Gradient Fractionation—RBL-2H3 cells were lysed at 3×10^7 cells/ml in 0.25% Triton X-100 in lysis buffer for 10 min on ice. The lysates were then brought to 40% sucrose by a 1:1 (v/v) dilution with an 80% sucrose stock solution, and 1 ml of this solution was applied to the bottom of an 11 \times 60-mm centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA). 2 ml of 30% sucrose and then 1 ml of 5% sucrose were layered above the lysate. Samples were ultracentrifuged as previously described for 12–18 h at $250,000 \times g$ (15). After ultracentrifugation, samples were fractionated to obtain lipid raft and nonlipid raft fractions. These fractions were diluted 2-fold with lysis buffer containing RIPA for subsequent immunoprecipitation of Lyn.

Lyn Immunoprecipitation and *in Vitro* Kinase Assay—Lyn was immunoprecipitated from 0.5–1 ml of detergent extracts by incubation with 2 μ g anti-Lyn mouse monoclonal antibody H6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1–2 h on ice and then rotated for 45 min with 35 μ l of ImmunoPure Immobilized Protein A (Pierce) at 4 $^{\circ}$ C. Immunoprecipitates were washed twice with lysis buffer without detergent and then once with kinase assay buffer (20 mM Tris, pH 7.6, 10 mM $MgCl_2$, and 1 mM Na_3VO_4). After washing, Lyn immunoprecipitates were subjected to *in vitro* kinase assays by adding 200 μ l of kinase assay buffer containing either 1 mM ATP, no ATP, or 1 mM ATP and 100 μ g of dephosphorylated α -casein (Sigma) as an exogenous substrate. Samples were then incubated at either 37 $^{\circ}$ C for samples without α -casein, or 30 $^{\circ}$ C for samples with α -casein, for 15 min. Reactions were quenched by the addition of 50 μ l of 5 \times nonreducing SDS sample buffer (15) followed immediately by boiling.

Immunoblotting—Samples were separated by electrophoresis on 10% acrylamide SDS gels under nonreducing conditions, and then electrophoresed proteins were transferred to an Immobilon P membrane (Millipore Corp., Bedford, MA) with a Panther semidry electroblotter (Owl Separation Systems, Inc., Portsmouth, NH). Anti-phosphotyrosine blotting was performed using 0.1 μ g/ml 4G10 mouse monoclonal antibody conjugated to horseradish peroxidase (Upstream Biotechnology, Inc., Lake Placid, NY) diluted in a 1:1 (v/v) mixture of Tris-buffered saline solution (TBST) (50 mM Tris, pH 7.6, 150 mM $NaCl$, and 0.1% (v/v) Tween 20) and StabliZyme SELECT (SurModics, Eden Prairie, MN). Blots were then stripped by incubation with 0.2 M NaOH for 5–30 min and quenched with TBST. Anti-Lyn blotting was performed on the stripped blots using 0.2 μ g/ml anti-Lyn rabbit polyclonal antibody 44 (Santa Cruz Biotechnology) and a horseradish peroxidase-labeled anti-rabbit Ig secondary antibody (Amersham Biosciences), both in a solution of 0.8% bovine serum albumin in TBST. All immunoblots were developed as previously described (15).

Quantitation of Lyn Basal Phosphorylation and Specific Activity—Western blots were scanned from film with an Epson Expression 1600 digital scanner (Epson, Long Beach, CA), and density was determined using UnScanIt software (Silk Scientific, Orem, UT); intensities of multiple samples on a single blot were normalized to a single sample in each blot. Lyn basal phosphorylation is defined as the amount of phosphorylation on immunopurified Lyn after an *in vitro* kinase incubation in the absence of ATP. Lyn specific activity is defined as the amount of phosphorylation on immunopurified Lyn following an *in vitro* kinase assay incubation in the presence of ATP minus the basal phosphorylation of a parallel sample. Values were calculated by dividing the normalized intensity of tyrosine phosphorylation on Lyn from the 4G10 blot by the normalized intensity of Lyn from the reprobed anti-Lyn blot in the same lane from nonsaturated film following Western blotting. Alternatively, specific activity was determined as the intensity of phosphorylated α -casein following an *in vitro* kinase assay incubation in the presence of ATP and α -casein in the kinase assay buffer, divided by the amount of Lyn in the same lane.

Phosphopeptide Mapping of Lyn—Lyn was immunopurified using 35 μ l of anti-Lyn conjugated to agarose beads (Santa Cruz Biotechnology) and $1-2 \times 10^8$ cell equivalents of RBL-2H3 cells lysed in the RIPA detergent buffer as above. Some Lyn samples were subjected to autophosphorylation in the *in vitro* kinase assay prior to elution and mapping. Lyn was eluted from the anti-Lyn beads by incubation with 0.1 M glycine HCl, pH 2.5. Next, the sample was dried using vacuum centrifugation (Thermo Savant, Hollbrook, NY) and exchanged to 70% formic acid containing 100 mg/ml cyanogen bromide (CNBr) (ICN Biomedicals, Inc., Aurora, OH) to digest the protein overnight at room temperature

