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From the Departments of Molecular and Experimental Medicine and Vascular Biology, The Scripps Research Institute, La Jolla, California 92037 and the Division of Biology and the Cancer Center, University of California at San Diego, La Jolla, California 92093

Jun Sun, Ji Zhao, Martin A. Schwartz, Jean Y. J. Wang, Therese Wiedmer, and Peter J. Sims

Phospholipid scramblase 1 (PLSCR1) is a plasma membrane protein that has been proposed to play a role in the transbilayer movement of plasma membrane phospholipids. PLSCR1 contains multiple proline-rich motifs resembling Src homology 3 (SH3) domain-binding sites. An initial screen against 13 different SH3 domains revealed a marked specificity of PLSCR1 for binding to the Abl SH3 domain. Binding between intracellular PLSCR1 and c-Abl was demonstrated by co-immunoprecipitation of both proteins from several cell lines. Deletion of the proline-rich segment in PLSCR1 (residues 1–118) abolished its binding to the Abl SH3 domain. PLSCR1 was Tyr-phosphorylated by c-Abl in vitro. Phosphorylation was abolished by mutation of Tyr residues Tyr69/Tyr74 within the tandem repeat sequence VYNQPYP77 of PLSCR1, implying that these residues are the likely sites of phosphorylation. Cellular PLSCR1 was found to be constitutively Tyr-phosphorylated in several cell lines. The Tyr phosphorylation of PLSCR1 was increased upon overexpression of c-Abl and significantly reduced either upon cell treatment with the Abl kinase inhibitor STI571, or in Abl−/− mouse fibroblasts, suggesting that cellular PLSCR1 is a normal substrate of c-Abl. Cell treatment with the DNA-damaging agent cisplatin activated c-Abl kinase and increased Tyr phosphorylation of PLSCR1. The cisplatin-induced phosphorylation of PLSCR1 was inhibited by STI571 and was not observed in Abl−/− fibroblasts. These findings indicate that c-Abl binds and phosphorylates PLSCR1, and raise the possibility that an interaction between c-Abl and plasma membrane PLSCR1 might contribute to the cellular response to genotoxic stress.

Phospholipid scramblase 1 (PLSCR1) is a member of a newly identified family of calcium-binding proteins that has been implicated in the transbilayer movement of plasma membrane phospholipids (PLs) under conditions of elevated cytoplasmic calcium (1, 2). The properties of PLSCR1 suggest its potential function in the redistribution of membrane PLs that plays a central role in activation of complement and coagulation systems of blood plasma and in recognition and phagocytic clearance of injured, aged or apoptotic cells (3–6). Four human PLSCR genes (HuPLSCR1–4) and their putative mouse orthologues (MuPLSCR1–4) have been identified (7). The PLSCR genes are conserved through evolution with recognizable orthologues in the Drosophila and Caenorhabditis elegans genomes.

The deduced amino acid sequence of HuPLSCR1 reveals a type-2 membrane protein containing 318 residues (35.1 kDa), with a predicted transmembrane helix (Lys289-Glu306) near the C terminus (1). The cytoplasmic domain includes an N-terminal proline-rich region (Met1-Pro97), an EF-hand-like Ca2+ binding segment (Asp273-Asp284), and a predicted PKC phosphorylation site (Thr161). Upon binding Ca2+, PLSCR1 undergoes a major conformational change and can mediate accelerated transbilayer movement of PLs in proteoliposomes containing this protein (8). There is also evidence that the activity of PLSCR1 may be regulated through palmitoylation at Cys thiols (9) and by phosphorylation at Thr161 by PKCδ (10). Phosphorylation of PLSCR1 by cellular PKCδ has recently been implicated in promoting the transbilayer movement of plasma membrane PLs that results in cell surface exposure of PS in apoptotic cells (10). Expression of cellular PLSCR1 is highly induced by IFNs, although the biologic significance of the marked transcriptional up-regulation of PLSCR1 by IFNs remains unknown (11). In particular, increased expression of PLSCR1 following IFN treatment was not accompanied by any detectable change in transbilayer distribution of plasma membrane PS, nor was any increase in transbilayer PL scrambling in response to either Ca2+ ionophore or to induction of apoptosis observed (11). This raises the question of whether the PL scramblase activity of PLSCR1 is under regulation by an obligatory co-factor that is unaffected by IFN, or whether the PL scramblase activity of the plasma membrane might be mediated by another factor. In mouse, a spontaneous mutation in PLSCR1 (designated TRA1) resulting in N-terminal truncation through codon 118 was associated with a leukemogenic transformation of monocytes, whereas a down-regulation of mutant TRA1 expression with induction of expression of the wild-type gene was found to occur upon induced differentiation of the leukemogenic monocytes to terminal macrophages (12). How this apparent association of a truncation mutation in PLSCR1 with leukemogenic transformation might relate to the putative

