Engagement of Phospholipid Scramblase 1 in Activated Cells

IMPLICATION FOR PHOSPHATIDYL SERINE EXTERNALIZATION AND EXOCYTOSIS

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Phosphatidylserine (PS) in quiescent cells is predominantly confined to the inner leaflet of the plasma membrane. Externalization of PS is a marker of apoptosis, exocytosis, and some nonapoptotic activation events. It has been proposed that PS externalization is regulated by the activity of PLSCR1 (phospholipid scramblase 1), a Ca2+-dependent endofacial plasma membrane protein, which is tyrosine-phosphorylated in activated cells. It is, however, unclear how the phosphorylation of PLSCR1 is related to its membrane topography, PS externalization, and exocytosis. Using rat basophilic leukemia cells as a model, we show that nonapoptotic PS externalization induced through the high affinity IgE receptor (FcRI) or the glycosylphosphatidylinositol-anchored protein Thy-1 does not correlate with enhanced tyrosine phosphorylation of PLSCR1. In addition, PS externalization in FcRI- or Thy-1-activated cells is not associated with alterations of PLSCR1 fine topography as detected by electron microscopy on isolated plasma membrane sheets. In contrast, activation by calcium ionophore A23187 induces changes in the cellular distribution of PLSCR1. We also show for the first time that in pervanadate-activated cells, exocytosis occurs even in the absence of PS externalization. Finally, we document here that tyrosine-phosphorylated PLSCR1 is preferentially located in detergent-insoluble membranes, suggesting its involvement in the formation of membrane-bound signaling assemblies. The combined data indicate that changes in the topography of PLSCR1 and its tyrosine phosphorylation, PS externalization, and exocytosis are independent phenomena that could be distinguished by employing specific conditions of activation.

Phosphatidylserine (PS) is an anionic aminophospholipid, predominantly confined to the cytoplasmic leaflet of the plasma membrane. The appearance of PS on the cell surface is a characteristic marker of apoptotic cells and participates in the recognition and elimination by macrophages of dying, injured, senescent, or necrotic cells (1, 2). In nonapoptotic cells, externalization of PS is associated with certain stages of cell development (3), cell fusion (4), blood clotting (5), secretory responses (6–9), cell infection (10), or neoplastic transformation (11). Externalized PS was found to regulate plasma membrane receptor-mediated cell signaling (12, 13) and cell-cell interactions (14). Translocation of PS to the external leaflet of the plasma membrane is presumably induced by simultaneous inhibition of lipid translocases, which maintain the transbilayer asymmetry of phospholipids in the plasma membrane, and activation of calcium-dependent PLSCR1 (phospholipid scramblase 1) (15–17). In activated cells, rapid transbilayer reorganization of the plasma membrane phospholipids, called lipid scrambling (18), is triggered through the elevation of concentration of free cytoplasmic calcium [Ca2+]i (16, 19). It has been suggested that binding of calcium to PLSCR1 leads to its conformational changes, self-aggregation, and phospholipid scrambling (20).

Apart from the scrambling activity, PLSCR1 also seems to function as a signal transduction molecule. PLSCR1 has been shown to be operative in cells activated through several plasma membrane receptors (21–23), during myelopoiesis and leukemogenesis (24, 25), growth of cancer cells (26), and response of hematopoietic cells to growth factors (27). Engagement of PLSCR1 in these activation events has been inferred from its tyrosine phosphorylation, plasma membrane or cellular redistribution, and/or its interactions with various signaling counterparts (21–23, 28–30).

The finding that aggregation of the high affinity IgE receptor (FcεRI) in rat basophilic leukemia (RBL) cells leads to a rapid tyrosine phosphorylation of PLSCR1 (22, 31) suggested that phosphorylation of this protein might be essential for the changes in the distribution of phospholipids in the

mAb, monoclonal antibody; αThy-1, MRCoX7 mAb specific for Thy-1.1; αFcεRI, anti-FcεRI α subunit-specific mAb, clone S.14; TNP, trinitrophenyl; DNP, dinitrophenyl; ToAb, polyclonal antibody; NTAL, non-T cell activation linker; LAT, linker for activation of T cells; GuM, goat anti-mouse; GuR, goat anti-rabbit; RuPLS, PLSCR1-specific rabbit pAb; αPY, phosphotyrosine-specific mAb, clone PY20; DiRl, donkey anti-rabbit; Cy3, cyanine 3; MuPLS*, mouse mAb 129.2 specific for PLSCR1; FITC, fluorescein isothiocyanate; DIM, detergent-insoluble membrane(s); GST, glutathione S-transferase; BSS, buffered saline solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline solution; BRA, bivariate Ripley's analysis; MuPLS, PLSCR1-specific mAb, clone TEC-23; GPI, glycosylphosphatidylinositol; Mes, 2-(N-morpholino)ethanesulfonic acid.
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It was, however, unclear whether tyrosine phosphorylation of PLSCR1 in mast cells is confined to FcεRI triggering and in what way it is related to PS externalization and exocytosis observed in activated mast cells. To address these issues, we studied the interrelationships among tyrosine phosphorylation of PLSCR1 and its plasma membrane topography, PS externalization, and degranulation in RBL cells triggered by various stimuli known to have different effects on protein tyrosine phosphorylation, [Ca^{2+}], and exocytosis. Our data indicate that the above events are independent phenomena, which can occur individually, depending on the activation methods employed.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were used: MRCOX7 monoclonal antibody (mAb) specific for Thy-1.1 (αThy-1) (32), anti-FcεRI α subunit-specific mAb, clone 5.14 (αFcεRI) (33), anti-FcεRI β subunit-specific mAb (34), tri-nitrophenyl (TNP)-specific IgE mAb (IGEL b4 1) (35), dinitrophenyl (DNP)-specific IgE mAb (36), rabbit polyclonal antibody (pAb) specific for non-T cell activation linker (NTAL) and linker for activation of T cells (LAT) (37), goat anti-mouse (GaM) IgG-10 nm, and goat anti-rabbit (GaR) IgG-5 nm gold particle conjugates (Amersham Biosciences). PLSCR1-specific rabbit pAb (RapPLS) was prepared by immunizing rabbits with recombinant PLSCR1. Phosphotyrosine-specific mAb, clone PY20 (αPY), conjugated to horseradish peroxidase, was purchased from BD Biosciences. Horseradish peroxidase-conjugated GaM IgG and horseradish peroxidase-conjugated GaR IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Donkey anti-rabbit (DA R) IgG-cyanine 3 (Cy3) conjugate was bought from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse mAb 129.2 specific for PLSCR1 (MoPLS*) was kindly provided by M. Benhamou (INSERM, Institut Pasteur, Paris, France). Annexin V-fluorescein isothiocyanate (FITC) was from BD Biosciences. Latrunculin B, EGTA, phenylmethylsulfonyl fluoride, calcium ionophore A23187, and all other chemicals were from Sigma.

