Phospholipid scramblase (PLSCR1) is a multiply palmitoylated, calcium-binding endofacial membrane protein proposed to mediate transbilayer movement of plasma membrane phospholipids. PLSCR1 is a component of membrane lipid rafts and has been shown to both physically and functionally interact with activated epidermal growth factor (EGF) receptors and other raft-associated cell surface receptors. Cell stimulation by EGF results in Tyr phosphorylation on tyrosines 69 and 74. In vitro interaction between PLSCR1 and Shc requires the Src-mediated phosphorylation on tyrosines 69 and 74. In vitro pull down studies, phosphorylated PLSCR1 was found to bind directly to Shc through the phosphotyrosine binding domain. Consistent with the potential role of PLSCR1 in growth factor signaling pathways, granulocyte precursors derived from mice deficient in PLSCR1 show impaired proliferation and maturation under cytokine stimulation. Using PLSCR1−/− embryonic fibroblasts and kidney epithelial cells, we now demonstrate that deletion of PLSCR1 from the plasma membrane reduces the activation of c-Src by EGF, implying that PLSCR1 normally facilitates receptor-dependent activation of this kinase. We propose that PLSCR1, through its interaction with Shc, promotes Src kinase activation through the EGF receptor.

Phospholipid scramblase 1 (PLSCR1) is an endofacial plasma membrane protein that has been proposed to play a role in the redistribution of plasma membrane phospholipids leading to cell surface exposure of phosphatidylinerse following cell activation, injury, or apoptosis (1–3). There are four distinct human PLSCR genes (hPLSCR1−4), corresponding orthologous genes in mouse (mPLSCR1−4), and putative orthologues in Drosophila, Caenorhabditis elegans, and Saccharomyces cerevisiae (4).

The amino acid sequence of human PLSCR1 reveals a type-2 membrane protein containing 318 residues with a predicted transmembrane helix near the C terminus (2–4). The cytoplasmic domain includes an EF hand-like calcium-binding domain and a predicted protein kinase C phosphorylation site (3, 4). PLSCR1 is multiply Cys-palmitoylated (5, 6) and has been shown to partition into lipid rafts, cholesterol and sphingolipid-rich membrane microdomains implicated in regulation and endocytic trafficking of receptor-signalong complexes (7). The N-terminal cytoplasmic domain of PLSCR1 contains multiple proline-rich motifs resembling both Src homology 3 (SH3) domain-binding sites and WW domain-binding motifs (4). PLSCR1 has been shown to bind c-Abl through its SH3 domain and to be a substrate of activated c-Abl tyrosine kinase (8).

Mice with targeted disruption of the PLSCR1 locus exhibit delayed fetal development of mature blood granulocytes and defective granulocytosis in response to stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) but not to interleukin 3 or granulocyte macrophage colony-stimulating factor (9). Consistent with these in vivo data, myeloid precursor cells from PLSCR1−/− mice in colony-forming assays display impaired proliferation and maturation when cultured with either SCF or G-CSF (9). Stimulation of myeloid precursor cells by either SCF or G-CSF was also found to induce marked increases in cellular expression of PLSCR1, suggesting that it is transcriptionally regulated through these growth factors (9). PLSCR1 is also one of the most highly interferon-induced genes and was shown to be transcriptionally regulated by signal transducer and activator of transcription 1 (STAT-1) and by an interferon-stimulated response element in its first exon (10).

In cells expressing receptors for epidermal growth factor (EGF), stimulation with EGF leads to an association of PLSCR1 with EGF-R and related growth factor receptors, we have examined EGF signaling in cells derived from PLSCR1−/− mice (9). Our data indicate that an activated Src kinase is responsible for the Tyr phosphorylation of PLSCR1 observed in EGF-treated cells and that this phos-
phorylation is required for the interaction of PLSCR1 with Shc upon EGF stimulation. Furthermore, our data suggest that deletion of PLSCR1 from the plasma membrane results in marked reduction in EGF-initiated activation of c-Src kinase.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The murine anti-PLSCR1 monoclonal antibody (mAb) 4D2 (specific for human PLSCR1) has been previously described (8) and recognizes a single protein of 37 kDa in cell lysates. The mAb IA8, recognizing mouse PLSCR1, has been developed in our laboratory and does not cross react with other PLSCR proteins (PLSCR2–4) (data not shown). Rabbit anti-EGF-R (1005) polyclonal IgG and HRP-conjugated anti-TyrP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cd2 electrode, purified recombinant human c-Src enzyme, and rabbit anti-Shc polyclonal IgG against p46, p52, and p66 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-c-Src mAb 327 was from Oncogene (San Diego, CA), and anti-c-Src mAb 2–17 antibody was a kind gift of Dr. Sally Parsons (University of Virginia, Charlottesville, VA). Appropriately HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-maltose-binding protein (MBP) rabbit polyclonal antibody was obtained from New England Biolabs (Beverly, MA).