PDGF, platelet-derived growth factor; DNA-PK, DNA-dependent protein kinase; mAb, monoclonal antibody.
role of this protein in apoptotic cells or to its effects on membrane PLs also remains unknown.

The N-terminal segment of PLSCR1 that is deleted in the TRA1 mutation is notable for being highly proline-rich and containing multiple PXXP motifs that might serve as binding sites for cellular proteins containing SH3 domains (13). This N-terminal segment of the PLSCR1 polypeptide is also notable for the relatively low conservation of amino acid sequence when the PLSCR1-4 polypeptides are aligned (7). Here we report evidence that c-Abl binds to this segment of PLSCR1 and phosphorylates PLSCR1 at two tyrosine residues (Tyr<sup>69</sup>Tyr<sup>74</sup>) within a tandem repeat sequence in the N-terminal region of PLSCR1. The Tyr phosphorylation of PLSCR1 is Abl-dependent and is induced by the DNA-damaging agent cisplatin.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The murine anti-PLSCR1 mAbs ID4, 4D2, and 219.1 were produced in our laboratory. ID4 and 4D2 recognize human PLSCR1, whereas 219.1 recognizes both human and murine PLSCR1. Rabbit anti-human PLSCR1 polyclonal antibody was raised against a synthetic peptide of human PLSCR1, and rabbit anti-murine PLSCR1 polyclonal antibody was raised against the N terminus of murine PLSCR1. The anti-Abl mAb 24-11, anti-Abl polyclonal antibody K-12, and anti-Tyr(P) mAb PY99 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Abl mAb 8E9 was purchased from PharMingen (San Diego, CA), and anti-MBP serum was from New England Biolabs (Beverly, MA). Appropriate horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Glutathione-Sepharose 4B and Protein G-Sepharose 4 Fast Flow were purchased from Amersham Pharmacia Biotech. The pSLX-c-Abl vector and the artificial Abl substrate GST-CDT-CRK fusion protein construct were described earlier (14). The c-Abl kinase domain fusion protein was kindly provided by Dr. W. Todd Miller (State University of New York at Stony Brook). Sera from Elizabeth Buchdunger (Novartis Pharma AG, Switzerland) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cells and Transfection—The Abl—/− mouse fibroblasts, the Abl+/+ mouse fibroblasts that were reconstituted with c-Abl cDNA by retroviral infection of the Abl−/− fibroblasts (15), and human embryonic kidney 293T cells were transfected with MBP-cDNA or MBP-Abl kinase domain fusion protein expressed in E. coli and was used to Tyr phosphorylate PLSCR1 in vitro. A total volume of 20 μl of reaction mixture, 4 μg of either MBP-PLSCR1 or GST-CDT-CRK fusion protein was used as substrate in kinase assay buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol (17). The reactions were initiated by addition of 1 μl of 0.8 μM ATP containing 20 μCi of [γ-<sup>32</sup>P]ATP or 1 mM cold ATP. After 30 min of incubation at room temperature, the reactions were terminated by adding SDS-PAGE sample buffer and boiling for 5 min. The proteins were resolved by SDS-PAGE and phosphorylation was detected by autoradiography or Western blotting with anti-Tyr(P) mAb PY99.