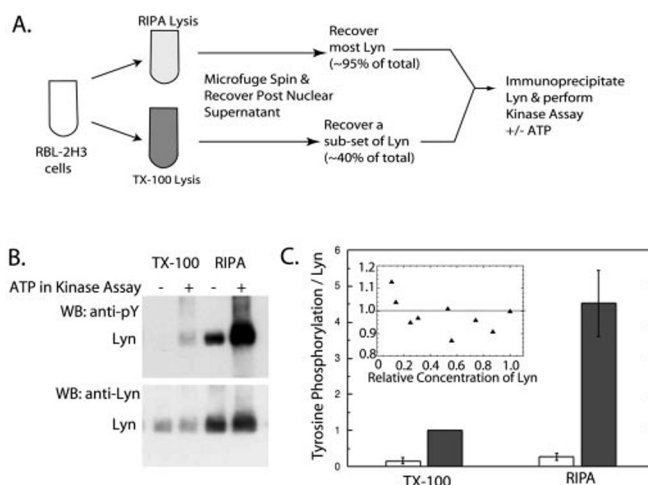


FIG. 1. Lyn recovered from RIPA PNS has a higher level of basal tyrosine phosphorylation and enhanced kinase activity compared with Lyn recovered from a Triton X-100 PNS. A, experimental design. Equal volumes of RBL-2H3 cells (5×10^6 /ml) were lysed with RIPA or 0.5% Triton X-100 buffers, and PNS were collected after a 20-min microcentrifuge spin at $14,800 \times g$. RIPA PNS contained $95 \pm 1\%$ of the total cellular Lyn, and Triton X-100 PNS contained $40 \pm 9\%$ of total cellular Lyn as determined by relative recoveries of Lyn after immunoprecipitation. B, sample blot showing detection of Lyn phosphorylation (top panel) and the corresponding reprobe for Lyn (bottom panel) following immunoprecipitation and electrophoretic separation. C, summary of relative specific activity (shaded bars) and basal phosphorylation (white bars) of Lyn from unstimulated RBL-2H3 cells. Bars, S.E.; $n = 7$. Inset, dependence of tyrosine phosphorylation/Lyn on the amount of Lyn per sample. RIPA PNS was diluted in lysis buffer at various concentrations prior to immunoprecipitation and *in vitro* kinase analysis of Lyn. The relative specific activity of Lyn was determined as above and plotted against the relative amounts of Lyn in each sample as determined by Western blotting.

in the dark. The following day, 500 μ l of H_2O was added to digests, and this mixture was then evaporated to dryness in the vacuum centrifuge. This wash step was repeated three more times, and dried samples were solubilized in 30–50 μ l of $1\times$ sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.2 mg/ml bromophenol blue). Half of the sample was run on a 16.5% acrylamide Tricine gel (Bio-Rad) and then transferred to an Immobilon P^{sq} membrane (Millipore Corp.) by semidry transfer. Membranes were subsequently blocked for 4 h with 4% bovine serum albumin in TBST and then probed with anti-phosphotyrosine 4G10, washed, and detected by chemiluminescence as described above.

RESULTS

Lyn Isolated from Triton X-100 Postnuclear Supernatants Has Reduced Specific Activity—Initial experiments compared the specific activity of Lyn isolated from RBL-2H3 cells lysed with 0.5% Triton X-100 with that for Lyn isolated from cells lysed with RIPA detergents. Unstimulated RBL-2H3 cells were lysed in the presence of phosphatase inhibitors, and Lyn was immunoprecipitated from the PNS. Western blotting with anti-Lyn showed that $95 \pm 1\%$ of the total cellular Lyn was recovered from the RIPA PNS, whereas only $40 \pm 9\%$ of Lyn was recovered from the Triton X-100 PNS (Fig. 1A). The amount of protein solubilized in each PNS is approximately equal for Triton X-100 versus RIPA lysis.² Additionally, equivalent amounts of uncross-linked Fc ϵ RI were solubilized by these two detergent preparations as determined by fluorescent IgE quantitation in PNSs.² By Western blotting with anti-phosphotyrosine monoclonal antibody 4G10, we determined both basal tyrosine phosphorylation of Lyn and *in vitro* kinase autophosphorylation of Lyn under conditions similar to those in a previous study from our laboratory (15). Blots were then stripped,

² R. M. Young, D. Holowka, and B. Baird, unpublished results.

