Plasma Membrane Phospholipid Scramblase 1 Is Enriched in Lipid Rafts and Interacts with the Epidermal Growth Factor Receptor†

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Received January 31, 2002; Revised Manuscript Received April 8, 2002

ABSTRACT: We have identified physical and functional interactions between the epidermal growth factor (EGF) receptor and phospholipid scramblase 1 (PLSCR1), an endofacial plasma membrane protein proposed to affect phospholipid organization. PLSCR1, a palmitoylated protein, was found to partition with the EGF receptor in membrane lipid rafts. Cell stimulation with EGF transiently elevated Tyr-phosphorylation of PLSCR1, peaking at 5 min. Although PLSCR1 is a known substrate of c-Abl [Sun, J., et al. (2001) *J. Biol. Chem. 276*, 28984−28990], the Abl inhibitor STI571 did not substantially affect its EGF-dependent phosphorylation, suggesting PLSCR1 is a substrate of the EGF receptor kinase, or another EGF-activated kinase. Coinciding with phosphorylation, there was a transient increase in physical association of PLSCR1 with both the EGF receptor and the adapter protein Shc, as determined by immunoprecipitation and Western blotting. Confocal immunofluorescence analysis revealed that EGF initiates rapid internalization of both the EGF receptor and PLSCR1, with trafficking into both distinct and common endosomal pools. These data also suggested that whereas the EGF receptor is ultimately degraded, much of the endocytosed PLSCR1 is recycled to the cell surface within 3 h after EGF treatment. Consistent with this interpretation, Western blotting revealed neither ubiquitination nor proteolysis of PLSCR1 under these conditions, whereas the ubiquitination and degradation of the EGF receptor were readily confirmed. Finally, stimulation with EGF was also found to markedly increase the total cellular expression of PLSCR1, suggesting that in addition to its initial interactions with activated EGF receptor, PLSCR1 may also contribute to posttranscriptional effector pathway(s) mediating the cellular response to EGF.

Phospholipid scramblases (PLSCR)[1] are a family of calcium-binding proteins that have been implicated in the transbilayer movement of plasma membrane phospholipids (*1*−*3*). Four human *PLSCR* genes (*PLSCR1*−*4*), the mouse orthologous genes (*muPLSCR1*−*4*) (*3*), and a rat *PLSCR1* orthologue (*4*) have now been identified. In addition, putative orthologues are present in *Drosophila*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. Thus, *PLSCR* genes appear to have been well conserved through evolution.

Comparison of the deduced amino acid sequences of the PLSCR proteins suggests that they are probably type-2b membrane proteins with a single predicted transmembrane helix near the C-terminus (*2*, *3*). A conserved Ca^{2+} binding site (Asp^{273}−Asp^{284}) has been identified in PLSCR1, immediately proximal to the transmembrane domain (*2*, *3*). The N-terminal segment of PLSCR1 is Pro-rich and contains multiple predicted binding sites for SH3- and WW-domain containing proteins.

Upon binding Ca^{2+}, PLSCR1 undergoes a conformational change and mediates accelerated transbilayer movement of phospholipids in proteoliposomes (*1*, *5*). The ability of PLSCR1 to induce the redistribution of phospholipids between membrane leaflets suggests that it may play a role in the activation of the complement and coagulation systems of blood plasma. In addition, it may be involved in the externalization of phosphatidylserine that is important in the recognition and phagocytic clearance of injured, aged, or apoptotic cells (*6*−*9*).

Although PLSCR1 is likely to play a role in the redistribution of plasma membrane phospholipids, the actual cellular function of PLSCR1 and related members of this gene family has not been definitively demonstrated. The putative role of PLSCR1 in mediating accelerated transbilayer migration of plasma membrane phospholipids is based on its ability to mediate this function in reconstituted membrane systems (*2*). In addition, there is a correlation between endogenous PLSCR1 expression in cells and the propensity of those cells to expose phosphatidylserine in response to an influx of Ca^{2+}

† Supported by National Institutes of Health Grants HL36946 (to P.J.S.), HL63819 (to P.J.S.), HL61200 (to T.W.), and GM56329 (to L.J.P.), by the Leukemia & Lymphoma Society Fellow Grant 5071-02 (to J.S.), and by the Stein Endowment Fund. This is manuscript #14658-MEM from The Scripps Research Institute.

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[1] Abbreviations: PLSCR, phospholipid scramblase; SH3, Src homology 3; SH2, Src homology 2; PTB, phosphotyrosine-binding domain; EGF, epidermal growth factor; mAb, monoclonal antibody; pTyr, phosphotyrosine; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; WB, Western blotting; MES, 2-(*N*-morpholino)ethanesulfonic acid.

10.1021/bi025610l CCC: $22.00
Published on Web 05/14/2002

(*10*). However, elevation of PLSCR1 expression by either plasmid transfection or through cytokine-induced transcription failed to increase surface exposure of plasma membrane phosphatidylserine in response to either Ca^{2+} ionophore or to apoptotic stimuli (*11*, *12*). Thus, the role of PLSCR1 in phosphatidylserine externalization remains unclear.

In yeast, the apparent PLSCR orthologue *YJR100C* is a stress-induced gene (*13*). Similarly, human PLSCR1 is highly induced by the interferons (*11*, *14*). These observations suggest a possible role for PLSCR in immune/stress responses, cell cycle regulation, or apoptosis. Consistent with these possibilities, activated c-Abl, a proto-oncogene with both pro-apoptotic and transforming potential, binds to and tyrosine phosphorylates plasma membrane PLSCR1 (*15*).