Production of New mAb against Plasma Membrane Microdomains and Identification of Their Target Antigens—RBL cells were lysed in ice-cold 1% Brij 96-containing lysis buffer (38). The lysate was subjected to fractionation on a sucrose density gradient, as described previously (38), and low density fraction, containing detergent-insoluble membranes (DIM), were pooled and used for immunization of BALB/c mice. Hybridoma cells were obtained after fusion of SPO2 mouse myeloma cells with spleen cells of immunized mice using standard procedures (39). The target antigens of the new mAb were immunoprecipitated from the lysate of resting RBL cells and separated by two-dimensional gel electrophoresis under reducing conditions. Alignment of immunoblotted and colloid silver-stained membranes allowed identification of silver-stained proteins corresponding to the target antigens. The corresponding in-gel silver-stained proteins were excised and destained as described previously (40). The proteins were digested by trypsin and analyzed by peptide mass mapping and/or peptide sequencing (41). Data base searches of the identified peptide fragments allowed determination of the target antigens.

DNA Constructs, Recombinant Proteins, and Immunoaffinity Purification of pAb—To prepare the N-terminal fragment of recombinant PLSCR1, full-length cDNA of rat PLSCR1 without initial ATG codon was cloned and cloned into Xmal and HindIII cloning sites of pQE30 expression vector (Qiagen, Hilden, Germany) using the forward (5'-AAACCGGGGAG-AAGCAGGAACCCAGAA-3') and reverse (5'-CCCCAGCCTGCTACCATACTCTGACCTTTG-3') primers. A DNA fragment of the cloned cDNA was excised by SpHi and BamHI restriction enzymes and cloned into the corresponding sites in pQE70 expression vector (Qiagen). To prepare the glutathione S-transferase (GST)-PLSCR1 fusion protein, full-length cDNA of rat PLSCR1 without the initial ATG codon was cloned into EcoRI site of pGEX-3X expression vector (Amersham Biosciences) using the following primers: forward, 5'-AAGGAATT-CGGAGAAGCAGGAACCCAGAA-3'; reverse, 5'-AAGG-AATTCTACCATACTCTGACCTTTG-3'. Recombinant proteins were expressed in bacteria Escherichia coli strain JM109. The N-terminal fragment of PLSCR1 was isolated from inclusion bodies as previously reported (42) and used to immunize rabbits to generate RapLS. The recombinant GST-PLSCR1 was affinity-purified on glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer’s protocol. The isolated GST-PLSCR1 or recombinant fragment of rat NTAL (37) was covalently bound to CNBr-activated beads (Sigma), which were then used for immunoaffinity purification of PLSCR1- or NTAL-specific pAb (37). In some experiments, isolated GST-PLSCR1 fusion protein was used at a concentration of 50 μg/ml to confirm the specificity of anti-PLSCR1 antibodies.

Cells and Their Activation—The origin of RBL cells, clone 2H3, and their culture conditions have been described (43, 44). Before activation, cells were harvested and washed with buffered saline solution (BSS; 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5.6 mM glucose), supplemented with 1.8 mM CaCl2 and 0.1% bovine serum albumin (BSA). The cells were sensitized or not with TNP-specific IgE (IGEL b4 1 ascitic fluid diluted 1:1000 in BSS/BSA), αFcεRI, or biotin-labeled αThy-1 (both at 2 μg/ml), washed, and resuspended in BSS/ BSA to 20 × 106/ml. The cells were activated at 37 °C for the indicated time intervals by adding equal amounts of twice concentrated activators in BSS/BSA. Final concentrations of the activators were as follows: TNP-BSA (1 μg/ml), αFcεRI or αThy-1 (2 μg/ml), streptavidin (10 μg/ml), calcium ionophore A23187 (3 μM), and pervanadate (0.2 mM sodium orthovanada- date, 1 mM hydrogen peroxide). To induce apoptosis by UV irradiation, cells grown in medium were exposed in open Petri dishes to UV-C (predominantly 254 nm) from a germicidal lamp (Philips TUV G30T8 30-W bulb) at 60 cm distance. After a 10-min exposure, fresh medium was added, and the cells were cultured for further 6 h before analysis. Apoptosis was confirmed by proteolytic cleavage of Lyn (45) and DNA laddering as previously described (46).

Sucrose Density Gradient Fractionation—Cells were lysed in ice-cold Brij 58 lysis buffer (25 mM Mes, pH 6.5, 100 mM NaCl, 2 mM EDTA, 0.5% Brij 58, phosphatase inhibitors (2

plasma membrane during cell activation and degranulation.
and then labeled for 1 h with immunoaffinity-purified RePLS (5 µg/ml) or rabbit anti-LAT serum (diluted 1:1000), followed by washing with PBS, blocking for 20 min with PBS/BSA, and incubation for 1 h with DaR IgG-Cy3 conjugate (10 µg/ml).

After another washing step, the cells were fixed for 10 min with 5% paraformaldehyde in PBS, washed, and mounted in p-phenylenediamine (1 mg/ml, 50% glycerol in PBS). Images were acquired with a Leica TCS NT/SP confocal system in conjunction with Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with oil objective ×100/1.4 numerical aperture.