Cell lysates containing 100–500 μg of protein were preincubated with 20 μl of a 50% slurry of Protein G-Sepharose 4 Fast Flow for 30 min with gentle rotation. The beads were washed with 2–10 μl of antibody for 2 h on ice and 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 1,000 × g for 2 min and washed extensively. Immunoprecipitates were either stored at −80 °C for use in the kinase assays, or solubilized with SDS-PAGE sample buffer (Ferpi, Rockford, IL).

**RESULTS**

**PLSCR1 Binds to the SH3 Domain of the Abl Tyrosine Kinase**—The deduced amino acid sequence of human PLSCR1 reveals that its cytoplasmic segment contains a proline-rich segment (residues 1–118), which includes multiple PXXP motifs with potential to interact with protein SH3 domains. In order to gain initial insight into what cellular SH3-containing proteins might interact with PLSCR1 through this proline-rich segment of the polypeptide, we screened 13 different SH3 domains expressed as GST fusion proteins for their ability to specifically bind to PLSCR1, as expressed in the fusion construct MBP-PLSCR1 (Fig. 1). This initial screen revealed a marked specificity of PLSCR1 for binding to the SH3 domain of the Abl tyrosine kinase, with >25% of the input MBP-PLSCR1 captured. We were unable to detect specific binding of PLSCR1 to...
any of the other SH3 fusion constructs tested, with the exception of a comparatively minor interaction with the spectrin SH3 domain. This apparent avidity of the MBP-PLSCR1 fusion construct for selectively binding to the Abl SH3 domain was also confirmed for native PLSCR1, purified from human erythrocyte membranes (data not shown).

**Plasma Membrane PLSCR1 Forms an Intracellular Complex with c-Abl**—The potential for the cellular PLSCR1 and Abl proteins to interact was next explored by immunoprecipitation of the proteins from detergent lysates of 293T cells co-transfected with plasmids containing PLSCR1 and c-Abl cDNAs. As shown in Fig. 2A, immunoprecipitation performed using mAb 1D4 specific for PLSCR1 resulted in the co-precipitation of c-Abl antigen, whereas immunoprecipitation performed using mAb 24–11 specific for c-Abl resulted in the co-precipitation of PLSCR1 antigen. In various experiments, ~1–5% of total cellular PLSCR1 and c-Abl were found to co-immunoprecipitate. Similar results were obtained by immunoprecipitation of c-Abl and PLSCR1 from the Raji S5 human cell line (data not shown).

The Philadelphia chromosome of chronic myelogenous leukemia produces the Bcr-Abl fusion oncoprotein with intact Abl SH3 domain. The physical association of PLSCR1 with Bcr-Abl was examined in K562 cells. Both Bcr-Abl and c-Abl were detected in the immunoprecipitated proteins with the cA polyclonal antibody against PLSCR1 (Fig. 2B). These results suggest that plasma membrane PLSCR1 forms an intracellular complex with cytoplasmic c-Abl and Bcr-Abl, presumably through interaction of the Abl SH3 domain with one or more PXXP motifs contained within the endofacial N-terminal segment of PLSCR1.

**c-Abl Tyrosine Kinase Phosphorylates PLSCR1 in Vitro**—The physical association of PLSCR1 and Abl proteins in mammalian cells suggested that PLSCR1 might also be a substrate for Abl tyrosine kinase. To test whether c-Abl has the capacity to phosphorylate PLSCR1, in vitro kinase assays were first performed using full-length c-Abl immunoprecipitated from c-Abl-transfected 293T cells. Under these conditions, c-Abl exhibited constitutive tyrosine kinase activity, resulting in phosphorylation of both the Abl model substrate GST-CTD-CRK and human PLSCR1 purified from erythrocyte membranes. No phosphorylation of PLSCR1 was observed in the control

**Immunoprecipitation (Fig. 3, left panel).** The phosphorylation of PLSCR1 on the Tyr residue(s) was confirmed by Western blotting using mAb PY99 specific for Tyr(P) (data not shown). By contrast, a recombinant construct containing only the c-Abl kinase domain phosphorylated the model substrate GST-CTD-CRK but failed to phosphorylate PLSCR1 from erythrocytes (Fig. 3, right panel). These results suggest that an interaction of PLSCR1 with another domain of c-Abl, presumably the SH3 domain, is required for its efficient phosphorylation by the enzyme.