Cell culture media and reagents were purchased from Invitrogen, and purified recombinant Protein Inhibitor Mixture Tablets (containing inhibitors for serine and cysteine proteases) was from Roche Applied Science. AG1478, an EGF-R tyrosine kinase inhibitor, and PP2, a Src family tyrosine kinase inhibitor, were obtained from Calbiochem (San Diego, CA), and EGF was from BD Biosciences (San Diego, CA). The Ab inhibitor, ST1571, was a gift of the Dr. Elizabeth Buchdunger (Novartis Pharma AG, Basel, Switzerland). The PLSCR family members, hPLSCR1, 2, 3, and 4, were produced as fusion proteins with MBP as described previously (2).

**Establishment of Fibroblast and Epithelial Cell Lines**—Human epidermal A431 cells were obtained from Dr. Linda Pike (Washington University, St. Louis, MO). Mouse embryo fibroblasts (MEFs) were isolated from embryos of PLSCR1 knockout (PLSCR1−/− mice) and immortalized by transfection with SV40 genomic DNA (11). Kidney immortalized by SV40 virus infection (12, 13). Mouse PLSCR1, human PLSCR1, and human Y699F/Y747F PLSCR1 mutant cDNAs were cloned into the MSCV-IRE6-GFP vector and used to transfect the virus packaging cell line, PT-67, to produce infectious virus. The immortalized MEFs and KECs were infected with either mouse mPLSCR1-MSCV-IRE6-GFP, human hPLSCR1-MSCV-IRE6-GFP, Y699F/Y747F-hPLSCR1-MSCV-IRE6-GFP, or MSCV-IRE6-GFP. Infected cells were sorted by flow cytometry using GFP as indicator to collect cells with optimal levels of transduction.

**Preparation of Cell Lysates and Immunoprecipitation**—Cells were starved in serum-free medium for 3 to 14 h prior to stimulation with EGF (100 ng/ml) for the indicated times. MEFs and KECs were infected with either mouse mPLSCR1–MSCV-IRE6-GFP or MSCV-IRE6-GFP. Cells were lysed with 2% Triton X-100 in 1× PBS, insoluble material was removed by centrifugation at 10,000 g for 10 min at 4 °C, and the supernatants were transferred to fresh tubes for further use. For immunoprecipitation, cell lysates containing 100–500 μg of protein were incubated with 2 μg of anti-EGF-R mAb 327 or mAb 2–17, 5 μg of anti-hPLSCR1 mAb 4D2, 2 μg of anti-Shc polyclonal IgG, or 2 μg of anti-EGF-R 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 at 1000 × g for 2 min and washed extensively, and immunoprecipitates were subjected to Western blotting.

**Western Blotting**—Proteins were resolved on 10% Tris glycine Novex gels and electrochemically transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% (w/v) Blotto, membranes were incubated for 1 h with primary antibody and 1 h with appropriate horseradish peroxidase-conjugated secondary antibodies. Blots were developed using SuperSignal chemiluminescence substrates (Pierce). The following concentration of antibodies were used in Western blotting: anti-hPLSCR1 4D2, 1 μg/ml; anti-mPLSCR1 IA8, 2 μg/ml; anti-EGF-R, 0.2 μg/ml; HRP-conjugated anti-TyrP (P) FY99, 1:20,000 dilution; anti-c-Src mAb 327, 8 μg/ml; anti-c-Src mAb 2–17, 1.4 μg/ml; anti-Shc, 4 μg/ml; anti-MBP, 1:20,000 dilution.