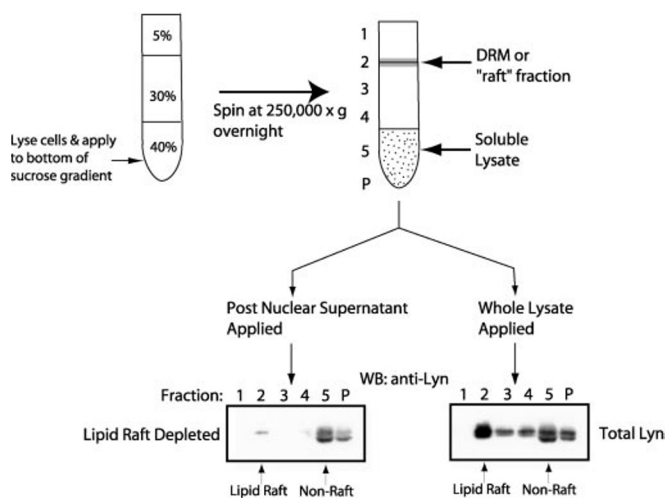


FIG. 2. The Triton X-100 PNS is depleted of lipid raft-associated Lyn. RBL-2H3 cells were lysed at 5×10^6 cells/ml with 0.5% Triton X-100 lysis buffer as in Fig. 1A. Lysates were either clarified by a 20-min microcentrifuge spin at $14,800 \times g$ to yield a PNS or not clarified; both samples were adjusted to 40% sucrose and applied to a sucrose gradient. After centrifugation at $250,000 \times g$ (for 12–18 h) gradients were divided into six fractions as indicated for analysis. Anti-Lyn Western blots of these samples are shown.

and the amount of Lyn present in each sample was determined by anti-Lyn Western blotting (Fig. 1B). Basal tyrosine phosphorylation of Lyn from both Triton X-100- and RIPA-solubilized Lyn (Fig. 1C, *white bars*) is small compared with tyrosine phosphorylation of Lyn following the incubation with ATP, although Lyn from RIPA PNS consistently has slightly higher levels of basal phosphorylation. The specific kinase activity for Lyn autophosphorylation (Fig. 1C, *shaded bars*) is 4.5-fold higher in Lyn recovered from RIPA PNS compared with Triton X-100 PNS. Thus, Lyn isolated following Triton X-100 solubilization represents a subset of total Lyn with relatively low specific activity that is less than 10% of the total cellular Lyn activity.

To investigate further the effects of the two different lysis conditions on Lyn specific activity, we performed additional control experiments. To test whether RIPA enhances Lyn kinase activity, we washed Triton X-100-solubilized, immunoprecipitated Lyn with RIPA prior to the *in vitro* kinase reaction. We found no difference in kinase activity of Lyn with and without the RIPA wash, indicating no differential effects of detergents on Lyn kinase activity.² Also, because of the large differences in Lyn recovery from RIPA and Triton X-100 PNS, we verified that results obtained were independent of the concentration of Lyn under the range of concentrations used for the *in vitro* kinase assay (Fig. 1C, *inset*).

The Triton X-100 Postnuclear Supernatant Is Depleted of Raft-associated Lyn—The insolubility of a substantial percentage of cellular Lyn following RBL-2H3 lysis in 0.5% Triton X-100 led us to examine the distribution of solubilized Lyn in the Triton X-100 PNS by sucrose gradient analysis. Fig. 2 compares the distribution of this Lyn, obtained as PNS (Fig. 1A), with the distribution of Lyn from cells lysed in the same concentration of Triton X-100 but directly loaded onto a sucrose gradient without precentrifugation. The representative blots shown in Fig. 2 illustrate that the Triton X-100 PNS is significantly depleted of lipid raft-associated Lyn; 4% of the Lyn from the PNS fractionates in the lipid raft region of the gradient, whereas 45% of the total cellular Lyn is found in the raft fraction under these conditions. Similar results were observed for lysis in 1% Triton X-100, indicating that Lyn solubility is not limited by the amount of detergent under these conditions.²

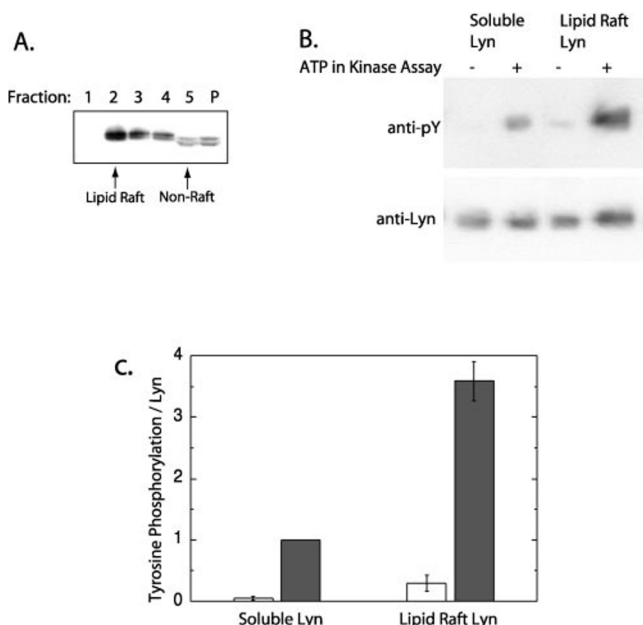


FIG. 3. Lyn recovered from the low density, lipid raft region of sucrose gradients has a higher level of basal tyrosine phosphorylation and enhanced kinase activity compared with Lyn recovered from the high density, nonlipid raft region of sucrose gradients. A, representative sucrose gradient distribution of Lyn from RBL-2H3 cells lysed at 30×10^6 cells/ml in 0.25% Triton X-100 and analyzed as in Fig. 2. Lipid raft-associated Lyn (fraction 2) and nonlipid raft Lyn (fraction 5) were diluted in RIPA buffer, and Lyn was immunoprecipitated and subjected to *in vitro* kinase analysis as in Fig. 1. B, representative Western blot showing *in vitro* kinase analysis. The top panel shows the anti-phosphotyrosine Western blot; the lower panel shows the anti-Lyn reprobe of the same blot. C, summary of relative specific activity (*shaded bars*) of Lyn autophosphorylation Lyn basal phosphorylation (*open bars*) for raft and nonraft Lyn isolated from resting RBL-2H3 cells. Error bars, S.E.; $n = 8$.