**Electron Microscopy**—Plasma membrane sheets were prepared as described (51, 52). Briefly, cells in complete culture medium were grown overnight on glass coverslips in the presence or absence of DNP-specific IgE (1 µg/ml). Adherent cells were activated or not by DNP-BSA (1 µg/ml) or other activators in BSS/BSA for the indicated time intervals at 37 °C, and the glass coverslips with cells were washed in ice-cold PBS and then inverted and briefly pressed onto pionoform-covered and poly-l-lysine-coated electron microscopy grids kept on ice; careful separation of coverslips from the grids leaves the plasma membrane sheets attached to the grids. The membranes were fixed immediately with 2% paraformaldehyde and then exposed to immunoaffinity-purified RePLS (10 µg/ml), rabbit anti-NTAL pAb (10 µg/ml), or anti-FcεRI-β mAb (3 µg/ml). Washed membranes were exposed to gold-conjugated secondary antibodies diluted 1:20 from commercial stocks. After additional fixation with 2% glutaraldehyde and staining with osmium tetroxide, tannic acid, and uranyl acetate, the samples were dehydrated and examined under a JEOL JEM 1200EX electron microscope operating at 60 kV. To obtain membrane sheets from cells with aggregated Thy-1, the cells were incubated for 15 min at room temperature with α-Thy-1 (1 µg/ml), washed, and then incubated at 37 °C for 10 min with GaM IgG-10-nm gold conjugate. In the case of Thy-1 labeling without aggregation, the cells were first fixed with paraformaldehyde and labeled for 15 min with α-Thy-1, followed by 10-min exposure to GaM IgG-10-nm gold conjugate. After the extracellular labeling, plasma membrane sheets were isolated as described above. Plasma membrane sheets from nonadherent apoptotic cells were prepared as described (53).

**Evaluation of the Results**—Means ± S.D. were computed from at least three independent experiments. Statistical significance of intergroup differences was calculated using an unpaired Student’s t test; each group consisted of four independent experiments. Evaluation of gold particle co-localization was performed by bivariate Ripley’s analysis (BRA) using the L(t)-r function as described (52). Pictures of plasma membrane sheets from two independent experiments were taken to cover ~50 µm² of plasma membrane surface and analyzed using Matlab software algorithms (52). Significant co-localization (p < 0.01) of the proteins occurred when the position of the L value curve (solid lines) surpassed the boundaries (dashed lines) predicted for random distribution of gold particles at a corresponding distance (Fig. 3, E and F and Fig. 5, D, and E). Cluster size was determined by the program GOLD (54).
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RESULTS

FceRI-induced Degranulation and PS Externalization Do Not Correlate with the Extent of PLSCR1 Tyrosine Phosphorylation—Antigen-mediated aggregation of the FceRI triggers signaling pathways leading to increased tyrosine phosphorylation of numerous proteins, including PLSCR1, enhanced [Ca^{2+}], degranulation, and transient nonapoptotic PS externalization (6, 7, 9, 22, 55). To elucidate the role of PLSCR1 tyrosine phosphorylation in these processes, we first prepared and characterized new PLSCR1-specific mAb and pAb. A panel of mAb was prepared after immunizing BALB/c mice with DIM isolated from RBL cells. One of the mAbs, clone TEC-23, reacted with two proteins of ~36 and 38 kDa under reducing conditions (Fig. S1A). These two bands were observed even after direct solubilization of the cells in reducing Laemmli sample buffer and immediate boiling; this makes it unlikely that the lower band is produced by proteolysis during cell solubilization. Under nonreducing conditions, only one band of 38 kDa was observed (not shown). Interestingly, mass spectroscopic analysis identified 10 peptides, with amino acid sequences identical in both proteins and corresponding to rat PLSCR1 (Fig. S1B). Furthermore, immunoblotting experiments showed that the binding of the PLSCR1-specific mAb, clone TEC-23 (MaPLS), to its target was completely inhibited by recombinant GST-PLSCR1 (Fig. S1C); the same recombinant protein also inhibited the binding of immunofinity-purified RoPLS and MaPLS* but had no effect on binding of anti-Lyn mAb to its target (Fig. S1C). Furthermore, material immunoprecipitated with RoPLS or MaPLS* reacted with MaPLS and MaPLS* or RoPLS, respectively (Fig. S1D). All of these data indicated that the newly prepared mAb MaPLS and rabbit pAb RoPLS could specifically detect PLSCR1.

Using the newly prepared antibodies, we confirmed previous data indicating that antigen-mediated FceRI triggering leads to enhanced tyrosine phosphorylation of PLSCR1 (22) and extended them by showing that this phosphorylation is transient, with a peak at about 10 min after triggering (Fig. 1A, left). To decide whether the observed phosphorylation is related to antigen-induced FceRI aggregation (56) and/or internalization of FceRI (53, 57), the cells were activated by αFceRI, which triggers signaling events comparable with those induced by extensive aggregation of FceRI-IgE complexes by multivalent antigen but lacking FceRI clustering or rapid clearance of FceRI from the plasma membrane (9, 58). As shown in Fig. 1A (right), cells activated by αFceRI also exhibited transient PLSCR1 tyrosine phosphorylation, indicating that extensive
clustering and/or internalization of FcεRI is not necessary for PLSCR1 tyrosine phosphorylation.

Secretory response and internalization of FcεRI are negatively regulated by filamentous actin (59–61). To determine whether FcεRI-induced PLSCR1 tyrosine phosphorylation and PS externalization are dependent on intact actin filaments, we analyzed the properties of RBL cells stimulated with αFcεRI in the presence or absence of 0.5 μM latrunculin B, an inhibitor of actin polymerization (59, 62). In accordance with our previous data (9), stimulation of RBL cells with αFcεRI in the presence of latrunculin B significantly enhanced PS externalization, determined by annexin V–FITC binding (Fig. 1, B and C), and degranulation, measured by the release of β-glucuronidase into supernatant (Fig. 1C). However, tyrosine phosphorylation of PLSCR1 did not significantly increase in latrunculin-treated cells (Fig. 1, D and E). Treatment of the cells with 0.5 μM latrunculin B in the absence of FcεRI triggering had no effect on PLSCR1 tyrosine phosphorylation (Fig. 1D), PS externalization, and β-glucuronidase release (data not shown). The combined data suggest that enhanced PS externalization and degranulation are independent of enhanced tyrosine phosphorylation of PLSCR1.

Externalized PS Does Not Always Co-localize with PLSCR1 in FcεRI-stimulated Cells—FcεRI-induced activation of RBL cells leads to rapid and transient increase in [Ca^{2+}],. It has been suggested that this results in enhanced binding of calcium to PLSCR1 and its conformational changes, leading to PLSCR1 clustering on the plasma membrane and activation of phospholipid scrambling (20). To test this hypothesis, we analyzed the distribution of externalized PS and PLSCR1 in FcεRI-activated cells by confocal microscopy. In order to induce a slower but more sustained increase in [Ca^{2+}],, (58), we activated RBL cells by αFcεRI and stained them with annexin V–FITC, followed by fixation, permeabilization, and staining with PLSCR1-specific pAb. Data presented in Fig. S2A show that recombinant GST-PLSCR1 inhibited the binding of RoPLS but not anti-LAT pAb, proving that the antibody is specific for PLSCR1 in permeabilized cells. Furthermore, binding of αFcεRI alone did not give any signal with the secondary DαR IgG-Cy3 conjugate used for detection of RoPLS binding (Fig. S2B). In nonactivated cells, no externalization of PS was detected by annexin V–FITC conjugate (Fig. 2, C–/Annexin V). In the same cells, PLSCR1 was dispersed over the whole plasma membrane, as detected by RoPLS (Fig. 2, C–/PLSCR1). When the cells were activated by αFcεRI, externalized PS was detected by annexin V–FITC in discernible clusters, whereas PLSCR1 remained mostly randomly distributed on the plasma membrane (Fig. 2, αFcεRI). At higher magnification, PLSCR1 was distributed in numerous patches over the whole plasma membrane in both FcεRI-activated cells (Fig. 2, αFcεRI/Zoom/PLSCR1) and nonactivated cells (not shown).