A membrane-bound pool of c-Abl kinase is activated following stimulation of cells with EGF and other growth factors (*16*). Therefore, we investigated the possibility that EGF and the EGF receptor may be involved in the regulation of PLSCR1. We report here that PLSCR1 co-localizes with the EGF receptor in lipid rafts. EGF stimulates the tyrosine phosphorylation of PLSCR1 in an Abl-independent manner and promotes the internalization of PLSCR1. These data suggest that PLSCR1 plays a role in the EGF-induced metabolic or mitogenic response.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents. Human oral epithelial carcinoma KB cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

The murine anti-PLSCR1 mAb 4D2 has been described earlier (*11*, *15*). Rabbit anti-EGF receptor polyclonal IgG, anti-ubiquitin mAb P4D1, anti-Abl mAb 24−11, anti-Abl polyclonal IgG K-12, and HRP-conjugated anti-pTyr mAb PY99 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Shc polyclonal IgG was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-β-actin polyclonal IgG was obtained from Sigma (St. Louis, MO). Anti-flotillin-1 mAb was from Transduction Laboratories (Lexington, KY). Anti-transferrin receptor antibody was from Zymed (South San Francisco, CA). Appropriate HRP-conjugated secondary antibodies, Cy3-conjugated goat-anti-rabbit IgG, and FITC-conjugated goat-anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

The artificial Abl substrate GST−CTD−CRK fusion protein construct was described earlier (*17*). STI571 was the gift of Dr. Elisabeth Buchdunger (Novartis Pharma AG, Switzerland). Cell culture media and reagents were purchased from Life Technologies (Grand Island, NY). Complete EDTA-free Protease Inhibitor Cocktail tablets (containing inhibitors for serine and cysteine proteases) was from Roche Molecular Biochemicals (Indianapolis, IN). All other chemicals, unless otherwise noted, were obtained from Sigma.

Cell Lysates, Immunoprecipitation, Western Blotting, and In Vitro Kinase Assay. Cells were starved in serum-free medium overnight before stimulation with 100 ng/mL of EGF for the indicated time. In some experiments, cells were incubated with 10 μM STI571 for 2 h before EGF stimulation. After treatment of the sample with EGF, the medium was aspirated and the cells were rapidly chilled by washing with ice-cold PBS. Cells were then lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM sodium orthovanadate, 5 mM EDTA, and protease inhibitor cocktail) on ice for 30 min. Cell lysates were centrifuged at 20000*g* for 10 min at 4 °C, and the supernatants were transferred to fresh tubes for further use.

Cell lysates containing 100−500 μg of protein were precleared by incubation with 20 μL of a 50% slurry of Protein G Sepharose 4 Fast Flow at 4 °C for 30 min with gentle rotation. The supernatants were incubated with 2 μg of anti-EGF receptor polyclonal IgG or 5 μg of anti-PLSCR1 mAb 4D2 or 2 μg of anti-Abl mAb 24−11 or 2 μg of anti-Shc polyclonal IgG for 2 h on ice. Protein G Sepharose 4 Fast Flow beads were added to precipitate the immune complex by rotating at 4 °C for 1 h. Beads were pelleted by centrifugation at 1000*g* for 2 min and washed extensively. Immunoprecipitates were subjected to in vitro kinase assay or Western blotting as previously described (*15*). The following concentration of antibodies were used in Western blotting: anti-PLSCR1 4D2, 1 μg/mL; anti-EGF receptor, 0.2 μg/mL; anti-ubiquitin P4D1, 0.2 μg/mL; anti-Abl K-12, 0.2 μg/mL; HRP-conjugated anti-pTyr PY99, 1:20000 dilution; anti-Shc, 1 μg/mL; anti-β-actin, 1:2000 dilution; anti-flotillin-1, 1 μg/mL; anti-transferrin receptor, 1 μg/mL.

Preparation of Lipid Rafts. Lipid raft fractions were prepared from KB cells using the detergent-free method of Smart et al. (*18*). Briefly, cells were homogenized in isotonic sucrose buffer and a postnuclear supernatant fraction prepared by centrifugation at 1400*g* for 5 min. Two milliliters of the postnuclear supernatant was applied to the top of 8 mL of 30% Percoll and centrifuged for 30 min at 84000*g*. The plasma membranes, which banded at the middle of this gradient, were collected and sonicated six times for 20 s. The sonicated plasma membranes were mixed with an equal volume of 50% OptiPrep and overlayered with an 8 mL gradient from 20 to 10% OptiPrep. The gradients were centrifuged for 90 min at 52000*g*. The top 5 mL from the OptiPrep gradient were collected, mixed with 4 mL of 50% OptiPrep, and overlayered with 2 mL of 5% OptiPrep. After centrifugation of the sample for 90 min at 52000*g*, the lipid raft fraction was visible just below the 5% OptiPrep layer. This material was collected and used as the concentrated lipid raft fraction. Fraction 11 from the first OptiPrep gradient was used as the nonraft fraction. In this study, flotillin-1 and transferrin receptor were used as markers for lipid raft and nonraft fractions, respectively.

Lipid rafts prepared by detergent extraction of cells were isolated by a modification of Hope & Pike (*19*). KB cells from one D150 plate were washed, scraped into phosphate buffered saline, and pelleted. Cell pellets were resuspended in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 0.5% Brij 58, and capped tubes were rotated end-over-end for 30 min at 4 °C. The resulting lysate was centrifuged for 10 min at 800*g* to remove debris. One milliliter of the supernatant was mixed with an equal volume of 80% sucrose in MES-buffered saline. Six milliliters of 30% sucrose and 4 mL of 5% sucrose in MES-buffered saline were layered over the sample. The tubes were centrifuged for 3 h at 175000*g*, and 1.2 mL fractions were collected.

Treatment with Methyl-β-cyclodextrin. KB cells grown to confluence in D150 plates were transferred into DMEM containing 25 mM Hepes, pH 7.2 and 1 mg/mL bovine serum albumin with or without 7.5 mM methyl-β-cyclodextrin.