The proline-rich N-terminal peptide of PLSCR1 (residues 1–118) was confirmed as the portion of the protein that is responsible for binding to the Abl SH3 domain, and as the segment of PLSCR1 that is also phosphorylated by c-Abl kinase (Fig. 4). Consistent with this interpretation, deletion of this segment of PLSCR1 abolished its binding to the Abl SH3 domain as well as abrogated phosphorylation of PLSCR1 by c-Abl (Fig. 4). Although the SH3 domain-binding site in PLSCR1 remains to be mapped, it presumably encompasses one or more of the five PAXP motifs within the segment spanning residues 1–118.

**Tyr Residues within the Tandem Repeat Sequence** 68VYN-QPVYNQQP77 of PLSCR1 Are Required for Phosphorylation by c-Abl—Analysis of the efficiency of Abl kinase for various peptide substrates has suggested a potential consensus sequence for the Abl-preferred phosphorylation site of -IYA- (Fig. 4). Whereas the PLSCR1 polypeptide contains no exact match to this putative Abl substrate consensus sequence, it does contain homologous sequence within the tandem repeat 68VYN-QPVYNQQP77. This suggested that Tyr69 and Tyr74 were the most likely site(s) of tyrosine phosphorylation by Abl kinase. We generated MBP-PLSCR1 fusion proteins containing double...
fusion proteins Met1-Trp318, Met1-Glu118, and Val119-Trp318 were present 25% of protein used for binding assays. Purified MBP-fusion proteins were loaded as input, subjected to SDS-PAGE, and detected by Western blotting with anti-MBP antibody. Bound proteins were eluted from beads, subjected to SDS-PAGE, and detected by Western blotting with anti-MBP antibody. Protein phosphorylation was detected by autoradiography.

FIG. 3. PLSCR1 is phosphorylated by full-length c-Abl but not by active recombinant Abl kinase domain in vitro. 293T cells were transfected with pSLX-c-Abl for 24 h and the cell lysates were immunoprecipitated with anti-Abl mAb 24-11 using normal mouse IgG as control. The immunoprecipitated full-length c-Abl (left panel) and a recombinant Abl kinase domain (right panel) were used to phosphorylate PLSCR1 purified from erythrocyte or the Abl-specific artificial substrate GST-CTD-CRK in kinase assays with [γ-32P]ATP. Proteins were resolved on a 12% Tris glycine Novex gel, and the phosphorylated proteins were detected by autoradiography.

FIG. 4. The N-terminal region M1-E118 of PLSCR1 binds to the Abl SH3 domain and is phosphorylated by c-Abl. The full-length Met1-Trp318, the N-terminal region Met1-Glu118 and the N-terminal truncation Val119-Trp318 of PLSCR1 were produced as MBP fusion proteins. A, the MBP fusion proteins were incubated with immobilized GST or GST-Abl SH3 domain fusion proteins as described under “Experimental Procedures.” Bound proteins were eluted from beads, subjected to SDS-PAGE, and detected by Western blotting with anti-MBP antibody. Purified MBP-fusion proteins were loaded as Input, and represent 25% of protein used for binding assays. B, the MBP-PLSCR1 fusion proteins Met1-Trp318, Met1-Glu118 and Val119-Trp318 were phosphorylated in vitro by c-Abl with [γ-32P]ATP. Equal input of MBP fusion proteins was confirmed by Western blotting with an anti-MBP antibody. Protein phosphorylation was detected by autoradiography.