Externalized PS was occasionally associated with these patches but was also found in regions with no preferential localization of PLSCR1 (green spots in Fig. 2, αFcεRI/Zoom/Overlay). These data indicate that FcεRI triggering does not induce aggregation of PLSCR1 and that patches of externalized PS do not always co-localize with patches of PLSCR1.

Aggregation of FcεRI Does Not Lead to Changes in the Fine Topography of PLSCR1—To attain higher resolution in determination of PLSCR1 topography in the plasma membrane, we used electron microscopy combined with immunogold labeling on isolated plasma membrane sheets (51, 63). In pilot experiments, specificity of PLSCR1 visualization was examined. Isolated plasma membrane sheets were labeled with RoPLS or anti-NTAL pAb in the absence or presence of recombinant GST-PLSCR1, followed by application of GoR IgG-5-nm gold conjugate. As shown in Fig. 3A, the recombinant protein completely blocked the binding of RoPLS, whereas it had insignificant effect on anti-NTAL pAb binding, confirming thus the suitability of the reagent for electron microscopy studies. Next we evaluated the topography of PLSCR1 in cells activated by FcεRI dimerization and found no significant difference in PLSCR1 cluster size (Fig. 3B, left) and particle density (Fig. 3B, right) between controls and αFcεRI-activated cells. These findings supported previous results obtained with confocal microscopy showing that cell activation through FcεRI dimerization does not affect the distribution of PLSCR1 in the plasma membrane.

We also analyzed the topography of PLSCR1 in cells with extensively aggregated FcεRI by multimeric antigen-IgE complexes. In accordance with previous data (58, 63), the FcεRI β chain was found dispersed individually or in small clusters in nonactivated cells (Fig. 3C), whereas in multimeric antigen (DNP-BSA)-activated cells, it was found in large clusters associated with osmiophilic regions (Fig. 3D). Importantly, extensive FcεRI aggregation induced no obvious changes in the topography of PLSCR1. Furthermore, statistical evaluation showed no increased co-localization of PLSCR1 and FcεRI in

**FIGURE 2.** Topography of PLSCR1 and externalized PS in nonactivated and FcεRI-activated cells as determined by confocal microscopy. The cells were nonactivated (C) or activated for 20 min with αFcεRI. Externalized PS was detected by annexin V–FITC (Annexin V). PLSCR1 was visualized in fixed and permeabilized cells using RoPLS, followed by DαR IgG-Cy3 conjugate. The merge of both labels is shown on the right (Overlay). Bar in αFcεRI/Overlay, 10 μm. Zoom images of the indicated regions of αFcεRI-activated cells are shown at the bottom. Representative experiments from at least three performed are shown.
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**FIGURE 3.** Topography of PLSCR1 in plasma membrane of FcεRI-activated cells as detected by electron microscopy. A, specificity of the reagents used. Fixed plasma membrane sheets were labeled on the cytoplasmic side with pAb specific for NTAL (black bars) or PLSCR1 (white bars) in the absence (rec-PLSCR1–) or presence (recPLSCR1+) of recombinant GST-PLSCR1. Bound antibodies were visualized with GxR IgG-5-nm gold particle conjugate, and particle density was determined and normalized to the rec-PLSCR1– samples. B, topography of PLSCR1 in cells activated by FcεRI-dimerizing αFcεRI. Cells were stimulated for 20 min with vehicle alone (C) or αFcεRI. PLSCR1 was visualized as described in A. Cluster size and particle density were determined. C–H, topography of PLSCR1 and FcεRI in cells activated by extensive aggregation of FcεRI. Cells were sensitized with DNP-specific IgE and then stimulated with vehicle alone (control) or with DNP-BSA. Plasma membrane sheets from control (C) or DNP-BSA (2 min)-stimulated cells (D) were isolated, fixed, and simultaneously labeled on the cytoplasmic face with RuPRLS (PLSCR1) and FcεRI-β subunit-specific mAb (FcεRI-β). Bound RuPRLS and FcεRI-β were visualized with GxR IgG-5-nm gold particle conjugate (arrowheads) and GxM IgG-10-nm gold particle conjugate (arrows), respectively. BRA of co-localization of PLSCR1 and FcεRI-β subunit in control (E) and activated (F) cells was determined. G and H, the cells were sensitized with DNP-specific IgE and then stimulated with DNP-BSA for the time intervals indicated. Visualization of PLSCR1 and FcεRI-β subunit on isolated plasma membrane sheets was carried out as described above. Cluster size (G) and particle density (H) of PLSCR1 and FcεRI-β subunit was evaluated for each time interval after triggering (0–40 min). Statistical differences, calculated between recPLSCR1– and recPLSCR1+ (A) or nonactivated and activated cells (G and H), are indicated (*, p < 0.05; **, p < 0.01). In C and D, representative experiments from at least three performed are shown.
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Induced PS externalization detected by annexin V-FITC but no changes in PLSCR1 distribution (Fig. 4D, αThy-1; compare with Fig. 2, C–/PLSCR1). At higher magnification, there was no preferential localization of PLSCR1 at regions of patches of externalized PS (Fig. 4D, αThy-1/Zoom). Finally, electron microscopy studies on isolated membrane sheets indicated that cluster size (Fig. 4E, left) and density of PLSCR1 (Fig. 4E, right) were not significantly different between control and αThy-1-treated cells. Collectively, these data indicate that PS externalization induced by dimerized Thy-1 does not require enhanced tyrosine phosphorylation of PLSCR1 and/or its topographical changes.