As observed previously (25), the alternatively spliced 53- and 56-kDa forms of Lyn are more highly resolved in the nonraft fractions in Fig. 2. These results, taken together, indicate that Triton X-100 lysis yields a PNS that is selectively depleted of lipid raft-associated Lyn.

Raft-associated Lyn Has Enhanced Kinase Activity—The results of Fig. 2 suggest that the difference in specific activity between Lyn from Triton X-100 and RIPA PNSs could be due to different amounts of raft-associated Lyn. This interpretation is consistent with studies by Ilangumaran *et al.* (22) and Kabouridis *et al.* (23) indicating that the Src family kinases Lck and Fyn are differentially regulated by their lipid environment. To investigate directly whether the lipid raft environment affects Lyn activity, we isolated Lyn from either lipid raft or nonraft fractions obtained from sucrose gradient separation of RBL cells lysed by Triton X-100. A high concentration of cells was used in these experiments to permit sufficient recovery of Lyn from gradient fractions for immunoprecipitation and *in vitro* kinase analysis. The ratio of detergent to cells chosen is similar to that used in previous studies to preserve the interactions between cross-linked IgE-FcεRI complexes and lipid rafts (11, 15). Under these conditions, uncross-linked IgE-FcεRI was fully solubilized, fractionating with the nonlipid raft components, indicating complete plasma membrane lysis.² The sucrose gradient distribution of Lyn is shown in Fig. 3A and is similar to that in Fig. 2 and in previous experiments carried out under these conditions (11).

Lipid raft-associated Lyn was recovered from the interface between the 5 and 30% sucrose layers (fraction 2; Fig. 2), and nonraft Lyn was recovered from the soluble lysate in the 40% sucrose fraction (fraction 5; Fig. 2). Both fractions were then

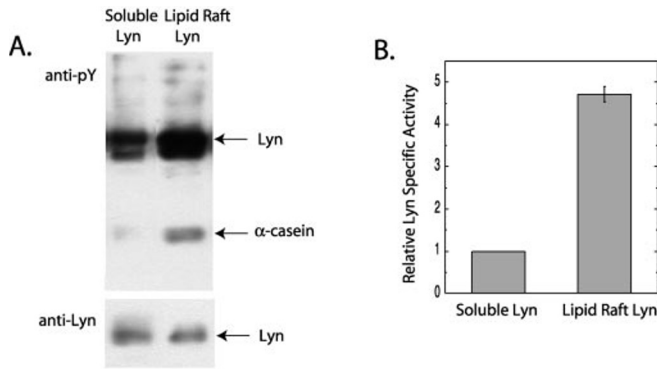


FIG. 4. **Lyn kinase specific activities with an exogenous substrate are consistent with autophosphorylation from sucrose gradient analyses.** A, representative Western blots showing *in vitro* phosphorylation of α -casein by Lyn fractionated and immunoprecipitated as in Fig. 3. B, summary of results from *in vitro* kinase assays using α -casein as substrate. The amount of α -casein phosphorylation was normalized to the amount of Lyn per sample as determined by anti-Lyn blotting on stripped and reprobed blots. Error bar, S.E.; $n = 3$.

diluted into RIPA buffer, and Lyn was immunoprecipitated and analyzed as in Fig. 1. Representative Western blots from these experiments are shown in Fig. 3B. Consistent with results in Fig. 1C, we find that Lyn isolated from lipid rafts has a 3.6-fold higher specific activity than Lyn isolated from nonraft fractions (Fig. 3C, shaded bars). Also, the levels of basal phosphorylation are 6-fold higher for Lyn from the detergent-resistant lipid raft environment compared with solubilized Lyn (Fig. 3C, open bars). Thus, lipid raft-associated Lyn has substantially more kinase activity than Lyn from a more disordered membrane environment in this autophosphorylation assay. Furthermore, this higher specific activity correlates with higher basal phosphorylation of Lyn.

To test the validity of conclusions based on *in vitro* Lyn autophosphorylation, we performed *in vitro* kinase assays using dephosphorylated α -casein as an exogenous substrate. We first determined that dephosphorylated α -casein is specifically phosphorylated by Lyn as indicated by a lack of phosphorylation in mock immunoprecipitations done without the Lyn antibody.² Activity was detected by quantitative Western blot analysis of the α -casein band with anti-phosphotyrosine and calculated per amount of Lyn detected by reprobing the blot as for the autophosphorylation assay. A representative Western blot is shown in Fig. 4A, and Fig. 4B summarizes the relative specific activities for Lyn from lipid raft and nonraft fractions. Consistent with the autophosphorylation results obtained in Figs. 1 and 3, raft-associated Lyn has a 4.7-fold higher specific activity toward α -casein than nonraft-associated Lyn.