Cells were then incubated for 30 min at 37 °C. At the end of the incubation, the treatment medium was removed, and nondetergent lipid rafts were isolated as outlined above.

Immunofluorescence Microscopy. KB cells were cultured on glass coverslips and serum-starved overnight. Cells were incubated for the indicated times at 37 °C in the absence or presence of EGF. At the end of the incubation, cells were fixed with 2.0% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 2% normal goat serum for 30 min. Cells were incubated with 2 μg/mL anti-EGF receptor polyclonal antibody and 4 μg/mL anti-PLSCR1 mAb 4D2 in blocking solution at 4 °C overnight and stained with 1 μg/mL Cy3-conjugated goat-anti-rabbit IgG and 4 μg/mL FITC-conjugated goat-anti-mouse IgG for 1 h at room temperature. For confocal microscopy analysis, immunofluorescent samples were scanned with an MRC 1024 Bio-Rad Laser Scanning Confocal microscope equipped with a Zeiss X60 objective.

RESULTS

PLSCR1 Partition into Membrane Lipid Rafts. The EGF receptor has been shown to be enriched in low-density membrane domains known as lipid rafts (*18*, *20*, *21*). Palmitoylated proteins such as p60Src also partition into lipid rafts. PLSCR has been shown to be palmitoylated (*22*). We therefore determined whether PLSCR1 was localized to the same low-density membrane domains as EGF receptor.

Lipid rafts were prepared from KB cells using a detergent-free method that involves centrifugation in density gradients prepared with OptiPrep (*18*). In the experiments shown in the left panel of Figure 1A, equal aliquots of the fractions from the first OptiPrep gradient were analyzed by SDS−PAGE followed by Western blotting. Flotillin-1 was used as a marker for lipid rafts, and flotillin-1 was found throughout the gradient with a portion of the material present in the low-density (top) fractions of the gradient. By contrast, the nonraft transferrin receptors were only present in the high-density (bottom) fractions of the gradient. Consistent with previous reports, the EGF receptor was distributed in a fashion similar to that of flotillin-1 indicating that it was present in lipid rafts. Interestingly, PLSCR1 also showed a distribution similar to flotillin-1 and the EGF receptor indicating that this protein is also present in lipid rafts.

The fractions from the first OptiPrep gradient were concentrated over a second step gradient. In the right-hand panel of Figure 1A, equal amounts of protein from the postnuclear supernatant, plasma membrane, lipid raft, and nonraft fractions were analyzed by SDS−PAGE and Western blotting. Flotillin-1, the EGF receptor, and PLSCR1 were all substantially enriched in the lipid raft fraction as compared to the plasma membrane fraction from which they were derived. By contrast, the nonraft transferrin receptor showed no enrichment in lipid rafts. These data suggest that like the EGF receptor, PLSCR localizes to lipid rafts.

PLSCR1 and the EGF receptor were also found to co-distribute into the low-density fraction when cells were solubilized in Brij 58. As shown in Figure 1B, the EGF receptor, flotillin, and PLSCR1 were all found in the low-density portion of a sucrose density gradient used to analyze the detergent lysate. As was found in the nondetergent preparation, the transferrin receptor was found at the bottom of the density gradient. Thus, by both detergent-containing and nondetergent methods, PLSCR1 and the EGF receptor were found to co-distribute in the lipid raft fraction.

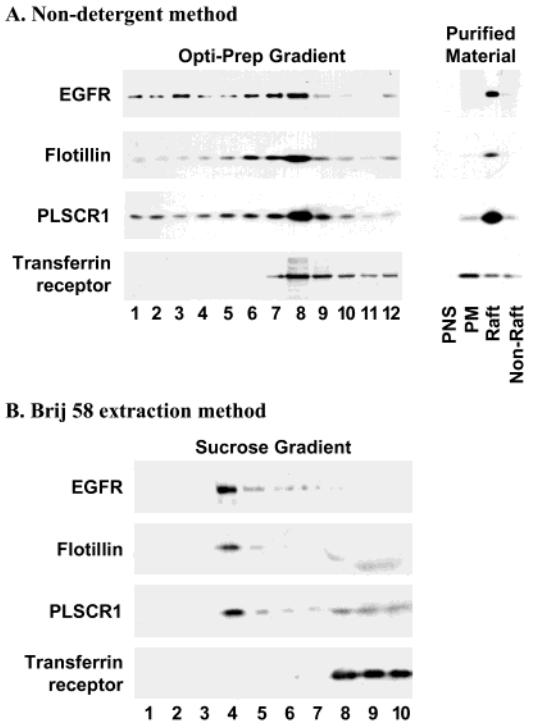

FIGURE 1: PLSCR1 and the EGF receptor both partition into membrane lipid rafts. (A) Lipid raft fractions were prepared from KB cells following the detergent-free method by Smart et al. (*18*). Fractions (1−12) were collected top to bottom from the OptiPrep gradient. Equal volumes of each fraction were resolved by SDS−PAGE and detected by Western blotting with anti-EGF receptor, anti-flotillin, anti-PLSCR1, and anti-transferrin receptor antibodies (left panels). Samples containing 20 μg of protein from various steps in the preparation of lipid rafts were resolved by SDS−PAGE and subjected to Western blotting with these four antibodies (right panels). PNS, post nuclear supernatant; PM, plasma membrane; Raft, lipid raft fractions by combining fractions 1−5; and Non-Raft, nonraft fractions by combining fractions 10−12. (B) Lipid rafts were prepared from KB cells by extraction in 0.5% Brij 58 as described in Materials and Methods. Sucrose gradients were fractionated into 10 fractions, and equal aliquots of each fraction were analyzed by SDS−PAGE and Western blotting as indicated above.