Thy-1 Multimerization Induces Enhanced Tyrosine Phosphorylation of PLSCR1—In contrast to Thy-1 dimerization, extensive aggregation of Thy-1 leads to enhanced [Ca^{2+}], and degranulation (64, 65). To find out whether this also induces changes in PLSCR1 tyrosine phosphorylation, we aggregated Thy-1 with biotin-labeled αThy-1-streptavidin complexes. Such treatment resulted in enhanced tyrosine phosphorylation of several cellular proteins peaking at 1 min after triggering for most of the proteins (Fig. 5A, top). Immunoprecipitation analyses showed that PLSCR1 was among the tyrosine-phosphorylated proteins (Fig. 5A, bottom) with a phosphorylation peak observed at 10 min after triggering. Although aggregated Thy-1 was easily detectable on isolated membrane sheets, its enhanced co-localization with PLSCR1 was detectable neither in resting nor activated cells (Fig. 5, B–E). These data indicated that increased tyrosine phosphorylation of PLSCR1 is not accompanied by enhanced co-localization with aggregated Thy-1.

Calcium Ionophore A23187 Induces Changes in the Topography of PLSCR1 and Apoptotic-like PS Externalization—Stimulation of the cells with calcium ionophores leads to a rapid elevation of [Ca^{2+}], followed by a sequence of Ca^{2+}-dependent signaling events, including externalization of PS, degranulation, and apoptosis (6, 66, 67). Furthermore, exposure of IgE-sensitized RBL cells for 30 min with calcium ionophore ionomycin has been reported to enhance tyrosine phosphorylation of PLSCR1 (31). To determine kinetics of calcium ionophore-induced phosphorylation, we studied tyrosine-phosphorylated proteins at various time intervals after A23187 triggering. Data in Fig. 6A show that the rate of phosphorylation of cellular proteins, including PLSCR1, is slower in A23187-activated cells than in FceRI-activated cells (compare with Fig. 1A). As to the extent of PS externalization, it was substantially higher in cells stimulated by A23187 and labeled in its presence (Iono+/-) than by FceRI dimerization (Fig. 6B,
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FIGURE 5. Properties of PLSCR1 in cells activated through extensively aggregated Thy-1. A, tyrosine phosphorylation of PLSCR1 after Thy-1 aggregation. Cells were sensitized or not (C−) for 30 min on ice with biotinylated αThy-1. The cells were washed and activated at 37 °C with streptavidin (αThy-1/Streptavd) for the time intervals indicated. Cell lysates were analyzed by immunoblotting (IB) for tyrosine-phosphorylated proteins with αPY, PLSCR1 was immunoprecipitated (IP) from the same cell lysates with R6PLS and analyzed by immunoblotting with αPY. The amount of immunoprecipitated PLSCR1 was determined by immunoblotting with MoPLS. Positions of PLSCR1, tyrosine-phosphorylated PLSCR1, and molecular weight standards are shown on the right. B–E, topography of PLSCR1 and Thy-1 as determined by electron microscopy on plasma membrane sheets prepared from nonactivated cells (B and D) or cells activated by extensive aggregation of Thy-1 (C and F). B, cells were fixed, and Thy-1 was labeled with αThy-1 and visualized with Gm IgG-10-nm gold particle conjugate (arrow). After this step, membrane sheets were isolated, and PLSCR1 (arrowhead) was labeled on the cytoplasmic face as described in the legend to Fig. 3A. C, nonfixed cells were exposed to αThy-1, and Thy-1-αThy-1 immunocomplexes were aggregated by Gm IgG-10-nm gold particle conjugate (arrow). The membrane sheets were then isolated and fixed, and PLSCR1 (arrowhead) was determined as described in B. D–E, BRA of co-localization of PLSCR1 and Thy-1 in nonactivated cell (D) or cells with extensively aggregated Thy-1 (E) were evaluated. In A–C, representative experiments from at least three performed are shown.

left and middle). However, the extent of PS externalization did not correlate with degranulation, which was substantially lower in A23187-triggered cells (Fig. 6B, right). The stability of A23187-induced PS externalization was tested by removing the drug from the cells during staining for PS (Iono+/−). The amount of bound annexin V-FITC decreased to levels comparable with those observed in FceRI-activated cells (Fig. 6C, left and middle). However, secretory response remained the same since NTAL, another protein with transmembrane domain (68), showed no significant decrease in immunolabeling on the same plasma membrane sheets (Fig. 6F). These findings confirm the confocal microscopy data and suggest that PLSCR1 is specifically released from plasma membrane in both A23187-activated and apoptotic cells.

Stimulation with Pervanadate Leads to Extracellular Calcium-independent Tyrosine Phosphorylation of PLSCR1 and
FIGURE 6. Changes in properties of PLSCR1 and PS externalization in calcium ionophore A23187-stimulated cells. A, tyrosine phosphorylation of PLSCR1. Cells were activated by A23187 at 37°C for the time intervals indicated. Cell lysates were prepared and analyzed by immunoblotting (IB) for tyrosine-phosphorylated proteins with αPY. Tyrosine phosphorylation of PLSCR1 was analyzed in RoPLS immunoprecipitates (IP) from the same cells and analyzed by immunoblotting with αPY. The amount of immunoprecipitated PLSCR1 was determined by immunoblotting with MαPLS. Positions of PLSCR1, tyrosine-phosphorylated PLSCR1, and molecular mass standards in kDa are shown on the right. B, PS externalization and degranulation. Cells were exposed for 15 min to A23187 (Iono+/−, thick line), αFcεRI (dashed and dotted line), or vehicle alone (C, thin line) and then labeled with annexin V-FITC for 15 min in the absence (αFcεRI or C) or presence (Iono+/−) of A23187. The cells were immediately analyzed by flow cytometry (left), and annexin V binding was normalized to maximal response obtained in each experiment (middle). In separate experiments, cells were treated for 30 min with A23187 or αFcεRI, and degranulation (β-gluc. release) was determined as described in the legend to Fig. 1C. C, cells were treated and analyzed as in B except that A23187 was absent during labeling with annexin V-FITC (Iono−/−, thick line), and degranulation was determined 15 min after triggering. D, topography of externalized PS and PLSCR1 in plasma membrane of A23187-stimulated or apoptotic cells as determined by confocal microscopy. A23187-stimulated and annexin V-FITC-labeled cells, as in B (Iono+/−, thick line), and degranulation was determined 15 min after triggering. D, topography of externalized PS and PLSCR1 in plasma membrane of A23187-stimulated or apoptotic cells as determined by confocal microscopy. A23187-stimulated and annexin V-FITC-labeled cells, as in B (Iono+/−, thick line), and degranulation was determined 15 min after triggering. E and F, topography of PLSCR1 on plasma membrane as determined by electron microscopy. Plasma membrane sheets from nonactivated (C−), A23187-stimulated (Iono+/−, 15 min), or apoptotic (Apo) cells were prepared and labeled as described in the legend to Fig. 3A with the exception that fixation of plasma membrane sheets was performed in the presence (Iono+/−) or absence (C−, Apo) of A23187. Cluster size of PLSCR1 (E) and particle density of PLSCR1 and control transmembrane adaptor protein NTAL (F) were normalized to nonactivated cells. Statistical differences between αFcεRI and Iono+/− (B), αFcεRI and Iono−/− (C), and controls (C−) and experimental groups (F) are shown (*, p < 0.05; **, p < 0.01). In A and D, representative experiments from at least three performed are shown.
Phospholipid Scramblase 1 and Phosphatidylserine Exposure

