

Phospholipid Scramblase 1 Modulates a Selected Set of IgE Receptor-mediated Mast Cell Responses through LAT-dependent Pathway^{*S}

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Engagement of the IgE receptor (FcεRI) on mast cells leads to the release of preformed and newly formed mediators as well as of cytokines. The signaling pathways responsible for these responses involve tyrosine phosphorylation of multiple proteins. We previously reported the phosphorylation on tyrosine of phospholipid scramblase 1 (PLSCR1) after FcεRI aggregation. Here, PLSCR1 expression was knocked down in the RBL-2H3 mast cell line using short hairpin RNA. Knocking down PLSCR1 expression resulted in significantly impaired degranulation responses after FcεRI aggregation and release of vascular endothelial growth factor, whereas release of MCP-1 was minimally affected. The release of neither leukotriene C4 nor prostaglandin D2 was altered by knocking down of PLSCR1. Analysis of FcεRI-dependent signaling pathways revealed that whereas tyrosine phosphorylation of ERK and Akt was unaffected, tyrosine phosphorylation of LAT was significantly reduced in PLSCR1 knocked down cells. Tyrosine phosphorylation of phospholipase Cγ1 and consequently the mobilization of calcium were also significantly reduced in these cells. In nonactivated mast cells, PLSCR1 was found in part in lipid rafts where it was further recruited after cell activation and was constitutively associated with Lyn and Syk but not with LAT or Fyn. Altogether, these data identify PLSCR1 as a novel amplifier of FcεRI signaling that acts selectively on the Lyn-initiated LAT/phospholipase Cγ1/calcium axis, resulting in potentiation of a selected set of mast cell responses.

Mast cells actively participate in the defense against bacterial and parasitic infections (1, 2) and are key players in the allergic

reaction. They express receptors that bind IgE with a high affinity (FcεRI) and that are aggregated by the cognate allergen when they are occupied by allergen-specific IgE (3). This results in a number of cellular responses, such as immediate release of granule contents (e.g. histamine and β-hexosaminidase), synthesis of the lipid mediators of inflammation leukotrienes (LT)⁶ and prostaglandins (PG), and delayed production and release of cytokines and chemokines (4).

The pathways leading from FcεRI aggregation to cellular responses have been extensively studied and depend on tyrosine phosphorylations (5, 6). The two main pathways involve tyrosine kinases belonging to the Src family, Lyn and Fyn (4). Lyn is constitutively associated with FcεRI β chain (7). Upon aggregation of the receptor it phosphorylates both the β and the γ chains of FcεRI on tyrosine residues within the immunoreceptor tyrosine-based activation motif that is found in their intracellular domains (8). This results in the recruitment of additional Lyn molecules and of the tyrosine kinase Syk to the phosphorylated receptors and in the activation of Syk (9–11). Lyn and Syk then phosphorylate a number of substrates including the scaffold proteins LAT (12) and LAT2 (13–15). Activation of PLCγ1 and PLCγ2 recruited to phosphorylated LAT leads to the release in the cytosol of inositol 1,4,5-trisphosphate (IP3) and of diacylglycerols. IP3 releases calcium from the intracellular stores with a subsequent influx of calcium from the extracellular milieu, thus participating in the calcium signal that is required for mast cell activation and, together with diacylglycerol, activates various protein kinase C.

Fyn is also constitutively associated with FcεRI and is activated after receptor aggregation (16, 17). It then phosphorylates the adaptor protein Gab2 leading to activation of PI3K and of its downstream effectors PDK1 and Akt (16, 18). It also results in activation of sphingosine kinases that phosphorylate sphingosine into sphingosine 1 phosphate, thus modulating calcium influx (19–21). Therefore, both the Lyn and the Fyn pathways participate in regulating the calcium signal in FcεRI-dependent mast cell activation. Production of LT and PG depends on the

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⁶ The abbreviations used are: LT, leukotriene; BSA, bovine serum albumin; DNP-HSA, dinitrophenyl coupled to human serum albumin; FcεRI, high affinity IgE receptor; IP3, inositol 1,4,5-trisphosphate; PG, prostaglandin; PLC, phospholipase C; PLSCR1, phospholipid scramblase 1; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; shRNA, small hairpin RNA; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; IP3R1, IP3 receptor 1.

production of their common precursor arachidonic acid following cleavage of membrane phospholipids by phospholipase A₂. Cytosolic phospholipase A₂ is activated downstream of the ERK/mitogen-activated protein kinase (MAPK) cascade (22) that depends on both the Lyn-initiated and the Fyn-initiated pathways (4). Similarly, both pathways participate in the activation of transcription factors leading to the synthesis of cytokines.

Recently, we reported the phosphorylation on tyrosine of phospholipid scramblase 1 (PLSCR1) after FcεRI aggregation (23). PLSCR1 has been initially proposed as the protein responsible for the rapid redistribution of phospholipids between the two leaflets of the plasma membrane after cell activation or apoptosis, leading to the disruption of their asymmetric distribution (24). To date however, contradictory results have been reported, and this issue is still unresolved (25, 26). Additional roles have been described for PLSCR1. Although PLSCR1 contains a consensus transmembrane domain, it requires palmitoylation to be stabilized at the plasma membrane (27). In the absence of palmitoylation, it is found in the nucleus where it can bind DNA and activate the transcription of the IP₃ receptor 1 gene (28, 29). When palmitoylated it participates in the epidermal growth factor receptor signaling (30) by amplifying the activation of the tyrosine kinase Src (31).

The tyrosine phosphorylation of PLSCR1 following FcεRI aggregation strongly suggested the involvement of this protein in FcεRI-dependent mast cell activation. Yet this role was still elusive (32). To address this question, we used an RNA silencing approach. We show that a mast cell line with a knocked down PLSCR1 expression had significantly impaired degranulation and VEGF release after FcεRI engagement that was preceded by a decreased tyrosine phosphorylation of LAT and PLCγ1 and a decreased calcium mobilization.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal anti-rat PLSCR1 antibodies 129.2 and 17.3 and anti-FcεRIβ monoclonal antibody 30.9 have been already described (23, 33). Antibody 17.3 recognizes also a p28 in addition to PLSCR1 (33). Rabbit anti-Syk polyclonal antibody has been described (34). Anti-phosphotyrosine monoclonal antibody 4G10 was used as a culture supernatant of the 4G10 hybridoma or as horseradish peroxidase-coupled antibody (Calbiochem, La Jolla, CA). The anti-DNP monoclonal IgE DNP48 was a kind gift from Dr. R. P. Siraganian (NIDCR, National Institutes of Health, Bethesda, MD). Mouse anti-LAT monoclonal IgG was a generous gift from Dr. P. Dráber (Laboratory of Signal Transduction, Institute of Molecular Genetics, Prague, Czech Republic). Anti-Fyn polyclonal IgG is a kind gift from Dr. S. Latour (INSERM U768, Necker Hospital, Paris, France). Other reagents were from the following sources: anti-Lyn polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA); Alexa Fluor 647 streptavidin, Lipofectamine 2000 (Invitrogen); anti-IP3R1 polyclonal antibody (Chemicon International, Temecula, CA); anti-phospho-ERK (Tyr²⁰²/Tyr²⁰⁴), -Akt (Ser⁴⁷³), -PLCγ1 (Tyr⁷⁸³), and -LAT (Tyr¹⁹¹ and Tyr¹³²), and anti-total-ERK, -Akt, -PLCγ1 polyclonal antibodies (Cell Signaling Tech, Danvers, MA); anti-PLCγ1 monoclonal antibody (Upstate Biotech Inc, Lake Placid,

NY); horseradish peroxidase-labeled secondary antibodies, Fura 2-AM, Triton X-100, SDS, bovine serum albumin (BSA), DNP-human serum albumin (DNP-HSA), proteases and phosphatases inhibitors (leupeptin, aprotinin, NaF, and Na₃VO₄), and polybrene (Sigma-Aldrich); Dulbecco's modified Eagle's medium, fetal calf serum, and other culture reagents as well as goat serum (Invitrogen); puromycin (Invivogen, San Diego, CA); and Vectashield mounting medium (Vector Labs, Burlingame, CA).