Specific Activity of Lyn Does Not Increase in Stimulated Cells—In previous studies, the total Lyn kinase activity associated with Fc ϵ RI was shown to increase following antigen stimulation, but the specific activity of receptor-associated Lyn toward an exogenous substrate was found to be unchanged (4, 24). To determine whether the specific activity of total cellular Lyn is altered by antigen stimulation, IgE-sensitized RBL-2H3 cells were stimulated with an optimal dose of antigen (0.9 μ g/ml dinitrophenylated-bovine serum albumin) for various times. Lyn was then immunoprecipitated from RIPA-lysed cells, and *in vitro* kinase activity was determined with α -casein as an exogenous substrate. The results are summarized in Table I. Consistent with previous results from Metzger and colleagues (4, 24), there is little change in Lyn specific kinase activity following stimulation for 2 min at 37 °C. Interestingly, Lyn specific activity was found to decrease by 60% after 5 min of stimulation (Table I); this may be related to the decline in

TABLE I
Antigen-stimulated changes in Lyn kinase specific activity

Time of stimulation	Relative Lyn specific activity ^a
min	
0	1.00
2	0.94 \pm 0.27 ^b
5	0.37 \pm 0.09

^a Determined for RIPA-solubilized Lyn using α -casein as in Fig. 4.

^b S.D. ($n = 3$).

Fc ϵ RI tyrosine phosphorylation observed at later times of antigen stimulation (25) (see "Discussion"). These results indicate that Fc ϵ RI cross-linking does not cause detectable activation of Lyn kinase *per se*. Rather, Lyn kinase activity in resting cells is sufficient for stimulated Fc ϵ RI phosphorylation that results from cross-linking by antigen. We conclude that the increase in Fc ϵ RI phosphorylation commonly observed after cross-linking results from changes in Fc ϵ RI proximity to active Lyn.

Tyrosine 397 Phosphorylation Is the Predominant Detectable Site of Tyrosine Phosphorylation on Lyn from Unstimulated RBL Cells—Results summarized in Figs. 1, 3, and 4 indicate that the higher specific activity of Lyn isolated from a lipid raft environment correlates with higher basal tyrosine phosphorylation. This higher specific activity is preserved following solubilization of Lyn away from the ordered lipids of the raft membranes by RIPA. Thus, it is likely that a covalent modification of Lyn, such as phosphorylation, is responsible for the higher activity state. Lyn, like other Src family kinases, has two major sites of tyrosine phosphorylation that regulate activity: one in the active site at residue 397 (Lyn A notation) and a second at the C terminus at residue 508 (26). Phosphorylation of Tyr³⁹⁷ has been reported to increase the specific activity of Lyn by 17-fold (27), whereas phosphorylation of the C-terminal regulatory site, Tyr⁵⁰⁸, reduces Lyn activity, similar to other Src family members (28).

To investigate the relative abundance of tyrosine phosphorylation at these sites both before and after the *in vitro* kinase assay, we performed CNBr peptide mapping. CNBr cleavage of Lyn is predicted to yield separate fragments containing the active site and C-terminal site tyrosines with sizes of 8.2 and 4.1 kDa, respectively (Fig. 5). For these experiments, RIPA-solubilized Lyn was immunoprecipitated and subjected to an *in vitro* kinase incubation, with or without ATP, as in previous experiments, followed by treatment with CNBr. As shown in Fig. 5, basal Lyn phosphorylation is readily detectable in an ~8-kDa fragment, consistent with the size expected for the minimal peptide containing Tyr³⁹⁷. Much less is detected in the ~4-kDa peptide that is the size of the C-terminal fragment, similar to previous results in B-cells (29). After the *in vitro* kinase incubation with ATP, Lyn autophosphorylation is again preferentially found in the ~8-kDa fragment, similar to trends observed in two previous studies (27, 44). Incomplete digestion of Lyn by CNBr makes it difficult to determine whether the 4.1-kDa C-terminal fragment is produced as efficiently as the 8.2-kDa fragment containing Tyr³⁹⁷, but the results indicate that phosphorylation at the active site loop contributes significantly, if not predominantly, to basal tyrosine phosphorylation of Lyn in RBL cells. Furthermore, the relative enhancements of phosphorylation following the *in vitro* kinase assay in this and in Figs. 1 and 3 show that only a small fraction of Lyn is phosphorylated in the basal state, suggesting that Lyn activity is regulated primarily by phosphorylation at Tyr³⁹⁷, and that Tyr⁵⁰⁸ phosphorylation plays a lesser role in the regulation of Lyn kinase activity in unstimulated RBL-2H3 cells.