**FIGURE 7. PLSCR1 tyrosine phosphorylation, degranulation, and PS externalization in pervanadate-stimulated cells.** A, cells were activated by pervanadate (Perv) for the time intervals indicated in calcium-free BSS/BSA supplemented with 1.8 mM CaCl₂ (Ca⁺⁺, +) or with 5 mM EGTA (Ca⁺⁺, –). Whole cell lysates were prepared and analyzed by immunoblotting (IB) with αPY. The amount of PLSCR1 was determined by immunoblotting on separate membranes with MuPLS. Positions of PLSCR1 and molecular weight standards are shown on the right. B, PLSCR1 was immunoprecipitated (IP) with RoPLS from cell lysates, prepared as in A, and analyzed by immunoblotting with αPY and MuPLS. Negative control (−) represents material from the lysate of stimulated cells (30 min) bound to the beads without RoPLS. Positions of PLSCR1 and tyrosine-phosphorylated PLSCR1 are shown on the right. C, PS externalization and cell degranulation in pervanadate-stimulated cells. Cells were activated for 15 min (left) or for the time intervals indicated (middle and right) with pervanadate (dotted line), αFcɛRI (thick line), or vehicle alone (thin line); PS externalization (Annexin V) and degranulation (β-gluc. release) was determined as described in Fig. 1, B and C, and normalized to maximal response attained in each experiment. D, cells were stimulated for 15 min with pervanadate or vehicle alone, and topography of PLSCR1 on plasma membrane sheets was determined by electron microscopy as described in the legend to Fig. 3B. PLSCR1 cluster size and particle density were normalized to nonstimulated cells. In C, statistical differences between maximal and other values among αFcɛRI- and pervanadate-activated cells are indicated (***, p < 0.001). In A–C, representative experiments from at least three performed are shown.

Degranulation without PS Externalization—Previous results suggested that tyrosine phosphorylation of PLSCR1 in calcium ionophore-activated cells is regulated downstream of the increase of [Ca⁺⁺]. (31). More information on the role of calcium in tyrosine phosphorylation of PLSCR1 was obtained in RBL cells stimulated by pervanadate in the absence or presence of extracellular calcium (Ca⁺⁺). Pervanadate is a compound triggering tyrosine phosphorylation of cellular proteins through inhibition of protein-tyrosine phosphatases (69). Depending on the concentration and time of exposure, it induces either cell activation or apoptosis (70, 71). For our purposes, we used pervanadate at a concentration that had previously been found to induce degranulation comparable with that induced by FcɛRI triggering (72). Using total cell lysates, we found rapid tyrosine phosphorylation of many cellular proteins in pervanadate-stimulated cells in the presence or absence of extracellular calcium (Fig. 7A, top). Immunoblotting on parallel membranes with MoPLS showed enhanced molecular weight of a fraction of PLSCR1 in pervanadate-activated cell (Fig. 7A, bottom), apparently reflecting its enhanced tyrosine phosphorylation. Immunoprecipitated PLSCR1 also showed strong tyrosine phosphorylation in the presence or absence of extracellular calcium (Fig. 7B). These data indicate that under these conditions, tyrosine phosphorylation of PLSCR1 is not dependent on extracellular calcium.

Next we examined whether pervanadate-induced degranulation was accompanied by PS externalization as was observed in FcɛRI-activated cells. To that aim, RBL cells were exposed to pervanadate or αFcɛRI (positive control) and concomitantly tested for PS externalization (Fig. 7C, left and middle) and degranulation (Fig. 7C, right). Surprisingly, exposure of the cells to pervanadate resulted in no significant PS externalization, although degranulation occurred as expected. There was no increase in the number of dead cells, determined by propidium iodide staining (not shown), arguing against the possibility that β-glucuronidase was released from cells killed by pervanadate. This finding demonstrates that, under certain conditions, externalization of PS cannot be considered as a reliable marker of cell degranulation, despite the fact that there is a correlation between PS externalization and degranulation (6–8, 73).

We also examined the topography of PLSCR1 in pervanadate-stimulated cells. Our observations from confocal microscopy (data not shown) indicated that pervanadate induced no redistribution of PLSCR1. Electron microscopy analyses of PLSCR1 topography in plasma membrane sheets isolated from controls or pervanadate-activated cells showed no significant changes in cluster size (Fig. 7D, left) or particle density (Fig. 7D, right). These data suggest that even hyperphosphorylation of PLSCR1 need not lead to its topographic changes.
Phospholipid Scramblase 1 and Phosphatidylserine Exposure

Tyrosine-phosphorylated PLSCR1 is localized mostly in DIM—PLSCR1, like other palmitoylated transmembrane proteins, has been reported to be enriched in low density membranes (23, 74). However, it remains questionable whether tyrosine-phosphorylated PLSCR1 in RBL cells is also associated with DIM and what changes in this association take place in the course of FceRI activation. When the cells were solubilized in a lysis buffer with 0.5% Brij 58 and then the whole cell lysates were fractionated by sucrose density gradient ultracentrifugation, a small amount (~10%) of PLSCR1 in DIM (fractions 1–3) was detectable by immunoblotting (Fig. 8A, top and bottom, left). This was much less than the Lyn kinase (~80%), which is considered to be a typical DIM-associated protein (38). Furthermore, when the cells were solubilized in lysis buffer with 1% Triton X-100, no PLSCR1 was recovered in DIM, whereas most of the Lyn still remained in DIM (not shown). In cells activated by IgE-TNP-BSA complexes, there was a ~60% decrease in the amount of PLSCR1 in DIM but only ~20% decrease in Lyn (Fig. 8A, top and bottom, right). Although only a small fraction of PLSCR1 was associated with DIM (see above), most of the tyrosine-phosphorylated PLSCR1 was located in DIM when PLSCR1 was immunoprecipitated from pooled low or high density fractions and analyzed by immunoblotting, and this amount further increased after FceRI triggering (Fig. 8B). The increase in phosphorylated PLSCR1 in high density fractions was even more striking. Activation of the cells with pervanadate resulted in an enhanced amount of phosphorylated PLSCR1 in both low and high density fractions (Fig. 8C). These findings support the concept that tyrosine phosphorylation of PLSCR1 is dependent on equilibrium between kinases and phosphatases.