Cells and Cell Lines—The rat mast cell line RBL-2H3 and human embryonic kidney cells HEK 293 were cultured in Dulbecco's modified Eagle's medium with Glutamax supplemented with 10% heat-inactivated fetal calf serum and with penicillin and streptomycin.

siRNA, shRNA Design, and Stable Expression—Three siRNA were selected that were specific for rat PLSCR1. These corresponded to the following sequences: siRNA1, 5'-GUG-GCU-UUC-CUG-UCC-AAC-A-3'; siRNA2, 5'-GGC-AGG-ACG-UUC-UAA-AGG-U-3'; and siRNA3, 5'-CCU-UGA-GGA-UCC-UGG-AUA-A-3'; control siRNA (scrambled siRNA3) was 5'-UGC-UAG-UGA-CCU-GCC-AUA-A-3'. The siRNA duplexes were transfected into RBL-2H3 cells by electroporation. Two days later, the cells were lysed. The presence of PLSCR1 in the lysates was examined by immunoblotting with the monoclonal antibody 17.3 that recognizes both PLSCR1 and a 28-kDa protein (p28). This protein served as a control both for loading and for silencing specificity during the screening test. The sequence of the most efficient siRNA (and of the control siRNA) were then selected to design a shRNA. The mammalian expression vector pSuper.retro.puro (Oligo-Engine, Seattle, WA) was used for expression of these shRNA in RBL-2H3 cells. The sequences were inserted between the HindIII and BglII sites of the vector, and after amplification the cloned sequences were confirmed. To generate retrovirus-containing supernatants, the packaging cell line HEK 293 was plated at 3 × 10⁶ cells/10-cm plate 48 h prior to transfection of the recombinant vectors with Lipofectamine. A green fluorescent protein expression system was used to control for transfection efficiency by flow cytometry. The culture medium was changed 24 h after transfection, and 48 h later the retrovirus-containing supernatant was collected, supplemented with polybrene (8 μg/ml), and added to RBL-2H3 cell cultures. The infected cells were resuspended 48 h later in fresh medium containing puromycin at 1 μg/ml for selection. Silencing of PLSCR1 expression was confirmed by Western blotting and confocal microscopy. For functional analyses, puromycin was omitted at least 72 h before experiments without detectable alleviation of PLSCR1 silencing.

Cell Stimulation and Measurement of β-Hexosaminidase Release—One or 1.5 × 10⁶ RBL-2H3 cells were incubated overnight in the presence of a 1:100 dilution DNP48 anti-DNP IgE ascitic fluid. After two washes in Tyrode's solution (10 mM Hepes, pH 7.3, 135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.5 mg/ml BSA), the cells were stimulated at 37 °C with DNP-HSA. Release of the granule marker β-hexosaminidase was measured in the supernatants as described (36).

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Prostaglandin and Leukotriene Measurements—Supernatants from cells stimulated in Tyrode's buffer containing fatty acid-free BSA were centrifuged at 1400 rpm for 5 min and used for the determination of LTC₄ and PGD₂ using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations.

VEGF and MCP-1 Release—Supernatants of cells stimulated in culture medium were centrifuged to eliminate cell debris. Secreted VEGF was measured using ELISA DuoSet kits (R & D Systems, Minneapolis, MN). MCP-1 was measured with the rat MCP-1 ELISA development kit from Peprotech (Paris, France).

Calcium Imaging— 5×10^5 IgE-sensitized RBL-2H3 cells were plated on coverslips and loaded with 1 μ l of pluronic acid F-127 (Invitrogen) and 1 μ M Fura 2-AM at 37 °C for 30 min. Fluorescence measurements were performed with a combination of a scanning monochromator and a CCD camera operated by TILLvisION software (Till Imago, TILL Photonics, Gräfelfing, Germany). The camera was mounted to an inverted microscope (Axiovert 100; Carl Zeiss, Jena, Germany) with a 40 $\times$ oil objective (Nikon). The cells were exposed to alternating excitation light pulses of 340- and 380-nm wavelengths of 120 μ s each. Fluorescence values of the collected emission images were corrected for background fluorescence before starting data collection. The results are compiled from three experiments, and per experiment up to 5–6 cells were analyzed simultaneously. The fluorescence intensity ratio was calculated by the ratio of the fluorescence values at 340- and 380-nm excitation. Fluorescence signals were plotted as $(F/F_0) - 1$ with F as fluorescence during the experimentation and F_0 as the initial level of fluorescence (37). The areas under the curve were calculated as arbitrary units.

Confocal Microscopy— 2.5×10^5 RBL-2H3 cells were plated on coverslips inside 24-well plates. After each step, the cells were washed three times with PBS. The cells were fixed in PBS, 3% paraformaldehyde for 15 min at 4 °C and incubated with 50 nM of NH₄Cl in PBS (to reduce aldehyde groups) at room temperature. After a 1-h saturation in A1 buffer (0.25% BSA, 0.025% saponin, and 10% of goat serum in PBS), the cells were incubated with biotinylated 129.2 antibody (20 μ g/ml) in A1 buffer for 1 h. The cells were then incubated with streptavidin coupled to AlexaFluor 647 (1:400 dilution) for 45 min in 0.25% BSA, 0.00125% saponin, 10% goat serum in PBS. Slides were mounted using Vectashield mounting medium and analyzed by confocal microscopy with a Zeiss LSM-5 confocal scanning laser microscope, equipped with argon and helium-neon lasers, using a plan-apochromat 63 $\times$ objective (numerical aperture, 1.40; oil immersion) and a crop $\times 2$, at room temperature. Red fluorescence was observed with a 560-nm long pass emission filter under 543-nm laser illumination. Images were collected every 0.4 μ m along the z -axis. Imaging was obtained using LSM imaging software.

FACS Analysis—The cells were washed twice in PBS containing 0.5% BSA and 0.01% NaN₃ (PBA) at 4 °C. After a 30-min incubation with 100 μ g/ml rabbit IgG in PBA, 5×10^5 cells were further incubated with or without 10 μ g/ml IgE at 4 °C for 1 h. After two washes in PBA, the cells were incubated for 1 h at 4 °C with goat anti-mouse IgE antibody coupled to fluorescein isothiocyanate. After two final washes in PBA, the cell-associated

fluorescence was analyzed with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometer.

Metabolic Labeling with [³H]Palmitate—One million RBL-2H3 cells were incubated overnight with 200 μ Ci/ml of [³H]palmitate (45 Ci/mmol; MP Biomedicals, Irvine, CA) in a fatty-acid free medium containing fetal calf serum dialyzed against PBS. The cells were then washed two times in PBS prior to lysis as described below.