Tyrosine 397 Phosphorylation Is Responsible for Increased Kinase Activity of Raft-associated Lyn—Lipid raft-associated Lyn has a higher level of basal phosphorylation than nonraft

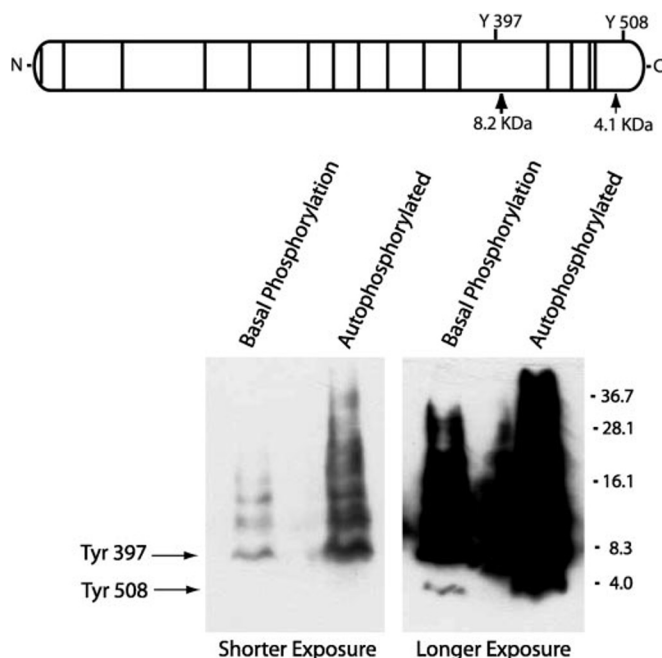


FIG. 5. Cyanogen bromide peptide mapping of Lyn indicates that tyrosine 397 is the predominant phosphotyrosine residue in unstimulated RBL-2H3 cells. CNBr map and representative CNBr digests of equal cell equivalents of Lyn from RBL-2H3 cells before (Basal Phosphorylation) and after *in vitro* kinase autophosphorylation (Autophosphorylated). Immunopurified Lyn from unstimulated RBL-2H3 cells lysed in RIPA buffer was cleaved with CNBr, and the fragments were separated on a 16.5% Tricine gel and then transferred to an Immobilon P^{8Q} membrane. Anti-phosphotyrosine Western blot detection shows that the peptides containing tyrosine 397 (~8.2 kDa) are predominantly phosphorylated in both samples. The left frame shows a short exposure, whereas the right shows a much longer exposure to reveal the phosphorylated Tyr⁵⁰⁸ peptide (~4.1 kDa).

Lyn (Fig. 3), and RIPA-solubilized Lyn appears to be phosphorylated primarily at Tyr³⁹⁷ (Fig. 5). We hypothesized that lipid raft-associated Lyn has a higher level of tyrosine 397 phosphorylation than nonraft Lyn in RBL cells, and this leads to its higher specific activity. We were unable to isolate sufficient Lyn from sucrose gradient fractions to directly evaluate CNBr digests of basally phosphorylated Lyn from lipid rafts. However, a prediction of our hypothesis is that the enhanced kinase activity of raft-associated Lyn should be selectively reduced by the actions of tyrosine phosphatases during cell lysis. To test this prediction, we compared the specific activities of lipid raft-associated and nonraft Lyn obtained from cells lysed either in the presence (as in previous experiments) or in the absence of the tyrosine phosphatase inhibitors NaVO₄ and β -glycerophosphate.

As shown in Fig. 6, lipid raft-associated Lyn isolated in the absence of phosphatase inhibitors (shaded bars) has a markedly decreased specific activity compared with Lyn isolated in the presence of phosphatase inhibitors (white bars). In fact, raft-associated Lyn isolated in the absence of phosphatase inhibitors has a kinase activity similar to that of Lyn isolated from nonraft environments. Thus, dephosphorylation of Lyn during cell lysis in the absence of tyrosine phosphatase inhibitors substantially reduces kinase activity of Lyn from lipid rafts, suggesting that this Lyn derives its higher specific activity from enhanced basal phosphorylation that is preserved during lysis in the presence of phosphatase inhibitors. Taken together with the phosphopeptide mapping results, these data imply that raft-associated Lyn is more active because it has a higher amount of phosphorylation at Tyr³⁹⁷. Conversely, the specific activity of soluble, nonlipid raft, Lyn is slightly in-

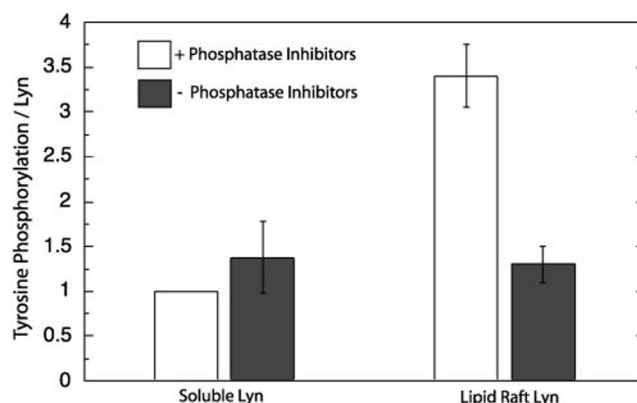


FIG. 6. The enhanced specific activity of Lyn kinase from lipid rafts is lost following lysis in the absence of phosphatase inhibitors. Lyn was isolated, and specific kinase activity was determined as described in Fig. 3, except that cells were lysed in either the presence (white bars) or absence (shaded bars) of the tyrosine phosphatase inhibitors (NaVO₄ and β -glycerophosphate). All values are normalized to the tyrosine phosphorylation of soluble Lyn in the presence of phosphatase inhibitors. Error bars, S.E.; *n* = 5.