Further support for the lipid raft localization of PLSCR1 was obtained by analyzing the effect of cholesterol depletion on PLSCR1 localization. Lipid rafts are enriched in cholesterol, and the depletion of cholesterol leads to the disruption of lipid rafts and the release of raft proteins into bulk plasma membrane (*23*). KB cells were incubated in the absence or presence of methyl-β-cyclodextrin for 30 min to deplete cholesterol. Plasma membranes were isolated using the nondetergent method and lipid rafts isolated by centrifugation in a 10−20% Opti-Prep gradient. As shown in Figure 2, depletion of cholesterol markedly reduced the fraction of PLSCR1, EGF receptor, and flotillin found in the low-density portion of the gradient. Thus, the localization of PLSCR1 to low-density membrane domains is sensitive to the depletion of cholesterol to the same extent as the EGF receptor and flotillin, two known lipid rafts proteins. These data are consistent with the conclusion that PLSCR1 is present in lipid rafts.

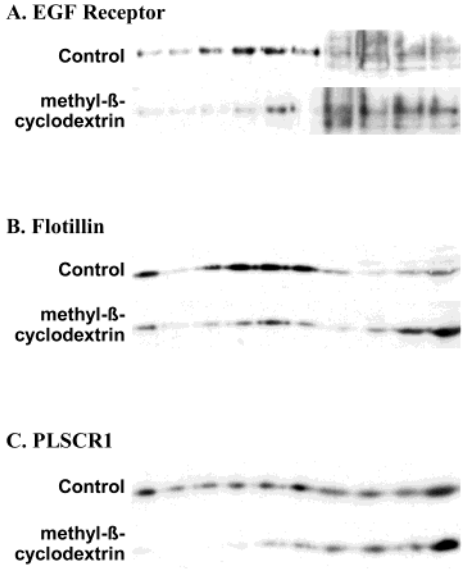

FIGURE 2: Effect of cholesterol depletion on localization of PLSCR1 to lipid rafts. KB cells were treated in the absence or presence of methyl-β-cyclodextrin as described in Materials and Methods. Lipid rafts were then prepared through the first Opti-Prep gradient of the nondetergent method. Equal aliquots of each fraction were analyzed by SDS−PAGE and Western blotting with (A) anti-EGF receptor antibodies; (B) anti-flotillin antibodies; and (C) anti-PLSCR1 antibodies.

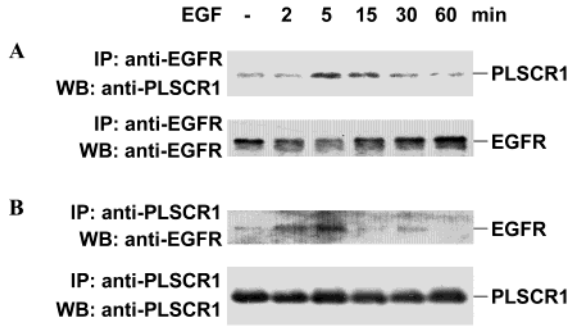

FIGURE 3: PLSCR1 is associated with the EGF receptor in response to EGF stimulation. KB cells were stimulated without or with 100 ng/mL EGF for 2, 5, 15, 30, or 60 min. Cells lysates were prepared. (A) The EGF receptor was immunoprecipitated with anti-EGF receptor polyclonal antibody. PLSCR1 co-immunoprecipitated with the EGF receptor was detected by Western blotting using anti-PLSCR1 mAb 4D2 (upper panel, A). Immunoprecipitated EGF receptor protein was detected with anti-EGF receptor polyclonal antibody (lower panel, A). (B) PLSCR1 was immunoprecipitated with anti-PLSCR1 mAb 4D2. The EGF receptor co-immunoprecipitated with PLSCR1 was detected by Western blotting with anti-EGF receptor polyclonal antibody (upper panel, B). Immunoprecipitated PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (lower panel, B).

PLSCR1 Associates with the EGF Receptor in Response to EGF Stimulation. The co-localization of the EGF receptor and PLSCR1 in lipid rafts suggested the possibility that there was a direct interaction between the two proteins. To examine this possibility, cells were stimulated with EGF and detergent lysates were immunoprecipitated with an anti-EGF receptor antibody. The immunoprecipitated proteins were analyzed by Western blotting for the presence of EGF receptor as well as PLSCR1 (Figure 3A). A small amount of PLSCR was found to co-immunoprecipitate with the EGF receptor under basal conditions. Stimulation of the cells with EGF led to a distinct increase in the co-immunoprecipitated PLSCR with the EGF receptor, which peaked 5 min after EGF stimulation and declined thereafter.

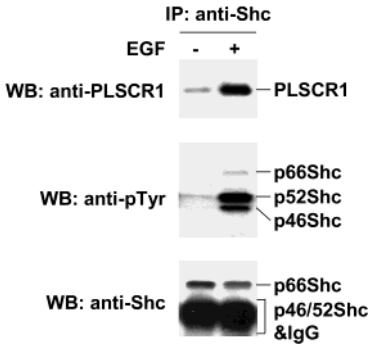

FIGURE 4: PLSCR1 is associated with Shc in response to EGF stimulation. KB cells were stimulated without or with 100 ng/mL EGF for 5 min. Cell lysates were prepared. Shc was immunoprecipitated with anti-Shc polyclonal antibody. PLSCR1 co-immunoprecipitated with Shc was detected by Western blotting with anti-PLSCR1 mAb 4D2 (top panel). Abundance of immunoprecipitated p66Shc proteins was detected with anti-Shc polyclonal antibody (bottom panel). The successful immunoprecipitation of all three Shc isoforms was confirmed by Western blotting with HRP-conjugated anti-pTyr mAb PY99 in the EGF-treated samples (middle panel).