**In Vitro Tyrrosine Kinase Assay**—EGF-R or c-Src immunoprecipitated from MEF, KEC, or A431 cell lysates, or purified recombinant c-Src as indicated in the figure legends, were used for in vitro tyrosine phosphorylation of PLSCR1. In a total volume of 30 μl of reaction mixture, 4 μg of MBP-PLSCR1 fusion protein or 1–4 μg of cdc2 peptide was used as substrate in kinase assay buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1.2 mM MnCl2, 2 mM sodium orthovanadate, 2 mM sodium fluoride, and 2 mM sodium orthovanadate. Reactions were initiated by the addition of 2 μl of 1 mM cold ATP or 2 μl of 8 μM ATP containing 10 μCi of [γ-32P]ATP. After incubation for 15 min at room temperature (for A431 cells), or 1 h at 37 °C (for MEFs, KECs, and purified recombinant c-Src), reactions were terminated by adding SDS-PAGE sample buffer and boiling at 100 °C for 5 min. Proteins were resolved by SDS-PAGE, and phosphorylation was detected by Western blotting with anti-TyrP (P) mAb FY99-HRP or autoradiography.

**In Vitro Binding of PLSCR1 to GST Fusion Proteins**—The GST fusion proteins of full-length p52 Shc were kindly provided by Dr. Ralph Bradshaw (University of California at Irvine, Irvine, CA) and those of the phosphotyrosine binding (PTB), collagen homology (CH), and SH2 domains of Shc by Dr. Kodi Ravichandran (Beirne Carter Center for Immunology Research, University of Virginia). The GST fusion proteins were expressed by induction overnight at 25 °C with 0.1 mM isopropyl-1-thio–β-D-galactopyranoside in BL21 DE3 (protein deficient) Escherichia coli and purified using glutathione-Sepharose 4B according to the manufacturer’s instructions.

**Results**

Phosphorylation of PLSCR1 in Response to EGF Is Mediated by a Src kinase—We had previously observed that cellular treatment with EGF results in a transient increase in Tyr phosphorylation of PLSCR1 (7). An increase in PLSCR1 phosphorylation was also observed for platelet-derived growth factor (PDGF) and SCF, suggesting that PLSCR1 might be a substrate of a tyrosine kinase(s) that is activated through multiple growth factor receptors (14) (data not shown). This indicated that PLSCR1 was a substrate of either the growth factor receptor tyrosine kinases themselves or of other tyrosine kinase(s) activated through EGF-R, platelet-derived growth factor receptor, or c-kit, respectively, including potentially the Src family of tyrosine kinases that are known to function prominently in the growth factor receptor activation pathways (15). As shown in Fig. 1, Tyr phosphorylation of PLSCR1 upon cell treatment with EGF was found to be sensitive to inhibition by the Src kinase inhibitor PP2 at concentrations of inhibitor that had only small effects on the autophosphorylation of EGF-R, suggesting that a Src kinase mediates phosphorylation of PLSCR1 under these conditions. Consistent with these data, when recombinant PLSCR1 was used as substrate for in vitro kinase assays of either immunoprecipitated EGF-R (Fig. 2A) or c-Src (Fig. 2B), only c-Src was found to mediate the phosphorylation of PLSCR1. Because Src kinases are known to phosphorylate...
Requires Phosphorylation of Tyr69 and Tyr74 in PLSCR1

We next set out to identify the sites of phosphorylation in PLSCR1. The sites of phosphorylation in PLSCR1 by c-Src were next mapped using Tyr mutants of the PLSCR1 polypeptide. We found that mutation at both Tyr69 and Tyr74 was required to eliminate phosphorylation by both Src kinases. Of the various domains of Shc, only full-length p52 Shc and the PTB domain of Shc were expressed as GST fusion proteins and used to interact with PLSCR1 that had been phosphorylated by c-Src. Of the various domains of Shc, only full-length p52 Shc and the PTB domain of Shc were observed to bind phosphorylated PLSCR1 (Fig. 6). Consistent with this, Tyr(P) PLSCR1 was depleted in the supernatants after pull down with full-length Shc and PTB, compared with the GST control and the other Shc domains tested. These results suggest that interaction of phosphorylated PLSCR1 with Shc is mediated predominantly by the PTB domain. In the absence of phosphorylation, some interaction of PLSCR1 with full-length Shc and its PTB domain was also observed (see “Discussion”).
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**Fig. 3.** In vitro phosphorylation of PLSCR1 by purified recombinant c-Src with MBP-PLSCR1 as substrate in the absence or presence of PP2. Proteins were resolved on a 10% SDS-PAGE gel, and the phosphorylated proteins were detected by Western blotting (WB) with HRP-conjugated anti-Tyr(P) mAb PY99 (top panel). MBP-PLSCR1 was detected with anti-MBP (bottom panel). Data shown are representative of 2 experiments performed. B, kinase assays were performed using purified recombinant c-Src with MBP-PLSCR1, 2, 3, or 4 as a substrate. Proteins were resolved on a 10% SDS-PAGE gel and the phosphorylated proteins were detected by Western blotting with HRP-conjugated anti-Tyr(P) mAb PY99 (top panel). MBP-PLSCR1 was detected with anti-MBP (bottom panel). Data shown are representative of 2 experiments performed.