creased when isolated from cells lysed in the absence of phosphatase inhibitors (Fig. 6). This suggests that the specific activity of Lyn outside the lipid raft environment may be negatively regulated by phosphorylation at Tyr⁵⁰⁸.

DISCUSSION

Our previous studies (11, 16) provided evidence that antigen-stimulated Fc ϵ RI phosphorylation by Lyn tyrosine kinase depends on lipid rafts, but it remained unclear from those studies how the raft environment facilitates this process. Because cholesterol depletion causes both Lyn and cross-linked Fc ϵ RI to lose their association with lipid rafts, inhibition of stimulated Fc ϵ RI phosphorylation by this treatment could be due to the loss of an ordered lipid environment for either Lyn or Fc ϵ RI or both. These considerations prompted us to investigate the role of the lipid raft environment on Lyn kinase activity.

Our present results demonstrate that the lipid raft environment regulates the specific activity of Lyn kinase. Specifically, Lyn from a lipid raft environment has a substantially higher *in vitro* kinase activity than nonlipid raft-associated Lyn (Figs. 1, 3, and 4). Furthermore, this activity measured with autophosphorylation or α -casein does not increase following antigen stimulation. Phosphopeptide mapping of Lyn indicates that Tyr³⁹⁷, the active site tyrosine, is a major site for tyrosine phosphorylation in unstimulated cells. This basal phosphorylation at Tyr³⁹⁷ is found primarily on Lyn isolated from lipid rafts, and dephosphorylation by omission of tyrosine phosphatase inhibitors during cell lysis reduces raft-associated Lyn kinase activity to levels similar to that of Lyn isolated from more fluid regions of the membrane. We conclude that co-compartmentalization of active Lyn with Fc ϵ RI in lipid rafts after antigen cross-linking results in increased receptor phosphorylation and thereby initiation of intracellular signaling.

Our initial experiments used different detergents to solubilize Lyn for immunoprecipitation and *in vitro* kinase analysis, and we found that Triton X-100 under standard lysis conditions resulted in recovery of less than half of the total cellular Lyn in the PNS. Octyl glucoside yielded much better recoveries (data not shown), and the best recoveries of Lyn kinase were with the RIPA mixture of Triton X-100, deoxycholate, and SDS. Furthermore, the specific activity of Lyn solubilized by Triton X-100 was found to be substantially less than that from RIPA-lysed cells, and sucrose gradient analysis showed that the Triton X-100 PNS was almost completely depleted of lipid raft-associated Lyn (Fig. 2). Because RIPA and octyl glucoside

solubilize lipid rafts (30) (data not shown), sucrose gradient fractionation is not useful for cells lysed in these detergents.

These initial experiments led us to compare the recovery and distribution of Lyn using standard sucrose gradient analyses of Triton X-100-lysed cells. In the absence of a clarification step to obtain a PNS, nearly half of the Lyn is recovered in the lipid raft floating fractions. The remainder is either in mixed micelles in the middle of the gradient or at the bottom of the gradient, where cytoskeletal and other large, detergent-insoluble structures are found. These results, together with sucrose gradient analysis of the PNS, indicate that most Lyn associated with lipid rafts pellets in the centrifugation step that immediately follows cell lysis with Triton X-100 in a standard immunoprecipitation protocol, possibly because of association of lipid rafts with the cytoskeleton at the time of cell lysis. During overnight centrifugation in the sucrose gradients, this interaction is lost, allowing Lyn and most raft-associated components to float at low densities. Previous studies showed that clustered lipid rafts on intact cells are major sites for attachment of the actin cytoskeleton to the plasma membrane (25, 31), and other studies indicated that Triton X-100-resistant rafts exist as a continuous network supported by the cellular cytoskeleton immediately following cell lysis (32, 33). Taken together, our results indicate that Lyn kinase activity from Triton X-100 PNS represents a subset of its total cellular activity that is depleted of lipid raft-associated Lyn.