Figure 3B shows the results of the reciprocal experiment in which detergent lysates were immunoprecipitated with anti-PLSCR1 antibodies and subsequently analyzed by Western blotting for both PLSCR1 and the EGF receptor. EGF receptors were found to co-immunoprecipitate with PLSCR1, and this co-immunoprecipitation also peaked 5 min after stimulation with EGF.

Because PLSCR1 and the EGF receptor do not contain identifiable domains that are now known to promote direct interaction of these two proteins, we examined the possibility that the association between PLSCR1 and the EGF receptor was mediated via the adapter protein Shc. KB cells were stimulated with or without EGF and detergent lysates of the cells were immunoprecipitated with an anti-Shc antibody. As shown in Figure 4, PLSCR1 was co-immunoprecipitated with Shc and this co-immunoprecipitation was markedly increased after EGF treatment. As anticipated, stimulation with EGF was found to enhance the tyrosine phosphorylation of Shc (Figure 4, middle panel).

PLSCR1 Is Tyr-phosphorylated in Response to EGF. To determine whether PLSCR might also be tyrosine phosphorylated in response to EGF, KB cells were stimulated with EGF for increasing lengths of time and detergent lysates were immunoprecipitated with anti-PLSCR1 antibodies. The initial lysates and anti-PLSCR immunoprecipitates were then analyzed by SDS−PAGE and subjected to Western blotting with an anti-phosphotyrosine antibody (Figure 5). Stimulation of cells with EGF led to a time-dependent increase in the autophosphorylation of the EGF receptor, which peaked about 2 min after treatment with EGF. Similarly, EGF stimulated an increase in the tyrosine phosphorylation of immunoprecipitated PLSCR. The increase in PLSCR1 tyrosine phosphorylation lagged somewhat behind that of the EGF receptor, peaking 5 min after EGF stimulation. These results suggest that PLSCR1 is a substrate for the EGF receptor tyrosine kinase itself or for another tyrosine kinase downstream of the EGF receptor.

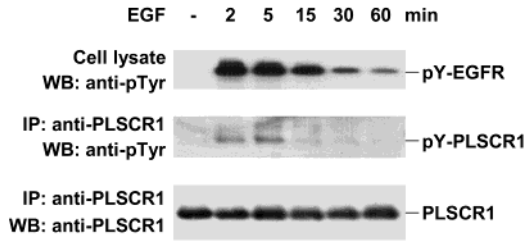

FIGURE 5: PLSCR1 is Tyr-phosphorylated in response to EGF stimulation. KB cells were stimulated without or with 100 ng/mL EGF for 2, 5, 15, 30, or 60 min. Cell lysates were prepared followed by immunoprecipitation of PLSCR1 with anti-PLSCR1 mAb 4D2. Tyr-phosphorylation of PLSCR1 was detected by Western blotting with HRP-conjugated anti-pTyr mAb PY99 (middle panel) and the level of immunoprecipitated PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (bottom panel). The Tyr-phosphorylation of the EGF receptor was examined by Western blotting with HRP-conjugated anti-pTyr mAb PY99 using cell lysates (top panel).

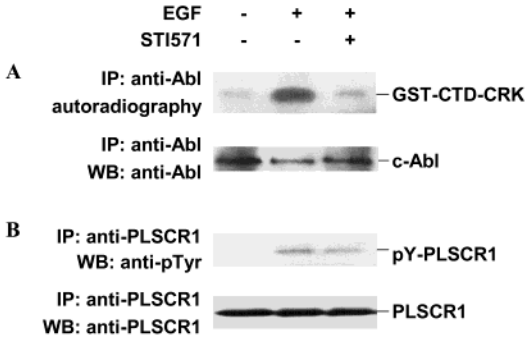

FIGURE 6: EGF-stimulated Tyr-phosphorylation of PLSCR1 is not c-Abl-dependent. KB cells were treated without or with 10 μM STI571 for 2 h prior to stimulation without or with 100 ng/mL EGF for 5 min. (A) c-Abl was immunoprecipitated from cell lysates with anti-Abl mAb 24−11 and subjected to kinase assays using Abl substrate GST−CTD−CRK with [^{32}P]-γ-ATP. Proteins were resolved on a 12% Tris-Glycine Novex gel and the phosphorylated proteins were detected by autoradiography (upper panel, A). Abundance of c-Abl protein in the kinase assays was detected with anti-Abl polyclonal K-12 antibody (lower panel, A). (B) PLSCR1 was immunoprecipitated from cell lysates with anti-PLSCR1 mAb 4D2. Tyr-phosphorylation of PLSCR1 was detected by Western blotting with HRP-conjugated anti-pTyr mAb PY99 (upper panel, B) and the level of immunoprecipitated PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (lower panel, B).

The EGF-stimulated Tyr-phosphorylation of PLSCR1 Is not c-Abl-dependent. c-Abl is activated by EGF (*16*) and can phosphorylate PLSCR1 (*15*). To examine the role of c-Abl in the EGF-induced phosphorylation of PLSCR1, KB cells were treated with or without Abl kinase inhibitor STI571 prior to stimulation with EGF for 5 min. As shown in Figure 6A, the activation of c-Abl kinase induced by EGF stimulation and the inhibition of c-Abl activation by prior STI571 treatment were assessed by an in vitro kinase assay that utilizes immunoprecipitated c-Abl and an artificial substrate, GST−CTD−CRK (see Materials and Methods). As shown in Figure 6B, the EGF-stimulated tyrosine phosphorylation of PLSCR1 was only marginally reduced when c-Abl kinase activity was inhibited by STI571. These results suggest that c-Abl is not the predominant kinase responsible for the increase in tyrosine phosphorylation of PLSCR1 observed following EGF stimulation.