FIG. 5. Tyr residues within the tandem repeat sequence 68VYNQPVYNQP77 of PLSCR1 are required for phosphorylation by c-Abl. The full-length Met1-Trp318, the Y69F/Y74F, Y69F, and Y74F mutations of PLSCR1 were produced as MBP fusion proteins. A, the MBP fusion proteins were incubated with immobilized GST or GST-Abl SH3 domain fusion proteins. Bound proteins were eluted from beads, subjected to SDS-PAGE, and detected by Western blotting with anti-MBP antibody. Purified MBP fusion proteins were loaded as Input, and represent 25% of protein used for binding assays. B, the MBP-PLSCR1 fusion proteins Met1-Trp318, Y69F/Y74F, Y69F, and Y74F were phosphorylated in vitro by c-Abl. Equal input of MBP fusion proteins was confirmed by Western blotting with an anti-MBP antibody. Protein phosphorylation was detected by Western blotting with the anti-Tyr(P) mAb PY99.

FIG. 6. Cellular PLSCR1 is constitutively Tyr-phosphorylated, and this phosphorylation markedly increases with overexpression of c-Abl. 293T cells were transfected with pSLX-c-Abl or vector control. Cells were harvested 24 h after transfection and extracted with lysis buffer containing 1% Nonidet P-40. Cell extracts were immunoprecipitated with anti-PLSCR1 mAb ID4 using normal mouse IgG as control. Tyr phosphorylation of PLSCR1 was detected by Western blotting with the anti-Tyr(P) mAb PY99 (upper panel). Equal loading of PLSCR1 was confirmed by Western blotting with anti-PLSCR1 mAb 4D2 (lower panel).

or single Tyr to Phe mutations at Tyr69 and/or Tyr74. As shown in Fig. 5, these MBP-PLSCR1 fusion proteins did not affect binding between Abl SH3 domain and PLSCR1. By contrast, mutation at either residue Tyr69 or Tyr74 in this segment of PLSCR1 markedly reduced phosphorylation by c-Abl, and the phosphorylation by c-Abl was eliminated when both residues Tyr69 and Tyr74 were mutated to phenylalanine. These results suggested residues Tyr69 and Tyr74 as the sites of phosphorylation by c-Abl. Alternatively, Tyr69 and Tyr74 are required for c-Abl-mediated phosphorylation of PLSCR1 at other Tyr residues within the polypeptide.

PLSCR1 Is Tyrosine-phosphorylated by c-Abl in Mammalian Cells—To explore whether intracellular PLSCR1 might also be Tyr-phosphorylated by either c-Abl or by other tyrosine kinase(s), PLSCR1 was immunoprecipitated from 293T cells and subjected to SDS-PAGE. The presence of Tyr phosphorylation in the PLSCR1 polypeptide was determined by Western blotting with PY99 antibody (Fig. 6). The extent of Tyr(P) modification detected in cellular PLSCR1 was markedly increased by prior transfection with cDNA for c-Abl (Fig. 6). Constitutive Tyr phosphorylation was also detected in PLSCR1 immunoprecipitated from Jurkat, K562, and Raji cells (data not shown). Whereas phosphorylation of PLSCR1 by activated protein kinase Cδ has been implicated to promote accelerated transbilayer movement of plasma membrane PS, we were unable to detect any change in cell surface exposure of PS in response to the Tyr phosphorylation of plasma membrane PLSCR1 by c-Abl (data not shown).

PLSCR1 is constitutively Tyr-phosphorylated and the extent of this phosphorylation is further increased by overexpression of c-Abl, implying that PLSCR1 might be a normal substrate of endogenous c-Abl kinase. In order to explore this possibility, we first determined whether the basal Tyr phosphorylation detected in PLSCR1 was affected by prior treatment of the cells with the Abl kinase inhibitor STI571. STI571 is a 2-phenylaminopyrimidine derivative that exhibits selectivity in inhibiting the Abl tyrosine kinase. STI571 has been reported to be essentially inactive against all Ser/Thr kinases and most Tyr kinases tested, with the exception of known inhibitory activity toward the PDGF receptor kinase and c-kit kinase (19, 20). As shown in Fig. 7A, prior incubation of 293T cells with this inhibitor effectively abrogated Tyr phosphorylation detected in PLSCR1, implying that PLSCR1 is constitutively
phosphorylated by c-Abl, or by another tyrosine kinase inactivated by STI571. Similar results were obtained in Jurkat T and Raji S5 cells (data not shown).