DISCUSSION

We have previously shown that dimerization of Thy-1 or some other GPI-anchored proteins induces nonapoptotic PS externalization in several cell types, including mast cells (9). It was an unexpected finding, because GPI-anchored proteins have no transmembrane and cytoplasmic domains, and therefore their effect must be mediated through their binding to other proteins possessing such domains or through poorly characterized interactions within lipid rafts where GPI-anchored and other signaling molecules are presumably localized (75, 76). Furthermore, when the cells were activated through dimerized GPI-anchored proteins, it was possible to get PS externalization even in the absence of degranulation (9). These data suggested that rational tests on cells activated under various conditions and by different activation pathways may possibly provide new information on the relationships between, on the one hand, PS externalization, enhanced [Ca^{2+}], and exocytic responses and, on the other, tyrosine phosphorylation and topography of PLSCR1, an enzyme that is phosphorylated in activated cells and thought to take part in phospholipid scrambling. For our experiments, we selected RBL cells, which are...
widely used as a model object for studies on surface receptor-mediated exocytosis and nonapoptotic PS externalization. These cells can be activated by various means, including dimerization of FcRI or Thy-1 by corresponding mAb, extensive aggregation of FcRI through IgE-multimeric antigen complexes, or aggregation of Thy-1 by biotinylated anti-Thy-1 mAb and streptavidin, calcium ionophore A23187, or phosphatases inhibitor, pervanadate. Data presented in this study and summarized in Table 1 show that many of the signaling events can be separately approached by employing various activation methods.

First, dimerization of Thy-1 could induce PS externalization in the absence of PLSCR1 tyrosine phosphorylation. Interestingly, the extent of PS externalization in cells activated in this way was comparable with that induced by extensively aggregated FcRI or Thy-1. This fact, together with our previous finding that the proliferation rate of cells cultured in the presence of anti-Thy-1 mAb is not impaired (9), indicate that tyrosine phosphorylation of PLSCR1 is not essential for nonapoptotic PS externalization. The conclusion is further corroborated by the observation that latrunculin B, an inhibitor of actin polymerization, known to enhance secretory responses (see also Refs. 59 and 61), also increased PS externalization without any significant effect on tyrosine phosphorylation of PLSCR1. Thus, the inhibitory effect of actin on both PS externalization and secretory response is not mediated through inhibition of PLSCR1 tyrosine phosphorylation.

Second, the above conclusion is consistent with our finding that activation by calcium ionophore A23187 induced delayed tyrosine phosphorylation of PLSCR1 but strong PS externalization. At the same time, degranulation in A23187-triggered cells was lower than in FcRI-activated cells; it strengthens the conclusion that PS externalization and secretory responses are independent phenomena.

Third, when activated with pervanadate, the cells exhibited clear secretory response and strong PLSCR1 tyrosine phosphorylation but no PS externalization. These observations provide the first evidence that degranulation can occur even in the absence of PS externalization and support the concept that phosphorylation of PLSCR1, PS externalization, and degranulation are disparate events. Comparable tyrosine phosphorylation of PLSCR1 was observed in cells activated by pervanadate in Ca\(^{2+}\)-supplemented medium or Ca\(^{2+}\)-free medium supplemented with EGTA. This was an unexpected finding, because a previous study showed that FcRI-mediated phosphorylation of PLSCR1 required Ca\(^{2+}\); in its absence, phosphorylation was markedly reduced (31). This difference could be related to the existence of preassembled complexes of PLSCR1, kinases, and phosphatases (see below) as well as to the fact that Ca\(^{2+}\) is required for uncoupling the phosphatases from the complexes under conditions of physiological triggering, such as through FcRI.

Fourth, it had been proposed that enhanced binding of metal ions to PLSCR1 caused its conformational changes, followed by PLSCR1 clustering and activation of phospholipid scrambling (20). It was therefore of interest to find out whether cell activation and PS externalization are indeed accompanied by changes in topography of PLSCR1. Confocal microscopy did not reveal any enhanced movement of PLSCR1 into regions of externalized PS. Although clusters of externalized PS and PLSCR1 were occasionally observed, such clustering was not found in other cases, suggesting that either clustering events are highly dynamic or clustering of PLSCR1 is not essential for PS externalization. Fine topography of PLSCR1 was studied with electron microscopy on isolated and paraformaldehyde-fixed plasma membrane sheets in combination with immunogold labeling, and again, no evidence of any clustering or other topographic changes of the protein was detected in cells activated by various means, with the exception of calcium ionophore A23187. This drug did not change the extent of PLSCR1 clustering in the plasma membrane but decreased the protein density in the membrane. This could be due to the masking of target epitopes or release of PLSCR1 from the plasma membrane. The finding of an enhanced amount of PLSCR1 in the cytoplasm of A23187-activated cells, detected by confocal microscopy, supports the release hypothesis. By studying the fine topography of NTAL adaptor protein on the plasma membrane sheets isolated from control and A23187-activated cells, we found that NTAL, unlike PLSCR1, did not exhibit any changes; this excluded the possibility of generally enhanced internalization of plasma membrane proteins by the drug. The observed decrease of PLSCR1 in the plasma membrane, coupled with strong expression of surface PS in A23187-activated cells, supports again the conclusion that PS externalization is probably not dependent on enhanced PLSCR1 clustering. Our data also suggested that PS externalization in A23187-treated cells is in part directly induced by an interaction of calcium ionophore with the plasma membrane and not just by increased

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**TABLE 1**

Summary data on PLSCR1 tyrosine phosphorylation, PS externalization, PLSCR1 topography, Ca\(^{2+}\) response, and degranulation in cells activated by various stimuli

| Parameter                        | FcRI Dimerized | Extensively aggregated | Thy-1 Dimerized | Extensively aggregated | A23187 | Pervanadate |
|----------------------------------|----------------|------------------------|-----------------|-----------------------|--------|-------------|
| PLSCR1 tyrosine phosphorylation  | +              | +                      | −               | +                     | +      | +           |
| PS externalization\(^a\)         | +              | +                      | −               | +                     | +      | +           |
| Changes in PLSCR1 topography     | +              | +                      | −               | +                     | +      | +           |
| Ca\(^{2+}\) response\(^b\)      | +              | +                      | −               | +                     | +      | +           |
| Degranulation\(^c\)             | +              | +                      | −               | +                     | +      | +           |

\(^a\) Ref. 6, data presented in this study, and our unpublished data.