Cell Lysis and Immunoprecipitation—One or 10×10^6 cells were lysed in 200 μ l or 1 ml of lysis buffer (50 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 0.5% Triton X-100, 50 mM Hepes, and anti-proteases: 10 μ g/ml leupeptin, 10 μ g/ml aprotinin) for 10 min at 4 °C and centrifuged to obtain the soluble fraction. Protein concentration was determined by the Bradford protein assay from Bio-Rad. For immunoprecipitation, rabbit polyclonal or mouse monoclonal anti-PLC γ 1 or anti-Fc ϵ RI β chain monoclonal antibody 30.9 was incubated with Protein A/G-agarose beads (VWR, Paris, France) and with 200 μ l of cell lysates on a rotating wheel for 2–3 h at 4 °C. The beads were washed six times with ice-cold lysis buffer, and the bound material was eluted by boiling for 5 min in 50 μ l of Laemmli sample buffer. For coimmunoprecipitation, Sepharose 4B beads coupled with anti-PLSCR1 mAb 129.2 or with a control antibody were used.

Western Blotting—Proteins resolved by SDS-PAGE were transferred onto polyvinylidene difluoride membranes that were then blocked by incubation in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 4% BSA. The blots were incubated for 1 h at room temperature or overnight at 4 °C with primary antibodies. After several washes in TBST, horseradish peroxidase-labeled anti-rabbit or anti-mouse IgGs were used for a 1-h incubation at room temperature. The blots were washed, and labeling was visualized using the super signal West Pico chemoluminescence kit from Pierce followed by exposure to X-Omat Kodak films. For immunoprecipitated PLC γ 1 samples, after first probing for tyrosine phosphorylation, the membranes were stripped by two 10-min incubations in 2% SDS and two 10-min incubations in 0.2 N NaOH followed by three washes in TTBS. The membranes were then reprobed with antibodies directed against PLC γ 1 for loading control.

For quantifications, control and PLSCR1 knocked down samples of individual experiments were resolved in the same gel and blotted on the same membrane. The bands were analyzed with the National Institutes of Health ImageJ software and were quantified relative to the PLC γ 1 or to the p28 band analyzed in the corresponding lane. For analysis of individual experiments, normalization was done by setting arbitrarily the value thus obtained for the unstimulated control as "1." For statistical analysis, a different procedure was followed. Each individual value obtained for control cells in a given experiment was arbitrarily set as "100," and the value obtained for the corresponding point in knocked down cells in the same experiment was normalized according to it, *i.e.* as the percentage of the value obtained with control cells for the corresponding experimental point. This procedure allowed elimination of the intrinsic variations in "absolute" values between experiments resulting from the ImageJ analysis procedure and to analyze the paired differ-

ences between control and PLSCR1 knocked down cells. Student's *t* test was then used with the values thus calculated.

Isolation of Lipid Raft Microdomains by Sucrose Gradient Centrifugation—Lipid rafts were isolated from freshly harvested cells. The procedure was adapted from that of Pombo *et al.* (38). Eight million RBL-2H3 cells were plated on 15-cm dishes, and 24 h later the cells were sensitized with a 1:10² dilution of the DNP-48 monoclonal IgE ascitic fluid for an overnight incubation. The cells were stimulated with 1 μ g/ml DNP-HSA in Tyrode's buffer for 30 min and washed two times in ice-cold PBS followed by two washes in ice-cold TNV buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). The cells were solubilized in 1 ml of ice-cold TNV containing 0.1% Triton X-100, 1 mM sodium orthovanadate, 1000 units/ml aprotinin, and 20 μ g/ml leupeptin and harvested by scraping. The lysate was transferred to a Dounce homogenizer and subjected to 15 strokes. The homogenate was mixed in an Ultra-clear TM centrifuge tube (Beckman) with an equal volume of 85% sucrose. This mixture was successively overlaid with 5 ml of 30% sucrose and 2 ml of 5% sucrose, neither of which contained Triton X-100. The tubes were then centrifuged at 200,000 $\times g$ without break at 4 $^{\circ}$ C, in a Beckman SW40Ti rotor for 16 h. Sequential pooled fractions 1–3 (1713 μ l), 4–6 (2499 μ l), 7–9 (2499 μ l), and 10–12 (3000 μ l) were harvested from the top of the gradient. An opaque band at the interface between the 5 and 30% layers was routinely harvested in fractions 4–6 and contained the lipid rafts. The samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated antibodies.

RESULTS

Knocking Down of PLSCR1 Expression—The function of PLSCR1 in mast cell activation through the Fc ϵ RI remained unclear, although its involvement was strongly suggested by its phosphorylation on tyrosine residues after Fc ϵ RI aggregation (23). To address this issue, we chose a siRNA approach. Three siRNA were tested for their ability to decrease PLSCR1 expression. For this screening we chose an immunoblotting procedure to follow protein expression. The siRNA2 was the most potent for silencing PLSCR1 expression (supplemental Fig. S1), whereas the other siRNA were somewhat less efficient. The sequences corresponding to siRNA2 and siRNA3 were selected to design shRNA. These sequences and a control shRNA corresponding to the scrambled sequence of siRNA3 were inserted into an expression vector that was transduced into cells with a retroviral system to allow for a stable and selectable decrease in PLSCR1 expression. By immunoblotting (Fig. 1A) the decrease in PLSCR1 expression reached over 99% in PLSCR1 shRNA-infected cells as compared with control shRNA-infected cells or to noninfected cells. This was confirmed by confocal analysis (Fig. 1B). By contrast, expression of Fc ϵ RI was unaffected in these cells (Fig. 1C).

PLSCR1 Amplifies Degranulation and Release of VEGF—Cell responses of mast cells in this virtual absence of PLSCR1 were then examined. Stimulation of the cells through Fc ϵ RI aggregation revealed a diminished ability of PLSCR1 knocked down cells to degranulate at all antigen concentrations and at all stimulation times considered (Fig. 2A). This decrease reached