The direct role of c-Abl in regulating Tyr phosphorylation of PLSCR1 was next examined in murine 3T3 cells deficient in the c-Abl kinase. Endogenous PLSCR1 was immunoprecipitated from either Abl+/+ or Abl−/− fibroblasts using antibody 4720 specific for murine PLSCR1, resolved by SDS-PAGE, and probed with either anti-Tyr(P) mAb PY99, or anti-PLSCR1 mAb 219.1 (Fig. 7B). As these data indicate, Tyr phosphorylation of PLSCR1 detected in the Abl+/+ fibroblasts was virtually abolished in the Abl−/− fibroblasts, implying that the c-Abl kinase accounts for most, if not all, of the Tyr phosphorylation of PLSCR1 detected in mammalian cells. Due to the relatively low level of Tyr phosphorylation detected in murine PLSCR1, in these experiments 0.1 mM pervanadate was added to the cell cultures prior to cell lysis and immunoprecipitation (see legend, Fig. 7).

Tyr Phosphorylation of PLSCR1 Is Induced by the DNA-damaging Agent Cisplatin. c-Abl kinase is activated in cells exposed to cisplatin for 2 h in the presence of 10 μM STI571 that was added 2 h before addition of cisplatin. Cells were harvested and extracted with lysis buffer containing 1% Nonidet P-40. Cell extracts were immunoprecipitated with anti-PLSCR1 mAb 1D4. Tyr phosphorylation of PLSCR1 was detected by Western blotting with anti-Tyr(P) mAb PY99 (top panel, A) and abundance of immunoprecipitated PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (lower panel, A). Raji S5 cells were treated either without or with 50 μM cisplatin for 2 h. Cells were harvested and extracted with lysis buffer containing 1% Nonidet P-40. c-Abl was immunoprecipitated with anti-Abl antibody K-12 and subjected to kinase assays using GST-CTD-CRK as substrate. Tyr phosphorylation of GST-CTD-CRK was detected by Western blotting with an anti-Tyr(P) mAb PY99 (upper panel, B). Abundance of c-Abl protein in the kinase assays was detected with anti-Abl mAb 8E9 (lower panel, B).

DISCUSSION

Our data establish that the Abl SH3 domain binds to the N-terminal proline-rich segment of PLSCR1 and that c-Abl phosphorylates Tyr residues of the PLSCR1 polypeptide, most likely Tyr69 and Tyr74 within the tandem repeat sequence 68VYNQPVYNQP78. The c-Abl tyrosine kinase appears to maintain a basal level of Tyr phosphorylation of plasma membrane PLSCR1 at steady state, and this level is increased by exposure of cells to genotoxic stress. Although the functional implications of this interaction remain to be clarified, it is of interest to note that both c-Abl and PLSCR1 have been implicated in apoptosis, whereas mutations affecting putative regulatory domains within both c-Abl and PLSCR1 have been as-

FIG. 7. Tyr phosphorylation of PLSCR1 is Abl-dependent. A, 293T cells were incubated in the presence or absence of 10 μM Abl kinase inhibitor STI571 for 18 h. During the last 20 min, cells were treated with 0.1 mM pervanadate for 20 min. Cell extracts were immunoprecipitated with anti-PLSCR1 mAb ID4. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane. Tyr phosphorylation of PLSCR1 was detected by anti-Tyr(P) mAb PY99 (upper panel, A). Abundance of loaded PLSCR1 was detected with anti-PLSCR1 mAb 4D2 (lower panel, A). B, Abl+/+ and Abl−/− mouse fibroblasts were treated with 0.1 mM pervanadate for 20 min. Cell lysates were immunoprecipitated with rabbit anti-murine PLSCR1 antibody 4720. Tyr phosphorylation of PLSCR1 was detected by Western blotting with anti-Tyr(P) mAb PY99 (top panel, B). Abundance of loaded PLSCR1 was detected with anti-PLSCR1 mAb 219.1 (middle panel, B). Expression of c-Abl in the fibroblasts was confirmed by Western blotting of total cell lysates with anti-Abl mAb 8E9 (bottom panel, B).