EGF-induced Endocytosis of PLSCR1. The binding of EGF to its receptor is known to induce rapid endocytosis of the receptor via clathrin-coated pits (*24*). In light of the apparent physical association of PLSCR1 and the EGF receptor, we investigated the possibility that EGF might induce the trafficking of PLSCR1 to an intracellular compartment. KB cells were stimulated with EGF for up to 180 min and fixed, permeabilized, and immunostained for both the EGF receptor and PLSCR1. As shown in Figure 7, prior to stimulation, PLSCR1 and the EGF receptor were predominately localized in the plasma membrane with little intracellular antigen detected. As early as 5 min after stimulation with EGF, the EGF receptor underwent rapid endocytosis and localized in distinct intracellular vesicles. The increase in intracellular EGF receptor antigen was accompanied by a corresponding reduction in the intensity of plasma membrane staining, consistent with endocytosis of the receptor into an endosomal compartment. By 3 h, there was net reduction of EGF receptor staining, presumably due to lysosomal degradation.

Over this 3 h period of EGF treatment, there was a partial redistribution of PLSCR into an intracellular compartment. As shown in the merged images, there was a small amount of co-localization of internalized PLSCR1 and EGF receptor; however, the majority of these proteins did not co-localize after internalization. By 3 h, the endosomal pool of PLSCR1 antigen diminished, with a relative increase in cell surface distribution suggesting a recycling of PLSCR1 to the plasma membrane. Following EGF stimulation, a portion of the EGF receptor is known to undergo ubiquitination by Cbl and consequent lysosomal degradation (*24*). Whereas we readily detected the ligand-induced ubiquitination of EGF receptor and the appearance of its proteolytic fragments, we detected neither ubiquitination nor evidence for proteolysis of PLSCR1 under these conditions (Figure 8).

EGF Increases Cellular Expression of PLSCR1. Interferon treatment resulted in transcriptional induction of PLSCR1 (*11*, *14*). The biologic activity of EGF is mediated in part through transcription of select genes that initiate mitosis and other cellular changes induced by this growth factor. To explore whether the cellular levels of PLSCR1 might also increase as part of the transcriptional response to the EGF receptor activation, the expression of PLSCR1 was monitored in KB cells stimulated by EGF (Figure 9). A marked increase in cellular PLSCR1 antigen was detected as early as 6 h, peaking substantially above basal levels at 12−18 h after receptor stimulation. These results suggest that in addition to its observed interaction with the EGF receptor, Shc, and potentially other components that mediate the initial signaling from activated EGF receptor, newly synthesized PLSCR1 may also contribute to posttranscriptional effector pathway(s) underlying the cellular response to this cytokine. As was previously reported for stimulation by interferon (*11*), EGF treatment resulting in increased expression of PLSCR1 was not found to increase plasma membrane PL scramblase activity or cell surface exposure of phosphatidylserine (data not shown).

DISCUSSION

Our data suggest that PLSCR1, a plasma membrane protein that was proposed to participate in transbilayer movement of phospholipids, is a component of membrane lipid rafts and of the activated EGF receptor complex. Our data also suggest that upon stimulation by EGF, there is (i)