**Fig. 4.** Tyrosine residues in the tandem repeat sequence GNYQVNYQNP77 of PLSCR1 are required for phosphorylation by c-Src. A, purified recombinant c-Src was used in kinase assays with either wild-type MBP-PLSCR1 or mutant MBP-Y69F/Y74F-PLSCR1 as substrate. Proteins were resolved on a 10% SDS-PAGE gel, and the phosphorylated proteins detected by Western blotting (WB) with HRP-conjugated anti-Tyr(P) mAb PY99 (top panel). MBP-PLSCR1 was detected with anti-MBP (bottom panel). Data shown are representative of 2 experiments performed. B, PLSCR1−/− KECs expressing either wild-type hPLSCR1, mutant Y69F/Y74F hPLSCR1, or vector alone were stimulated with 100 ng/ml EGF for 5 min. Cell lysates were prepared, and Shc was immunoprecipitated with anti-Shc polyclonal antibody (upper panel). Abundance of immunoprecipitated p66 Shc protein was detected with anti-Shc polyclonal antibody (lower panel). Data shown are representative of 3 experiments performed.

**Fig. 5.** EGF-dependent association between PLSCR1 and Shc requires phosphotyrosine residues Tyr69 and Tyr74 of PLSCR1. PLSCR1−/− KEC expressing either wild-type or Y69F/Y74F hPLSCR1 were stimulated with 100 ng/ml EGF for times shown. Cell lysates were prepared, and Shc was immunoprecipitated with anti-Shc monoclonal antibody. The amount of PLSCR1 co-immunoprecipitated with Shc was detected by Western blotting (WB) with anti-PLSCR1 (upper panel). Phosphorylation of Shc proteins was detected with anti-Tyr(P) antibody (middle panel). Abundance of immunoprecipitated p66 Shc protein was detected with anti-Shc polyclonal antibody (lower panel). Data shown are representative of 3 experiments performed.

**DISCUSSION**

We have previously shown that upon EGF stimulation, PLSCR1 is Tyr phosphorylated and associates with both the Shc adaptor protein and the EGF receptor (7). The present study establishes that 1) the EGF-induced phosphorylation of PLSCR1 involves a Src kinase, 2) the sites of Src phosphorylation in PLSCR1 involve Tyr69 and Tyr74; 3) the interaction between PLSCR1 and Shc requires phosphorylation of Tyr69 and Tyr74 on PLSCR1; 4) Shc interacts with phosphorylated PLSCR1 through its PTB domain, and 5) deletion of PLSCR1 substantially attenuates the growth factor-dependent activation of c-Src, suggesting that PLSCR1 normally facilitates c-Src activation through EGF-R and potentially other related growth factor receptors.

Because many of Src’s substrates bind through either the SH2 or SH3 domains (15, 16, 21–23), we postulated that PLSCR1 might interact with Src in a similar manner, as it contains several PXXP motifs that could potentially serve as binding sites for the SH3 domain of Src. Nevertheless, we were unable to demonstrate association of PLSCR1 with Src through co-immunoprecipitation studies (data not shown), suggesting either a low affinity interaction that is disrupted under the conditions of immunoprecipitation, or that another adaptor molecule may be required as a bridge between PLSCR1 and Src.
PLSCR1 binds to Shc through the PTB domain in vitro. PLSCR1 was phosphorylated in vitro using purified recombinant c-Src (see “Experimental Procedures”). After incubation of either phosphorylated or unphosphorylated PLSCR1 with Shc domains immobilized on glutathione 4B-Sepharose beads, an aliquot of the supernatant was retained and the bound proteins were eluted. These samples were subjected to Western blot analysis. Phosphorylated PLSCR1 was detected with anti-Tyr(P) antibody, and total PLSCR1 with anti-PLSCR1 mAb 4D2. Amounts of GST fusion proteins immobilized on Sepharose 4B beads were detected by Ponceau stain. Stars denote GST-fusion proteins of correct molecular weight (p52 and CH domain contained proteolytic breakdown products) (29). Data shown are representative of 2 experiments performed.