Using RIPA solubilization to analyze total cellular Lyn, we find that antigen does not cause a detectable increase in total Lyn tyrosine kinase activity after 2 min of stimulation but rather results in a decrease in this activity at subsequent time points (Table I). Honda *et al.* (34) reported that a rapid, transient increase in Lyn kinase activity, which preceded maximal phosphorylation of Syk and other phosphotyrosine substrates, occurs at 1–3 min in wild type RBL-2H3 cells. Because they solubilized cells in a mixture of Triton X-100 and deoxycholate, it is unclear whether they analyzed a subset depleted in lipid raft-associated Lyn. Recent results by Ohtake *et al.* (35) indicate that, upon stimulation via Fc ϵ RI, Csk is recruited to lipid rafts via its binding to Cbp/PAG, and this results in a decrease in Fc ϵ RI tyrosine phosphorylation. The decrease in specific activity of Lyn we observe following antigen stimulation may result from the C-terminal phosphorylation of raft-associated Lyn by recruited Csk, and this may contribute to the time-dependent decrease in stimulated Fc ϵ RI phosphorylation that occurs. We previously showed that inhibition of stimulated actin polymerization by cytochalasin causes transient Fc ϵ RI phosphorylation to become more sustained in response to antigen (25). Thus, it will be interesting to investigate whether actin polymerization regulates Lyn kinase activity under stimulating conditions and whether Csk is involved in this process.

Together, our results indicate that tyrosine phosphorylation of the active site is a major determinant of Lyn kinase activity, both before and immediately after antigen stimulation. Our results are consistent with a role for Csk-dependent C-terminal phosphorylation as a mechanism to down-regulate Lyn kinase activity at longer times of stimulation. A previous study in RBL-2H3 mast cells indicated that overexpression of membrane-associated Csk can increase negative regulation of Lyn by enhancing C-terminal phosphorylation (28). Expression of mutated Lyn missing its C-terminal Tyr in these cells caused elevated basal phosphorylation of Fc ϵ RI and a stimulated increase by antigen. These results indicated that C-terminal phosphorylation of Lyn contributes to its regulation but is not the primary determinant of antigen-stimulated Fc ϵ RI phosphorylation. For Src, the prototype of this family, it is clear that C-terminal phosphorylation plays a dominant role in regulat-

ing its basal activity in mammalian cells (36), but this kinase exhibits only a very modest 2-fold increase in activity due to phosphorylation of its active site loop (37). In contrast, the large, 17-fold increase in Lyn kinase activity that results from Tyr³⁹⁷ phosphorylation makes this phosphorylation site much more important in determining the overall regulation of Lyn kinase activity (27).

Based on our results, we propose a model for signal initiation in which Lyn in an ordered lipid raft environment is protected from dephosphorylation at its active site. In this environment, Lyn would have little contact with transmembrane tyrosine phosphatases, such as CD45, which is excluded from lipid rafts (38). CD45 itself is not expressed detectably on most RBL-2H3 cells (39), but it represents a large family of receptor-like transmembrane phosphatases, one or more of which might serve a negative regulatory role on Lyn kinase activity. In our model, cross-linking of IgE-Fc ϵ RI by antigen coalesces small, dynamic lipid raft domains containing active Lyn into larger more stable domains containing active Lyn and cross-linked Fc ϵ RI. Within this environment, active Lyn phosphorylates ITAM sequences on Fc ϵ RI β and γ subunits to initiate the signaling cascade. The kinetics of this process parallels lipid raft association of Fc ϵ RI determined by sucrose gradient analysis (11, 25). Proteins in these coalesced lipid raft domains would thus be protected from transmembrane phosphatases, which are excluded and prevented from dephosphorylating Lyn or Fc ϵ RI.

Metzger and colleagues (40, 45) have shown that Fc ϵ RI and other substrates of Lyn or Syk kinase are not protected from dephosphorylation when ongoing stimulation is halted by monovalent hapten. Based on these and related results, they argue that lipid rafts fail to protect from the action of tyrosine phosphatases. However, in breaking up cross-links, monovalent hapten reverses lateral immobilization of cross-linked receptors (41), and this probably reflects dispersal of clustered lipid rafts. In another recent study, Kovarova *et al.* (42) indicated that Lyn with a single acyl chain failed to fractionate detectably with lipid rafts, but antigen-stimulated phosphorylation of Fc ϵ RI was preserved with this construct. Lyn with a single saturated acyl chain would still be expected to associate significantly with an ordered lipid environment, but its interaction energy would be reduced, and thus it would probably be less able to withstand the perturbation of Triton X-100 solubilization (43). Furthermore, the construct tested in these experiments contained a green fluorescent protein domain attached to its C terminus, so it is unclear whether this construct can be negatively regulated by C-terminal phosphorylation, which may contribute to the negative regulation of wild type Lyn in the fluid membrane environment (Fig. 6).

It is not yet clear whether the phosphatase(s) responsible for Fc ϵ RI dephosphorylation that occurs after cross-linking are also effective in dephosphorylating the active site phosphotyrosine of Lyn; these may represent distinct molecular species having differential interactions with lipid rafts. Testing this model critically will require identification and co-reconstitution of these phosphatase(s) with Fc ϵ RI and Lyn under conditions in which antigen-stimulated Fc ϵ RI phosphorylation can be observed. Our working model predicts that stimulated Fc ϵ RI phosphorylation depends on co-segregation of cross-linked receptor and Lyn from a phosphatase that is excluded from lipid rafts. The role of lipid rafts to coordinate interactions between active Lyn and cross-linked Fc ϵ RI is central to regulating the initial events of signal transduction in RBL-2H3 mast cells.

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