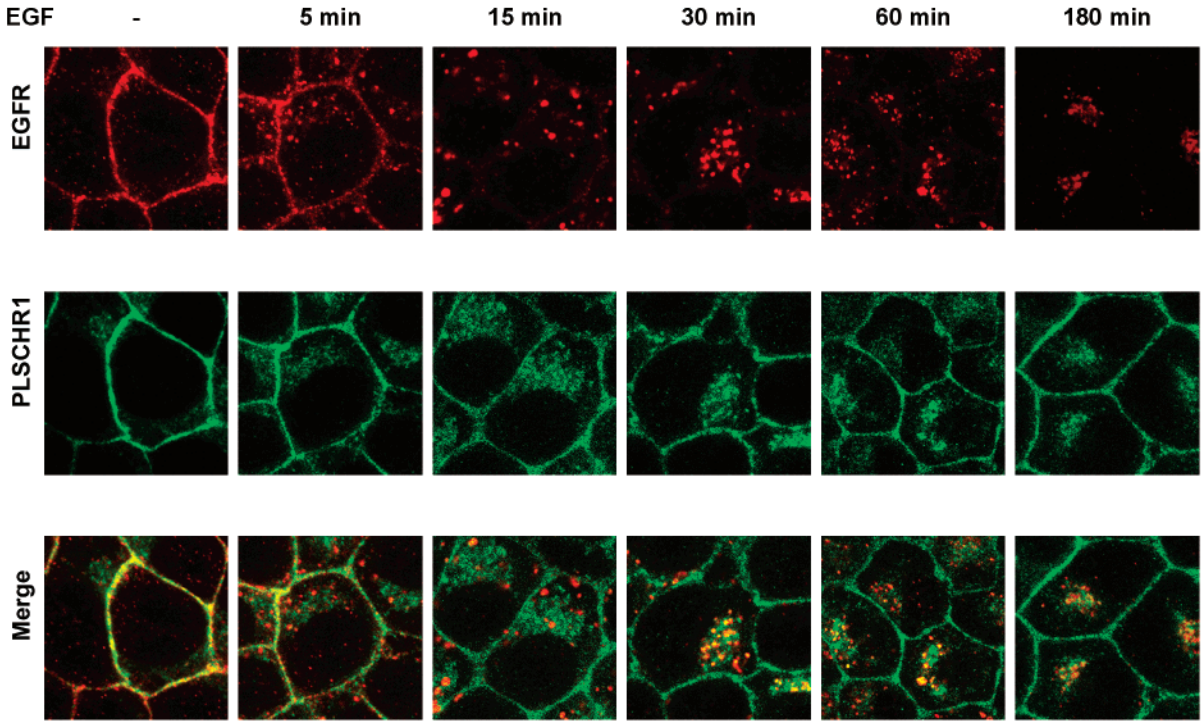

FIGURE 7: PLSCR1 undergoes endocytosis in response to EGF stimulation and co-localizes with internalized the EGF receptor. KB cells cultured on glass cover slips were stimulated without or with 100 ng/mL EGF for 5, 15, 30, 60, or 180 min. Cells were fixed, permeabilized, and stained simultaneously with anti-EGF receptor polyclonal antibody and anti-PLSCR1 mAb 4D2. The antigens were visualized with Cy3-conjugated goat-anti-rabbit IgG for the EGF receptor and FITC-conjugated goat-anti-mouse IgG for PLSCR1. Immunofluorescent samples were scanned with confocal microscope with constant exposure time. Negative staining by control IgGs and minimum leakage between fluorescent filters were confirmed separately by experiments.

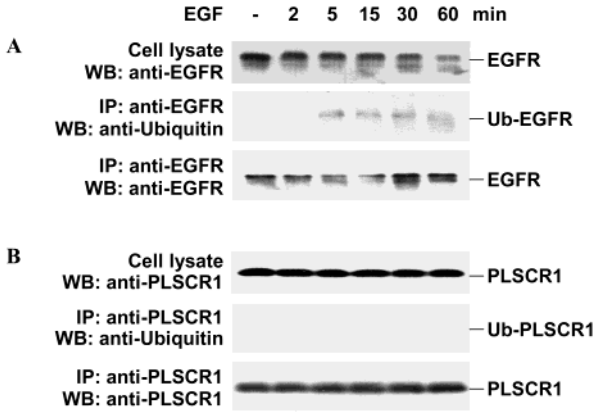

FIGURE 8: EGF receptor, but not PLSCR1, is ubiquitinated and degraded upon EGF stimulation. KB cells were stimulated without or with 100 ng/mL EGF for 2, 5, 15, 30, or 60 min. Cells lysates were prepared. (A) The EGF receptor was immunoprecipitated with anti-EGF receptor polyclonal antibody. Ubiquitination of the EGF receptor was detected by Western blotting with anti-ubiquitin mAb P4D1 (middle panel, A). Abundance of immunoprecipitated EGF receptor protein was detected with anti-EGF receptor polyclonal antibody (bottom panel, A). Degradation of EGF receptor in cell lysates was detected with anti-EGF receptor polyclonal antibody (top panel, A). (B) PLSCR1 was immunoprecipitated with anti-PLSCR1 mAb 4D2. Anti-ubiquitin mAb P4D1 did not detect any ubiquitination of PLSCR1 (middle panel, B), whereas abundant PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (bottom panel, B). No degradation of PLSCR1 in cell lysates was observed with anti-PLSCR1 mAb 4D2 (top panel, B).

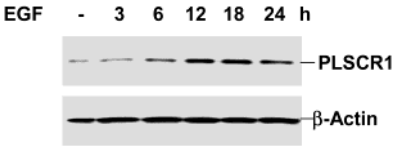

FIGURE 9: EGF induces the expression of PLSCR1. KB cells were stimulated without or with 100 ng/mL EGF for 3, 6, 12, 18, or 24 h. Cell lysates were prepared and resolved by SDS−PAGE. Expression levels of PLSCR1 in cell lysates were detected by Western blotting with anti-PLSCR1 mAb 4D2 (upper panel). Equal loading of total protein in each sample was confirmed with anti-actin polyclonal antibody (lower panel).

a physical association of PLSCR1 with both the adaptor protein Shc and the EGF receptor; (ii) an EGF-induced Tyr-phosphorylation of PLSCR1; (iii) endocytic trafficking of both PLSCR1 and the EGF receptor into distinct endosomal pools; and (iv) an EGF-induced synthesis of new cellular PLSCR1. These results identify PLSCR1, a protein not previously implicated in cell signaling, as being involved in EGF-dependent signal transduction and provide a framework for future studies to further define the cellular function of the PLSCR proteins.

A variety of proteins involved in cell signaling have been shown to be localized to low density, cholesterol-enriched plasma membrane domains termed caveolae or lipid rafts, including the EGF receptor (*19*, *21*, *25*, *26*). PLSCR1 is enriched in lipid rafts (Figures 1 and 2), implying a role for PLSCR1 in cell signaling events initiated from these low-density plasma membrane subdomains. While a subset of raft proteins are resistant to extraction with Triton X-100, both PLSCR1 and EGFR are soluble in Triton X-100 (*1*, *2*, *5*, *27*). Palmitoylation has been shown to target many signaling proteins to lipid rafts (*28*). PLSCR1 is multiply palmitoylated, a posttranslational modification that was shown to be required for normal phospholipid scramblase activity when reconstituted in proteoliposomes (*22* and

unpublished data). It remains to be explored whether the localization of PLSCR1 in plasma membrane lipid rafts is also regulated by its palmitoylation, and whether there are circumstances that alter the extent of thiolesterification of this protein in situ.