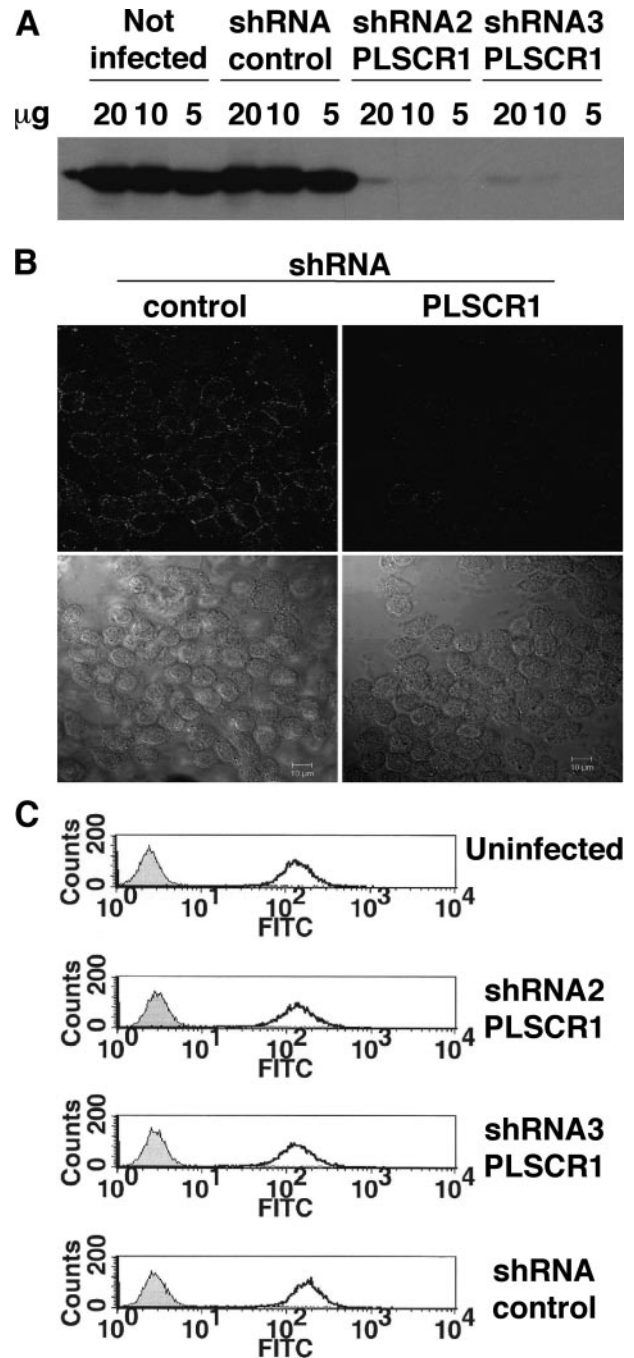


FIGURE 1. Knocked down PLSCR1 expression in RBL-2H3 cells. A, 20, 10, or 5 μ g of cell lysates from the indicated cell populations were analyzed by immunoblotting for PLSCR1 expression with 1 μ g/ml of mouse monoclonal antibody 129.2. B, control shRNA-treated (left panels) or shRNA2-treated (right panels) cells were immunostained with the biotinylated anti-PLSCR1 antibody 129.2 and streptavidin coupled to AlexaFluor 647. Mounted slides were analyzed by confocal microscopy. The lower panels show phase contrast images of the corresponding fields. C, cell surface Fc ϵ RI expression of the indicated cell populations was analyzed by FACS with or without 10 μ g/ml IgE followed by goat anti-mouse IgE antibody coupled to fluorescein isothiocyanate.

between 60 and 70% (*n* = 6) at optimal time and antigen concentration with either PLSCR1-specific shRNA but not with the scrambled shRNA, indicating that this effect was due to PLSCR1 silencing. These observations demonstrated that PLSCR1 is a significant modulator of Fc ϵ RI signaling.

PLSCR1 in IgE Receptor Signaling

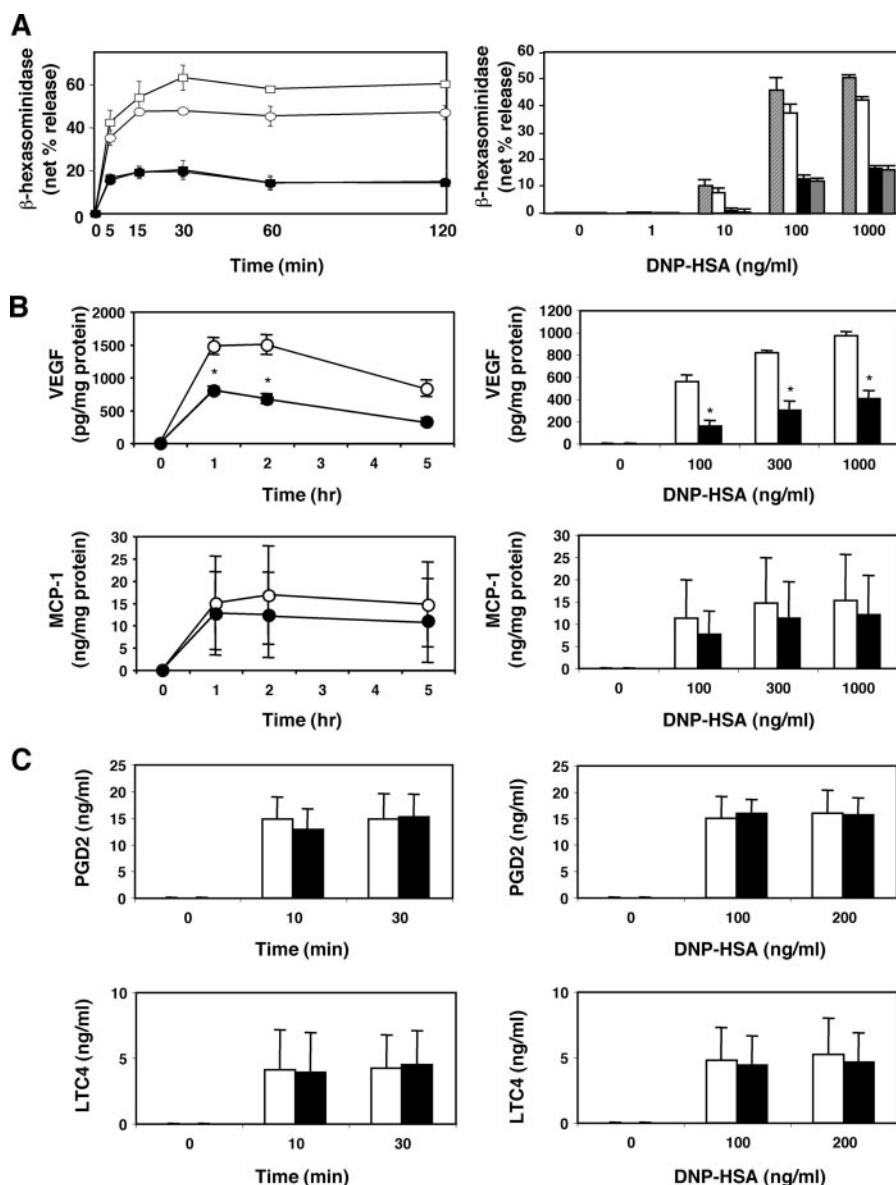


FIGURE 2. Cell responses in PLSCR1 knock-down RBL-2H3 cells. Time courses (left panels) and dose responses (right panels) were performed. Open squares and hatched bars, uninfected cells; open circles and open bars, control shRNA-expressing cells; closed circles and closed bars, PLSCR1 shRNA2-expressing cells; closed squares and shaded bars, PLSCR1 shRNA3-expressing cells. A, impaired degranulation in PLSCR1 knocked down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-2H3 cells were stimulated with 100 ng/ml of DNP-HSA for 0–120 min (left) or stimulated for 30 min with the indicated DNP-HSA concentration (right), and β -hexosaminidase released in the supernatants was measured. B, VEGF and MCP-1 production in PLSCR1 knocked down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-2H3 cells were stimulated in culture medium with 1 μ g/ml of DNP-HSA for 0 to 5 h (left) or with the indicated DNP-HSA concentration for 5 h (right). VEGF and MCP-1 released in the supernatant were measured by ELISA. Spontaneous release was subtracted. The released amounts are expressed after normalization per mg of cell proteins. C, no change in PGD₂ and LTC₄ release in PLSCR1 knocked down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-2H3 cells were stimulated with 200 ng/ml of DNP-HSA for 0–30 min (left) or with the indicated DNP-HSA concentration for 30 min (right). Determination of PGD₂ and LTC₄ release was done with an EIA kit. The data are the means $\pm$ S.E. of three experiments.

The production of cytokines was also investigated in these cells. In a first step, a screening of the cytokines released by the cells was performed. Thus, RBL-2H3 cells were stimulated with IgE plus antigen, and multiple cytokines released in the supernatants were analyzed with a cytokine array. Only VEGF and MCP-1 were detected after Fc ϵ RI aggregation (supplemental Fig. S2). Interestingly, ELISA analysis demonstrated that, like β -hexosaminidase release, VEGF

production was dramatically impaired, reaching on average in PLSCR1 knocked down cells 50% of the amount released by control cells, whatever the stimulation time or the antigen dose considered (Fig. 2B, upper panels). By contrast, MCP-1 production was minimally affected by the knocking down of PLSCR1 (Fig. 2B, lower panels). Stimulation of mast cells also leads to LT and PG synthesis and release in cell environment (39–41). Here, no difference in PGD₂ and LTC₄ release was observed between activated cells expressing either PLSCR1 shRNA or control shRNA (Fig. 2C). Altogether, these data demonstrate that the knocking down of PLSCR1 expression did not affect the cell reactivity as a whole but only a restricted set of cell responses and identify PLSCR1 as a significant modulator of mast cell responses to Fc ϵ RI aggregation.