Upon EGF stimulation, increased phosphorylation of PLSCR1 and its association with both EGF-R and the adaptor protein Shc was observed (7). Our present data now suggest that Tyr<sup>69</sup> and Tyr<sup>74</sup> of PLSCR1, which are required for phosphorylation by Src, are also required for PLSCR1’s interaction with Shc (Fig. 5), and we demonstrate that phosphorylated PLSCR1 interacts with Shc through Shc’s PTB domain (Fig. 6). Because tyrosine phosphorylation is a reversible, dynamic process controlled by the activities of protein kinases and the competing actions of protein tyrosine phosphatases, many SH2/PTB domain-phosphotyrosine-mediated interactions are short-lived. This is also the case for EGF-induced phosphorylation of PLSCR1 (Fig. 1), and its consequent recruitment into the EGF-R signaling complex mediated through its transient association with Shc (Fig. 6). Although we also noted some binding of unphosphorylated PLSCR1 to the PTB domain of Shc in <i>in vitro</i> pull down assays using recombinantly expressed proteins, such interaction does not appear to occur to a significant extent within the cell (Fig. 5). Because Shc isoforms are predominantly localized to the cytoplasm in quiescent cells and translocate to the plasma membrane only upon EGF stimulation (24, 25), increased association of plasma membrane-bound Shc with membrane PLSCR1 after EGF stimulation might be expected simply due to their increased proximity. Their interaction appears to be not only dependent on co-localization at the plasma membrane, but also on phosphorylation of PLSCR1 at Tyr<sup>69</sup> and Tyr<sup>74</sup>, because intracellular association of mutant Y69F/ Y74F PLSCR1 with Shc was not observed (Fig. 5). One possibility is that upon EGF stimulation, phosphorylated PLSCR1 initially recruits Shc to the plasma membrane through this interaction with the Shc PTB domain, and then promotes transfer of Shc to its phosphotyrosine-binding site in EGF-R (Tyr<sup>1173</sup>/Tyr<sup>1148</sup>) in EGF-R) (26). Alternatively, phosphorylated PLSCR1 might serve to displace Shc from the activated receptor through its competition for the Shc PTB domain, and thereby promote throughput of new substrate that is delivered to the receptor kinase.

It is of interest that in addition to attenuated activation of c-Src upon EGF stimulation, cells deficient in PLSCR1 consistently exhibited reduced basal Src kinase activity (Fig. 7). Presumably, this either reflects incomplete receptor inactivation through serum starvation, or some direct effect of PLSCR1 on the constitutive activity of c-Src. In this context, plasma membrane-recruited Shc has recently been implicated in the direct activation of c-Src (27). One possibility is that the small pool of PLSCR1 that is constitutively phosphorylated by c-Abl on Tyr<sup>69</sup> and Tyr<sup>74</sup> (∼1) may serve to recruit Shc to the plasma membrane in quiescent cells, thereby facilitating interactions between Shc and membrane-localized c-Src, increasing basal Src kinase activity. Upon EGF stimulation, further activation of c-Src is thought to be promoted through interaction of phosphorylated EGF-R with the Src SH2 domain (14). This activated c-Src is then capable of phosphorylating EGF-R (15), Shc (28), and PLSCR1 (Figs. 1–4). After EGF stimulation, the increased pool size of phosphorylated PLSCR1 may then recruit more Shc to the plasma membrane, leading to further activation of Src by Shc. Thus, a reduced amount of plasma membrane-localized Shc might account for the attenuated activity of c-Src observed in the PLSCR1 deficient cells.

As previously noted, cells deficient in PLSCR1 have been
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observed to exhibit defects in proliferative and maturational responses to selective growth factors, suggesting that this protein might play some role in signaling through growth factor receptors (9). Our present data suggest that at least one explanation for the phenotype that has been observed in PLSCR1−/− cells relates to a specific role of PLSCR1 in promoting the kinase activity of cellular c-Src.

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