FIG. 8. Tyr phosphorylation of PLSCR1 is induced by the DNA-damaging agent cisplatin. A, Raji S5 cells were treated either without or with 50 μM cisplatin for 1, 2, or 4 h, or treated with 50 μM cisplatin for 2 h in the presence of 10 μM STI571 that was added 2 h before addition of cisplatin. Cells were harvested and extracted with lysis buffer containing 1% Nonidet P-40. Cell extracts were immunoprecipitated with anti-PLSCR1 mAb ID4. Tyr phosphorylation of PLSCR1 was detected by Western blotting with anti-Tyr(P) mAb PY99 (upper panel, A) and abundance of immunoprecipitated PLSCR1 protein was detected with anti-PLSCR1 mAb 4D2 (lower panel, A). B, Raji S5 cells were treated either without or with 50 μM cisplatin for 2 h. Cells were harvested and extracted with lysis buffer containing 1% Nonidet P-40. c-Abl was immunoprecipitated with anti-Abl antibody K-12 and subjected to kinase assays using GST-CTD-CRK as substrate. Tyr phosphorylation of GST-CTD-CRK was detected by Western blotting with an anti-Tyr(P) mAb PY99 (upper panel, B). Abundance of c-Abl protein in the kinase assays was detected with anti-Abl mAb 8E9 (lower panel, B).
cisplatin is Abl-dependent. 

Abl+/- and Abl+/+ mouse fibroblasts were treated with or without 50 μM cisplatin for 2 h. During the last 20 min, cells were treated with 0.1 mM pervanadate. Cell lysates were immunoprecipitated with rabbit anti-murine PLSCR1 antibody 4720. Tyr phosphorylation of PLSCR1 was detected by Western blotting with anti-Tyr(P) mAb PY99 (upper panel). Abundance of immunoprecipitated PLSCR1 protein in each lane was detected with anti-PLSCR1 mAb 219.1 (lower panel).

**FIG. 9.** The induction of Tyr phosphorylation of PLSCR1 by cisplatin is Abl-dependent. Abl+/+ and Abl+/- mouse fibroblasts were treated with or without 50 μM cisplatin for 2 h. During the last 20 min, cells were treated with 0.1 mM pervanadate. Cell lysates were immunoprecipitated with rabbit anti-murine PLSCR1 antibody 4720. Tyr phosphorylation of PLSCR1 was detected by Western blotting with anti-Tyr(P) mAb PY99 (upper panel). Abundance of immunoprecipitated PLSCR1 protein in each lane was detected with anti-PLSCR1 mAb 219.1 (lower panel).

Associated with oncogenic transformation and cell proliferation (10, 12, 22–24).

As previously described, PLSCR1 has been shown to promote a Ca2+- and pH-dependent transbilayer movement of PLs in liposomes reconstituted with this protein that mimics the intrinsic PL scramblase activity of the plasma membrane (8, 25). However, the identity of PLSCR1 as the protein that mediates the intrinsic PL scramblase activity of the plasma membrane remains unresolved. Whereas our results suggest an interesting link between c-Abl tyrosine kinase and PLSCR1, tyrosine phosphorylation of PLSCR1 does not appear to directly stimulate plasma membrane PL scramblase activity. Moreover, the rapid increase in PLSCR1 tyrosine phosphorylation following cisplatin exposure does not temporally correlate with the delayed apoptotic response and consequent cell surface PS exposure in cisplatin-treated cells. Thus, either the PL scramblase activity of PLSCR1 is regulated through mechanism(s) other than Tyr phosphorylation by c-Abl, or accelerated transbilayer movement of plasma membrane PLs may be mediated by another protein, possibly another member of the PLSCR gene family. Potentially divergent biological roles of the various members of the PLSCR gene family is suggested by marked differences in their tissue distribution (7), by the marked differences in amino acid sequence in the proline-rich N-terminal segments of these proteins (7), and by the observation that only PLSCR1 appears to be under transcriptional regulation by IFN among the four known members of the human PLSCR gene family.2 In this context, it is also of interest to note that the tandem repeat sequence ENYQQPVYNDQ of PLSCR1 is not found in the other PLSCR family members, and we were unable to detect any evidence for Tyr phosphorylation of PLSCR2–4 by Abl (data not shown).