PLSCR1 Amplifies the LAT/PLC γ 1/Calcium Axis—The above described observations suggested that PLSCR1 amplified selected signaling pathways. To rule out any unexpected nonspecific effect on expression of signal effector molecules, the expression level of a number of proteins was examined. As described for Fc ϵ RI expression (Fig. 1C), no decrease in the expression of Lyn, Fyn, Syk, LAT, Akt, ERK1/2, PLC γ 1, or actin was observed, whereas PLSCR1 expression was drastically decreased (supplemental Fig. S3). Therefore, experiments were carried out to identify the pathways functionally affected by PLSCR1. To that effect, we examined the tyrosine phosphorylation of various effector molecules chosen for their implication at critical steps of the major signaling pathways activated by Fc ϵ RI engagement and the expression of which was unaltered in PLSCR1 knocked down cells. Two main pathways have been reported in Fc ϵ RI-dependent signal transduction, and both pathways contribute to ERK tyrosine phosphorylation (4). Here, IgE receptor-induced tyrosine phosphorylation of ERK was not significantly different between PLSCR1 knocked down and control cells (supplemental Fig. S4).

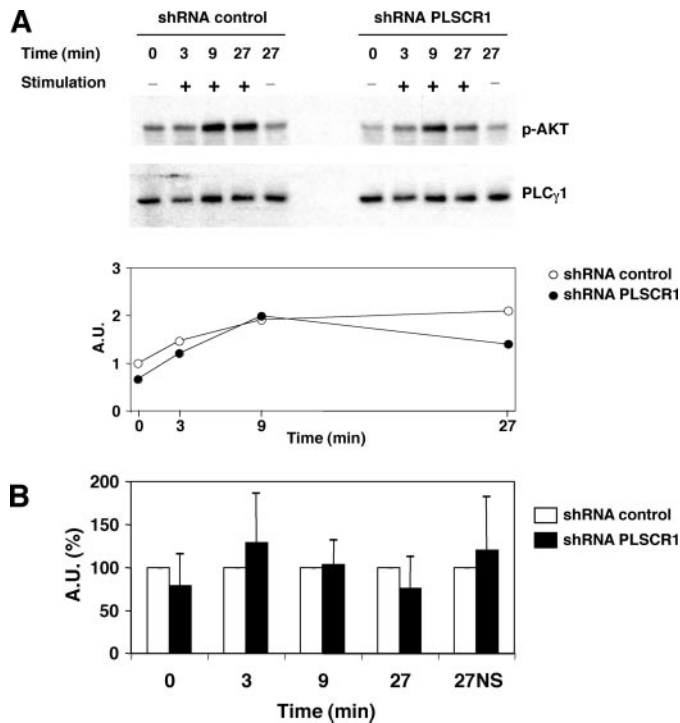


FIGURE 3. Unaltered Akt phosphorylation in PLSCR1 knocked down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-2H3 cells were stimulated with 1 μ g/ml of DNP-HSA for the time indicated. *A*, upper panels, lysates from these cells were resolved by SDS-10% PAGE and transferred onto polyvinylidene difluoride membranes that were cut in three slices. The middle portion was probed with rabbit polyclonal anti-phospho-Akt antibody, and the upper portion was probed with rabbit polyclonal anti-PLC γ 1 for loading control. The lower portion was analyzed for LAT Tyr191 phosphorylation (see next figure). *Lower panel*, quantification of Akt phosphorylation for the experiment shown in the upper panels was performed relative to the PLC γ 1 bands in arbitrary units (A.U.) after normalization with the unstimulated control cells. *B*, statistical analysis. For each time point, the value obtained in PLSCR1 knocked down cells was normalized relative to the control. The Student's *t* test was used for statistical analysis. The data are the means $\pm$ S.D. of four experiments.

The Fyn-dependent pathway leads to PI3K recruitment and phosphorylation (16) and eventually to modulation of calcium influx through the activation of sphingosine kinases (20, 21). PI3K activation allows recruitment of Akt to the plasma membrane where it becomes available for phosphorylation by various protein kinase C isoforms (4, 18). Here, no significant difference was observed between both cell populations for Akt phosphorylation after Fc ϵ RI engagement (Fig. 3).

The Lyn-dependent pathway leads to LAT phosphorylation and to calcium release from intracellular stores that eventually induce calcium influx. Investigation of this pathway through analysis of LAT tyrosine phosphorylation revealed that Fc ϵ RI-induced tyrosine phosphorylation of LAT was significantly lower ($p < 0.05$; $n = 4$) in PLSCR1 knocked down cells compared with control cells with a higher effect observed on the phosphorylation of tyrosine 132 (Fig. 4).

Tyrosine phosphorylation of LAT is required for recruitment of downstream effector molecules to the LAT adaptor protein. One major downstream signaling event is the recruitment mainly through phosphorylated tyrosine 132, phosphorylation, and activation of PLC γ 1 (12, 42). Analysis of the tyrosine phosphorylation of PLC γ 1 revealed that it was dramatically impaired in the absence of PLSCR1 (Fig. 5). The expected con-

sequence of the down-modulation of PLC γ 1 phosphorylation is the down-modulation of calcium mobilization. Indeed, this enzyme releases IP3 from phosphatidylinositol 4,5-bisphosphate. IP3 then induces release of calcium from intracellular stores after binding to its intracellular receptor. Depletion of these stores then leads to a calcium release-activated calcium inward current (I_{CRAC}) through store-operated calcium channels (4). RBL-2H3 cells, like mast cells in tissues, are adherent cells, and all above described data had been collected in an adherence setting. Therefore, the calcium mobilization was also examined in this setting. Single cell analysis revealed that the calcium signal was dramatically impaired by 45% during the first 5 min of stimulation in PLSCR1 knocked down mast cells. Interestingly, the more drastic effect occurred during the early stage of calcium mobilization (Fig. 6A).

It has been reported that PLSCR1 can modulate transcription of IP3R1 (29). Therefore, IP3R1 expression was examined to determine whether a down-regulation of its expression subsequent to PLSCR1 knocking down could also participate in the dampening of the calcium signal. No difference in IP3R1 expression was observed between both cell populations (Fig. 6B), demonstrating that the amplification by PLSCR1 of the calcium mobilization did not involve an increased expression of IP3R1.

PLSCR1 Is Associated with Lyn and with Syk—Lyn is upstream the LAT/PLC γ /calcium axis (16). In addition, Lyn and LAT are both localized in lipid rafts that serve as signaling platforms for Fc ϵ RI (43, 44). Therefore, we investigated the presence of PLSCR1 in these membrane microdomains. PLSCR1 was found in lipid rafts under unstimulated conditions, where it was further recruited after Fc ϵ RI aggregation (Fig. 7A). This localization in rafts has been related to palmitoylation of PLSCR1 in other cell types (30). Likewise, PLSCR1 was found to incorporate radiolabels when the RBL-2H3 cells were cultured in the presence of [^{3} H]palmitate (supplemental Fig. S5), suggesting that PLSCR1 localization in rafts in mast cells likewise was due to its palmitoylation. In confocal microscopy analysis, Lyn and PLSCR1 were in punctuated regions of the plasma membrane where they were colocalized (Fig. 7B). Coimmunoprecipitation experiments revealed that Lyn was associated with PLSCR1. This association was not modulated by Fc ϵ RI aggregation (Fig. 8). By contrast, no physical association of PLSCR1 with LAT was observed (Fig. 8), demonstrating that the Lyn-PLSCR1 association was not an experimental artifact resulting from their being in the same lipid raft domain. Also, PLSCR1 was not associated with Fyn (Fig. 8), confirming that its association with Lyn was specific. Because the Fc ϵ RI itself is a specific substrate for tyrosine phosphorylation by Lyn, we examined whether this phosphorylation was altered in PLSCR1 knocked down cells. As seen in the supplemental Fig. S6, there was no alteration of the phosphorylation of the Fc ϵ RI β chain in these cells, suggesting that PLSCR1 did not modulate the activation of Lyn.