Additional evidence for the involvement of PLSCR1 in cell signaling, specifically signaling by EGF, was obtained in experiments that tested for potential interactions between PLSCR1 and the EGF receptor, another resident lipid raft protein. Our data demonstrate that PLSCR1 co-immunoprecipitates with the EGF receptor and that this interaction is enhanced after stimulation with EGF (Figure 3). It is well established that ligand-induced dimerization and subsequent autophosphorylation of distinct tyrosine residues in the EGF receptor creates docking sites for various membrane-targeted adaptor proteins or enzymes containing SH2- or PTB-domains (*29*, *30*). Whereas PLSCR1 contains neither SH2- nor PTB-domains, the N-terminal Pro-rich region of PLSCR1 provides multiple potential binding sites for SH3- or WW-domain containing proteins, motifs that are not identified in the EGF receptor. This suggests that an adapter protein with avidity for both the EGF receptor and PLSCR1 mediates the observed association between these membrane proteins. The dramatic increase in co-immunoprecipitation of PLSCR1 with Shc upon EGF stimulation (Figure 4) suggests that the phosphorylation of PLSCR1 may expose a binding site for the Shc SH2-domain, while the Shc PTB-domain binds to the activated EGF receptor through its phosphotyrosine residues. Whereas our data implicate Shc as the adaptor that mediates the physical interaction of PLSCR1 with the EGF receptor, it is also possible that PLSCR1 binds to the EGF receptor through another adaptor protein and co-immunoprecipitates with Shc through its allosteric association with the EGF receptor. The confirmation of Shc as the adaptor protein for PLSCR1 and EGF receptor interaction is under investigation.

PLSCR1 is tyrosine phosphorylated in response to stimulation with EGF, consistent with a potential role for PLSCR1 in the diverse cell signaling events mediated by this growth factor (Figure 5). Ligand-induced autophosphorylation of the EGF receptor precedes the phosphorylation and physical association of PLSCR1 and the EGF receptor. Inhibition of c-Abl kinase activity with STI571 did not blunt the observed EGF-induced increase in tyrosine phosphorylation of PLSCR1 (Figure 6). Thus, the tyrosine phosphorylation of PLSCR1 does not appear to be mediated by c-Abl, a kinase shown to constitutively phosphorylate cellular PLSCR1 (*15*). This suggests that PLSCR1 is a substrate either of the EGFR kinase, or of another EGF-activated Tyr-kinase besides c-Abl. Although our data suggest that at least a portion of the PLSCR1 is found in a complex with the EGF receptor in intact cells, we have not been able to detect EGF-stimulated phosphorylation of PLSCR1 in isolated plasma membrane or lipid raft preparations in vitro (data not shown). Identification of the tyrosine kinase responsible for phosphorylating PLSCR as well as the residues phosphorylated in PLSCR is currently under investigation.

In addition to inducing the rapid phosphorylation of preexisting PLSCR1, cellular stimulation by EGF also induced a marked increase in total PLSCR1 protein expression (Figure 9). A comparable increase in PLSCR1 expression was previously observed in cells treated with interferon α (*11*) and in response to other interferons (*14*), suggesting that multiple cytokines may regulate the *PLSCR1* gene.

EGF is known to promote the rapid internalization of the EGF receptor via coated pits and its subsequent degradation in lysosomes. We observed rapid internalization of the EGF receptor after addition of EGF (Figure 7). Surprisingly, our results demonstrate that EGF also stimulates the internalization of PLSCR1. However, several features of this process appear to differ from those involved in the EGF receptor internalization. First, internalization of PLSCR1 was less extensive than that of the EGF receptor, with the internalized pool accounting for at most half of the total cellular PLSCR1. In addition, the merged images of PLSCR1 and the EGF receptor showed only limited and variable overlap in the location of these two proteins once they had been internalized into the cells (Figure 7). Finally, there appears to be a difference in the ultimate disposition of internalized PLSCR1 and the EGF receptor. The EGF receptor is both ubiquitinated and largely degraded after internalization. However, after an initial EGF-induced redistribution of a portion of plasma membrane PLSCR1 from cell-surface to internal, by 3 h, the amount of cell-surface PLSCR1 detected appeared to return to initial levels, suggesting that internalized PLSCR1 is ultimately recycled, rather than degraded. Consistent with this interpretation, we detected neither the ubiquitination nor proteolytic breakdown of PLSCR1 by Western blotting of EGF-treated cells (Figure 8). After EGF stimulation, total cellular content of PLSCR1 did not rise detectably until 6−12 h (Figure 9), well beyond the time course of the internalization studies, suggesting that newly synthesized PLSCR1 did not contribute to the apparent trafficking of PLSCR1 antigen between plasma membrane and endosomal compartments observed in Figure 7. The apparent differences in the EGF receptor and PLSCR1 internalization could arise from multiple sources. For example, the EGF receptor and PLSCR1 may both be internalized via coated pits, but then differentially sorted in early endosomes leading to recycling of PLSCR1 but degradation of the EGF receptor. Alternatively, since caveolae/lipid rafts are known to be involved in the internalization of a variety of proteins, it is possible that the differences in PLSCR1 and the EGF receptor internalization and trafficking are due to the former being internalized and recycled via lipid rafts and the latter being internalized via coated pits. Additional experiments will be required to clarify the mechanism of PLSCR1 internalization.

The rapid phosphorylation and redistribution of PLSCR1 observed in cells treated with EGF combined with subsequent induction of newly synthesized PLSCR1 implies that this protein may participate both in signaling events initiated by activated EGF receptor as well as in an effector pathway(s) that underlies the cellular responses to this cytokine, potentially including cell proliferation, differentiation, adhesion, migration, or apoptosis (*29*, *30*). In addition to this interaction with the EGF receptor, and its known regulation by interferons, PLSCR1 was also observed to be a substrate of cellular protein kinase C δ (*31*), c-Abl tyrosine kinase (*15*), and an IgE Fc receptor type I receptor-linked tyrosine kinase (*4*), diverse kinases that are also implicated in regulation of cell proliferation, differentiation, and apoptotic responses. It remains to be clarified how PLSCR1's interactions with the EGF receptor and these various other receptor and nonreceptor kinases are related to its apparent localization

in lipid rafts, to its transcriptional regulation by multiple cytokines, and to its putative role in promoting transbilayer movement of plasma membrane phospholipids.

ACKNOWLEDGMENT

The superb technical assistance of Lilin Li and Hongfan Peng is gratefully acknowledged.

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BI025610L