\(^b\) Refs. 44 and 58 and our unpublished data.

\(^c\) Refs. 44 and 85, data presented in this study, and our unpublished data.
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[Ca\(^{2+}\)]\(_i\). This stems from our prior data showing an extensive increase in fluorescence of merocyanine 540-labeled cells after triggering of RBL cells with ionomycin but not FceRI (9) as well as with the finding that thapsigargin, an inhibitor of sarco-plasmic/endoplasmic-reticulum Ca\(^{2+}\)/ATPases did not induce rapid phospholipid scrambling, although it was capable of initiating enhancement of [Ca\(^{2+}\)]\(_i\) (37, 77). It should be noted that A23187-induced changes in PS externalization and PLSCR1 internalization were similar to those observed in apoptotic cells. However, only A23187-triggered cells showed partial reversion of the process, adding another piece of evidence that the continuous presence of A23187 is required for its effect.

Finally, no simple relationship was found between the extent of PS externalization and secretory response. Strong degranulation was observed in FceRI-activated cells, whereas no degranulation took place in cells activated via dimerized Thy-1. However, both of these activation pathways resulted in comparable exposure of PS. On the other hand, A23187-activated cells showed extremely high PS externalization but weak degranulation when compared with other activation stimuli.

Using a biochemical approach, a distinct fraction of PLSCR1 was found in DIM if weak detergent (0.5% Brij 58) was employed. The more potent nonionic detergent, 1% Triton X-100, solubilized PLSCR1 completely. Under the same solubilization conditions, 80 and 60% of Lyn was recovered in 0.5% Brij 58- and 1% Triton X-100-resistant DIM, respectively, indicating that PLSCR1 and Lyn, although both are supposedly associated with lipid rafts, differ in detergent solubility. Because palmitoylation is often responsible for localization of proteins in DIM (78), it is possible that only a small fraction of membrane-associated PLSCR1 is palmitoylated.

Interestingly, two forms of PLSCR1 (36 and 38 kDa) were found in both DIM and high density fractions. It has been suggested that retarded migration of a fraction of PLSCR1 in PAGE could reflect multiple palmitoylation of PLSCR1 on clusters of cysteines that are present in the molecule (22). Palmitoylation of cysteines is often responsible for protein localization to DIM (75). However, because the two forms of PLSCR1 were present in similar ratios in both low and high density fraction (Fig. 8A), it is unlikely that multiple palmitoylation is responsible for retarded migration of the upper band. It is still possible that some other post-transcriptional modifications account for the observed size heterogeneity of PLSCR1. These poorly defined modifications could be responsible for different ratios between the 36 and 38 kDa bands observed in some experiments.

After FceRI-mediated activation, the amount of PLSCR1 in DIM was even reduced. In this respect, PLSCR1 differs from some other proteins with a transmembrane domain that exhibited enhanced association with DIM in activated cells (79). The finding that a clear decrease in amount of PLSCR1 in DIM observed in FceRI-activated cells is not accompanied by detectable changes in the distribution of PLSCR1 in plasma membrane sheets suggests that changes in biochemical parameters do not necessarily reflect topographical transpositions. This conclusion is supported by previous studies indicating that molecules assumed to be localized within and outside the lipid rafts exhibited similar topographic distribution on the plasma membrane and vice versa (52, 58, 80). Furthermore, these data support previous studies indicating that signaling domains formed on the plasma membrane could be very small, perhaps confined to individual protein molecules (58, 81).

Although the amount of PLSCR1 in DIM isolated from resting cells was relatively low, most of the tyrosine-phosphorylated PLSCR1 was localized in DIM. Soluble PLSCR1, recovered in high density fractions, showed only weak tyrosine phosphorylation, suggesting that palmitoylation and presumable localization in lipid rafts protect PLSCR1 from dephosphorylation. After FceRI triggering, the amount of tyrosine–phosphorylated PLSCR1 increased not only in DIM but mainly in the fraction of soluble proteins. Treatment with pervanadate, which inhibits enzymatic activity of protein-tyrosine phosphatases and thereby increases the intracellular equilibrium between phosphorylation and dephosphorylation rates (82), resulted in an enhanced amount of tyrosine-phosphorylated PLSCR1 in both low density (DIM-containing) and high density fractions, suggesting that a week phosphorylation of PLSCR1 in high density fractions in resting cells is attributable to enzymatic activity of protein-tyrosine phosphatases. When this activity is inhibited, even in the absence of any detectable movement of the PLSCR1 into new membrane domains, kinases mediate the phosphorylation of PLSCR1. The observed tyrosine phosphorylation of PLSCR1 in pervanadate-activated cells supports the model that signaling assemblies containing protein-tyrosine phosphatases and kinases are preassociated before triggering and that in resting cells the kinase activity is counterbalanced by active phosphatases (83). Although human PLSCR1 has been shown to bind to c-Abl tyrosine kinase through its Src homology 3 domain and is phosphorylated most likely at Tyr\(^{\text{i-69}}\) and Tyr\(^{\text{i-74}}\), the identities of kinases that phosphorylate PLSCR1 in mast cells remain to be determined.

In conclusion, our results indicate for the first time that changes in topography of PLSCR1 and its tyrosine phosphorylation, PS externalization, and secretory responses are independent phenomena that could be separated by employing various activation methods. Based on data in this study, we propose that enhanced tyrosine phosphorylation of PLSCR1 or its topographic changes in the plasma membrane are not required for PS externalization and degranulation. These data are in line with studies documenting that genetic removal of PLSCR1 does not lead to a decrease in phospholipid scrambling (27) and support the notion that PLSCR1 has other roles in cell signaling events affected through its interactions with various signaling molecules, such as epidermal growth factor receptor (23), β-secretase (30), and/or proteinase 3 (84).

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