Syk is another tyrosine kinase that is downstream of Lyn and that plays an important role in the tyrosine phosphorylation of LAT (45). Therefore, it was of interest to investigate whether PLSCR1 could also interact with Syk. Coimmunoprecipitation experiments demonstrated that Syk is physically associated

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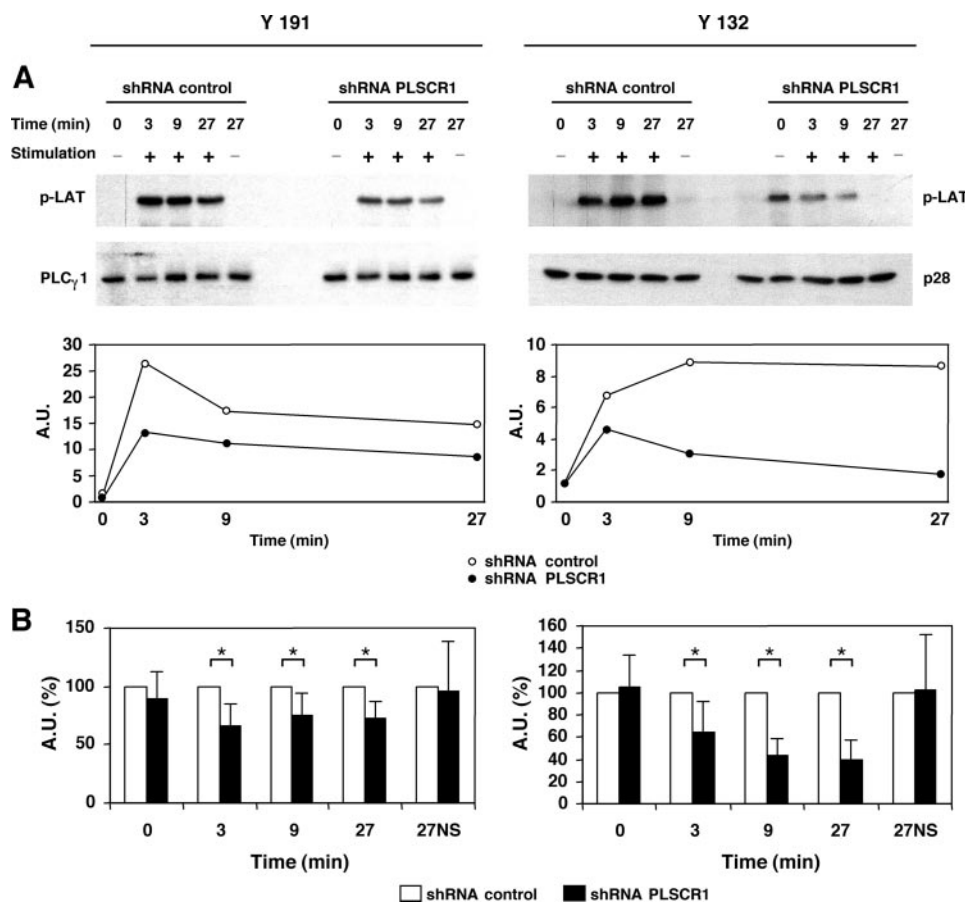


FIGURE 4. Lower tyrosine phosphorylation of LAT in PLSCR1 knocked down RBL-3H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-3H3 cells were stimulated with 1 μ g/ml of DNP-HSA for the time indicated. *A*, upper panels, lysates from these cells were resolved by SDS-10% PAGE and transferred onto polyvinylidene difluoride membranes that were cut in three slices. The lower portion was probed with rabbit polyclonal anti-phospho-LAT antibody (Tyr¹⁹¹, Y 191), and the upper portion was probed with rabbit polyclonal anti-PLC γ 1 (left panels) for loading control. Alternatively (right panels) the membrane were cut in two slices, the upper portion being probed with anti-phospho-LAT (Tyr¹³², Y 132) antibody, and the lower portion with anti-p28 monoclonal antibody (right panels) for loading control. Lower panels, quantification of LAT phosphorylation for the experiments shown in the upper panels were performed relative to the PLC γ 1 or p28 bands in arbitrary units (A.U.) after normalization with the unstimulated control cells. *B*, statistical analysis. For each time point, the value obtained in PLSCR1 knocked down cells was normalized relative to the control. The Student's *t* test was used for statistical analysis. The data are the means $\pm$ S.D. of four experiments (*, $p < 0.05$).

with PLSCR1 (Fig. 8) and that this association was not modulated by Fc ϵ RI stimulation. Altogether these results demonstrate that PLSCR1 is constitutively associated with both of the kinases that phosphorylate LAT (*i.e.* Lyn and Syk) but not with LAT itself.

DISCUSSION

The present study identifies PLSCR1 as a potent modulator of mast cell degranulation and VEGF production. This conclusion was made possible by the almost complete abolishment of PLSCR1 expression by the shRNA approach. Our data strongly suggest that PLSCR1 regulates the Lyn-dependent pathway and are in agreement with a proposed model whereby PLSCR1 amplifies the tyrosine phosphorylation of LAT on selected tyrosine residues leading to optimal recruitment of PLC γ 1 and eventually to an optimal calcium mobilization. This would affect a set, but not all, of cell responses.

The knocking down of PLSCR1 strongly affected the release of VEGF, whereas it demonstrated at best a minimal effect on

MCP-1 production. It has been reported that production of MCP-1 by IgE/antigen-stimulated mast cells is optimal when cells are activated with a weaker signal than that required for optimal release of non allergy-promoting cytokines (46). Here, MCP-1 release reached a plateau at the lower antigen concentration of 100 ng/ml, whereas VEGF did not (Fig. 2*B*, right panels). The present observations suggest that PLSCR1 may preferentially influence responses requiring high signal intensity.

We also observed that PLSCR1 did not impact on leukotriene and prostaglandin release. This is in agreement with the observed unaltered tyrosine phosphorylation of ERK. Indeed, the common precursor of these lipid mediators is the arachidonic acid released through the cleavage of membrane phospholipids by cytosolic phospholipase A₂, and activation of this enzyme following Fc ϵ RI engagement occurs downstream of the ERK cascade (22).