c-Abl tyrosine kinase was identified as the cellular homologue of the Gag-v-Abl oncogene in Abelson murine leukemia virus (26). The c-Abl gene is ubiquitously expressed and encodes two 145-kDa isoforms differing in their N termini (27, 28). The human c-Abl gene on chromosome 9 is translocated to form the Philadelphia chromosome that is characteristic of chronic myelogenous leukemia (29). The Philadelphia chromosome produces an abnormal Bcr-Abl fusion protein with a constitutively active tyrosine kinase (30). In leukemia cells, Bcr-Abl tyrosine kinase has been linked to a large number of signal transduction pathways that are normally regulated by receptor tyrosine kinases (24). By contrast to the transforming properties of v-Abl and Bcr-Abl, overproduction of c-Abl does not transform cells, but rather can inhibit cell growth and induce cell death (31, 32).

The c-Abl protein is localized in both nucleus and cytoplasm (33). As expected of a cytoplasmic tyrosine kinase, c-Abl has been linked to the transduction of extracellular signals through interaction with cell surface receptors. The c-Abl tyrosine kinase is regulated by integrins (17), as well as the platelet-derived growth factor (34). The precise function of c-Abl in these receptor-regulated signaling is not understood. The c-Abl tyrosine kinase contains three nuclear localization signals and a nuclear export signal, and it can shuttle between the nuclear and extranuclear compartments (35). Recent results have suggested a role for the nuclear c-Abl tyrosine kinase in the regulation of apoptosis (22). Indeed, even the oncogenic Bcr-Abl tyrosine kinase can activate apoptosis when it is trapped in the nucleus of leukemia cells (36). The pro-apoptotic function of nuclear Abl tyrosine kinase has been shown to contribute to the cytotoxic activity of cisplatin, presumably through the activation of p73 (14, 37), which is a p53-independent inducer of apoptosis (38, 39). Our results indicated that the Tyr phosphorylation of PLSCR1 by c-Abl was also substantially increased by cisplatin treatment implying an interaction with activated c-Abl kinase that translocates to the plasma membrane under these conditions. It remains to be determined how this interaction between PLSCR1 and activated c-Abl kinase contributes to the overall cellular response to DNA damage.

Mutations of both c-Abl and PLSCR1 have been associated with oncogenic transformation. Deletion of the N-terminal proline-rich region (codons 1–118) of PLSCR1 resulted in the loss of binding to the Abl SH3 domain (Fig. 4). Such truncation of PLSCR1 mimics a spontaneous mutation in murine PLSCR1 (designated as TRA1), which has been linked to a leukemogenic transformation in the MM monocytic cell line (12). In this study, transfection of cDNA encoding this mutant PLSCR1 to nonleukemogenic monocyteic cells was reported to induce a leukemogenic transformation of these cells (12). The actual role of PLSCR1 in the regulation of cell proliferation, and how this might relate to interaction with the Abl kinases, also remains to be explored. Both of the oncogenic proteins Bcr-Abl and v-Abl show an extranuclear localization (33). Myristoylation of the v-Abl polypeptide has been suggested to facilitate its transforming activity for fibroblasts, implying a possible relationship of a membrane localization to its transforming potential (40). It is therefore conceivable that PLSCR1 might modulate the transforming activities of extranuclear Abl kinases by affecting localization within the cell, including access to other substrates within the plasma membrane.

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