Our results are in agreement with a modulation of the Lyn-initiated signaling pathway by PLSCR1. Indeed, PLSCR1 did not modulate the phosphorylation of Akt on tyrosine, which is a strong argument that PLSCR1 does not modulate PI3K activation that is under control of the Fyn-initiated pathway (16). In addition, LAT tyrosine phosphorylation, which depends on the Lyn-initiated pathway, was affected by the knocking down of PLSCR1. LAT has been demonstrated as an important signal intermediate in IgE-mediated mast cell activation. In the absence of LAT, IgE-dependent mast cell degranulation is dramatically impaired as well as cytokine production (12), in agreement with our observations of decreased degranulation and VEGF production together with a lower tyrosine phosphorylation of LAT. The reported independence of the transcription of the MCP-1 gene from LAT (42) is also in agreement with our observations. Yet LAT controls also, at least in part, ERK phosphorylation and activation (12). We hypothesize that ERK activation is less sensitive to the reduced LAT phosphorylation that occurs in the absence of PLSCR1 than are degranulation and VEGF production. A basis for this hypothesis is that LAT can be phosphorylated on a number of tyrosines, allowing it, as a scaffold protein, to control various downstream effectors. Whether LAT phosphorylation acts as a sort of rheostat maintaining a different threshold for each downstream signaling event is an intriguing possibility.

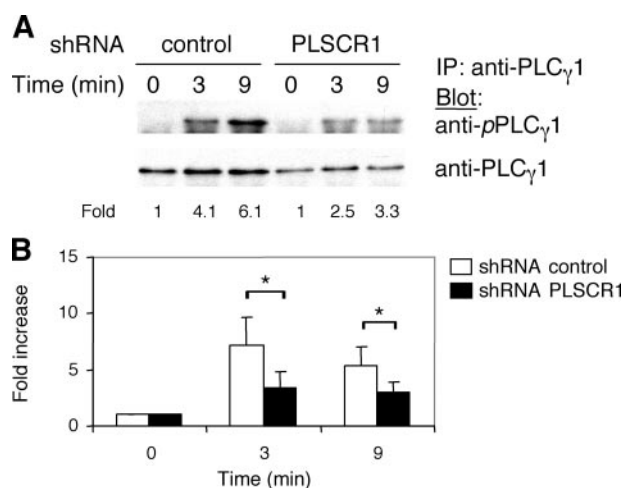


FIGURE 5. Impaired PLC γ 1 phosphorylation in PLSCR1 knocked down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked down RBL-2H3 cells were stimulated with 1 μ g/ml of DNP-HSA for the time indicated, and immunoprecipitated samples from these cells were resolved by SDS-10% PAGE, and transferred onto polyvinylidene difluoride membranes. **A**, a membrane from anti-PLC γ 1 immunoprecipitation was probed with anti-phospho-PLC γ 1 (pPLC γ 1) polyclonal antibody, stripped, and reprobed with anti-PLC γ 1 rabbit polyclonal antibody for loading control. The phosphorylation relative to the total amount of PLC γ 1 is indicated below each lane (Fold). **B**, quantitative analysis of six independent experiments. The mean fold increase was relative to unstimulated cells $\pm$ S.D., $n = 6$ (*, $p < 0.05$).

Recent studies using selected mutations of its individual tyrosines revealed that indeed, signaling events and cell responses downstream of LAT depended on which tyrosines were being phosphorylated because a given combination of these phosphorylations resulted in a particular set of cell responses (42, 47). It is not clear so far whether the decreased tyrosine phosphorylation of LAT that we observed resulted from a decreased number of LAT molecules being phosphorylated on tyrosine or whether all LAT molecules had an altered phosphorylation of one or more selected tyrosine.

Downstream of LAT, the early stages of calcium mobilization were profoundly affected by PLSCR1 knocking down. This is in agreement with our observation of a decreased phosphorylation of PLC γ 1, which is expected to affect in priority the release of calcium from intracellular stores. In addition, in agreement with our present observations, mast cells from PLC γ 2 knockout mice show significantly lower degranulation and cytokine production but normal ERK phosphorylation after Fc ϵ RI engagement (48). Thus, PLSCR1 appears to act through modulation of the LAT/PLC γ 1/calcium axis, thus resulting in reduced degranulation and VEGF production.

Whereas in the absence of Lyn some phosphorylation of LAT is still detected after Fc ϵ RI aggregation (16), the absence of Syk results in a complete ablation of LAT phosphorylation (45). This suggests that Syk, which is downstream of Lyn in Fc ϵ RI signaling, could be the main kinase responsible for LAT phosphorylation. In this respect, it is noteworthy that PLSCR1 is shown here to interact with both Lyn and Syk and to modulate the tyrosine phosphorylation of LAT. Whether this interaction is a direct physical interaction or the result of their confinement in the same nonraft membrane microdomains remains to be determined. Coimmunoprecipitation experiments with octyl-glucoside did not allow us to answer this question because of

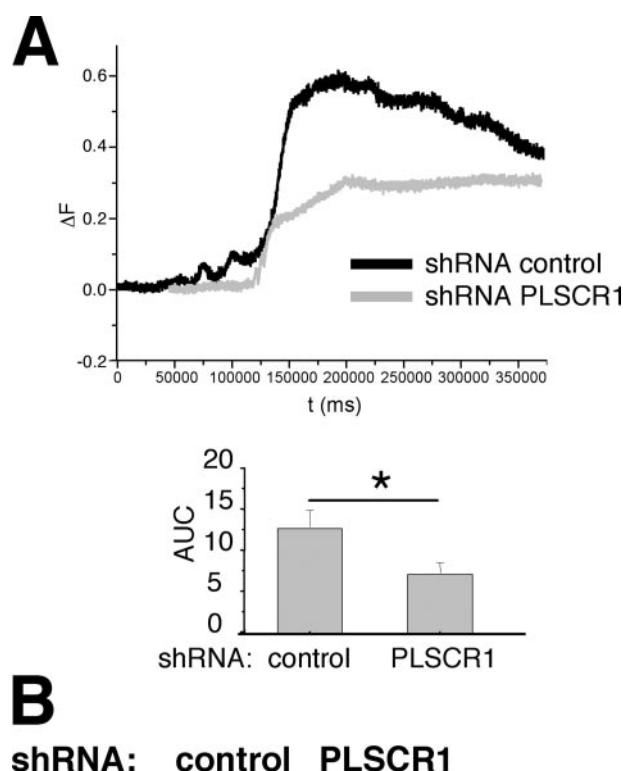


FIGURE 6. Impaired intracellular calcium mobilization in PLSCR1 knocked down RBL-2H3 cells. **A**, control and PLSCR1 knocked down cells were sensitized with IgE, plated on coverslips, and loaded with 1 μ l of pluronic acid F-127 and 1 μ M of Fura 2-AM at 37 $^{\circ}$ C. **Upper panel**, fluorescence measurement was performed with a scanning monochromator and a CCD camera as described under "Experimental Procedures." The results represent the mean values obtained from 15–18 cells collected from three experiments. **Lower panel** for statistical analysis, areas under the curve calculated for each individual cell were compiled and analyzed with analysis of variance test (*, $p < 0.05$). **B**, IP3R1 was examined by immunoblotting using lysates from control and PLSCR1 knocked down cells. Immunoblot was performed using a rabbit polyclonal anti-IP3R1. The membranes were stripped and reprobed with rabbit polyclonal anti-PLC γ 1 antibody for loading control. One of two experiments is shown.

high nonspecific interactions. Nevertheless, our observation that the Fc ϵ RI-dependent tyrosine phosphorylation of PLSCR1 is abolished in the absence of Lyn and greatly impaired in the absence of Syk⁷ demonstrates that these interactions are functionally relevant. The facts that Lyn activation upon Fc ϵ RI aggregation does not appear to be affected by PLSCR1 knocking down and that both PLC γ 1 and LAT are substrates of Syk suggest that Syk is the prime target of PLSCR1 for the modulation of Fc ϵ RI signaling.

⁷ N. Charles *et al.*, manuscript in preparation.

PLSCR1 in IgE Receptor Signaling

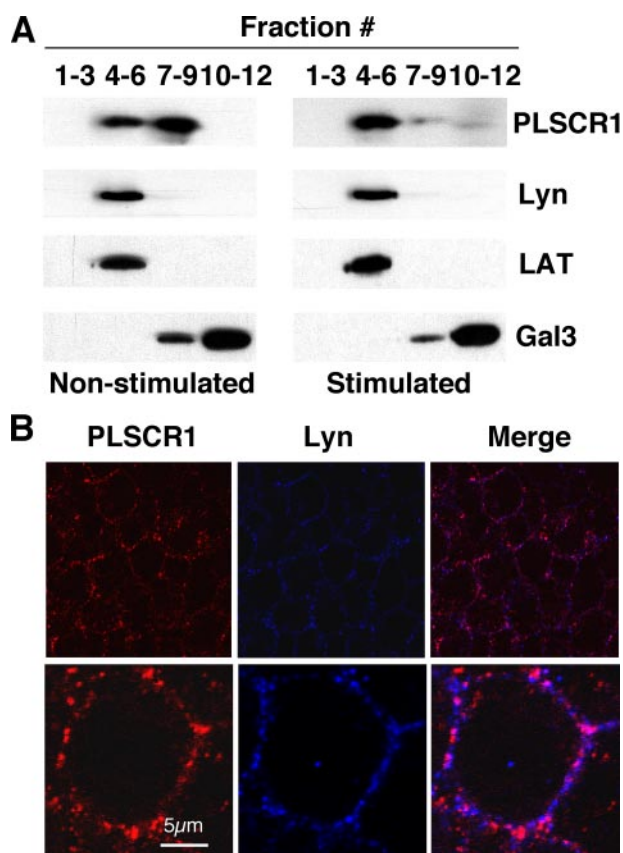


FIGURE 7. PLSCR1 is present within lipid rafts and colocalized with Lyn. *A*, lipid rafts analysis. RBL-2H3 cells were stimulated or not for 30 min and lysed in 0.1% Triton X-100. The lysates were fractionated by ultracentrifugation on a discontinuous sucrose gradient, and 12 fractions were collected from the top of the gradient and pooled into four sets (fractions 1–3, 4–6, 7–9, and 10–12). An aliquot from each set was run in SDS-10% polyacrylamide gel and analyzed by immunoblotting. Anti-Lyn and anti-LAT antibodies were used as specific markers of rafts, and anti-galactin 3 antibody was used to detect nonraft fractions (57, 58). One of four experiments is shown. *B*, cells were immunostained with the biotinylated anti-PLSCR1 monoclonal 129.2 and rabbit anti-Lyn antibodies followed by streptavidin coupled to AlexaFluor 568 (red) and anti-rabbit IgG coupled to AlexaFluor 647 (blue) staining. The mounted slides were analyzed by confocal microscopy. The lower panels show a digital zoom of a random cell from the corresponding field. One of three experiments is shown.

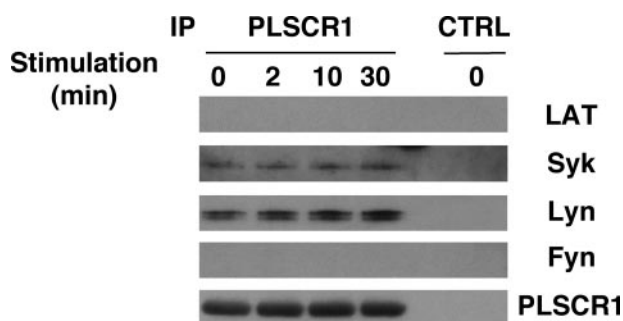


FIGURE 8. Lyn and Syk are associated with PLSCR1 in mast cells. Two million IgE-sensitized noninfected RBL-2H3 cells were stimulated or not stimulated with antigen for the time indicated. The cell lysates were immunoprecipitated (IP) with anti-rat PLSCR1 mAb 129.2-coupled beads or with beads coupled with an irrelevant mAb as a control (CTRL) as indicated. The eluates were analyzed by immunoblotting. To that effect, the membrane was cut in three pieces and was probed with anti-LAT, anti-Fyn, and anti-Syk antibodies and after stripping of the membranes, with mAb 129.2 (anti-PLSCR1) and anti-Lyn antibodies as indicated. One of three experiments is shown.

Recent findings demonstrated that although phosphatidylserine externalization and degranulation are correlated in both FcεRI-stimulated and calcium ionophore-stimulated mast cells (32, 49), they are disconnected under certain conditions such as stimulation with pervanadate (32). In addition, tyrosine phosphorylation of PLSCR1 and phosphatidylserine externalization appear to be independent events in mast cells (32). The involvement of PLSCR1 in the early steps of FcεRI signaling (our data described herein) and its late tyrosine phosphorylation (32)⁸ suggest that PLSCR1 phosphorylation plays either a role in another compartment of mast cell activation or that it serves to down-regulate PLSCR1 function. Analysis with mutant molecules will answer this question.

Thus, PLSCR1 arises as a modulator of selected mast cell responses following FcεRI aggregation. FcεRI signal amplifiers, defined as molecular “devices” that modulate quantitatively, but not qualitatively, cell responses, have been reported in the past. The most important of those is the β chain of FcεRI that acts as a general amplifier of the activation signal (50). In addition, other molecules such as SHIP1 (51) and SHIP2 (52) have been reported to dampen FcεRI signaling. However, fine-tuning of mast cell responses may involve more targeted regulatory mechanisms. For instance, chemokine production can be achieved with weaker signal intensity than that required for degranulation or cytokine production (46). Therefore, besides effector molecules required to initiate and propagate the signal (and that would constitute the framework of the signaling pathways), other molecules could intervene at strategic steps as regulators, either amplifying or dampening the signal, whether in a general manner or in a more targeted way, thus allowing a precise fine-tuning. Based on the collected evidence, PLSCR1 is proposed here to be such a molecule that serves as an amplifier allowing the signal to reach the required intensity for optimal degranulation and cytokine production. This amplification is implemented through modulation of the LAT/PLCγ1/calcium axis.

PLSCR1 is also capable of amplifying the epidermal growth factor receptor signaling (30, 31). It is also possible that such a signal amplifying mechanism might explain the potentiation by PLSCR1 of the antiviral activity of interferon α (53). Thus, this molecule could fulfill different functions depending on its subcellular localization, *i.e.* transcription of genes when it is present in the nucleus, and amplification of signaling pathways when it is expressed at the plasma membrane. Interestingly, overexpression of PLSCR2, another member of the PLSCR family, in RBL-2H3 cells inhibits FcεRI-dependent degranulation (54), and Lyn^{−/−} mast cells show both increased degranulation and decreased PLSCR2 expression (55). This suggests that PLSCR1 and PLSCR2 might play opposite functions as amplifiers and down-modulators of cell activation signaling, respectively. Whether the other members of the PLSCR family (PLSCR3 and 4) (56) could also play a role as signal regulators remains to be explored.

⁸ O. Amir-Mozzami, C. Alexia, N. Charles, P. Launay, R. C. Monteiro, and M. Benhamou, unpublished observations.

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Supplemental material: Omid Amir-Moazami et al. Phospholipid scramblase 1 amplifies IgE receptor signalling in mast cells

Supplemental Figure 1. Comparison of three PLSCR1 siRNA efficiency. RBL-2H3 cells were transfected by electroporation with three different PLSCR1 siRNA or control siRNA. After 48 hours cells were lysed and lysates were analyzed for the presence of PLSCR1 by immunoblotting of 20, 10 or 5 µg proteins with monoclonal antibody 17.3 that recognizes both PLSCR1 and a p28 protein.

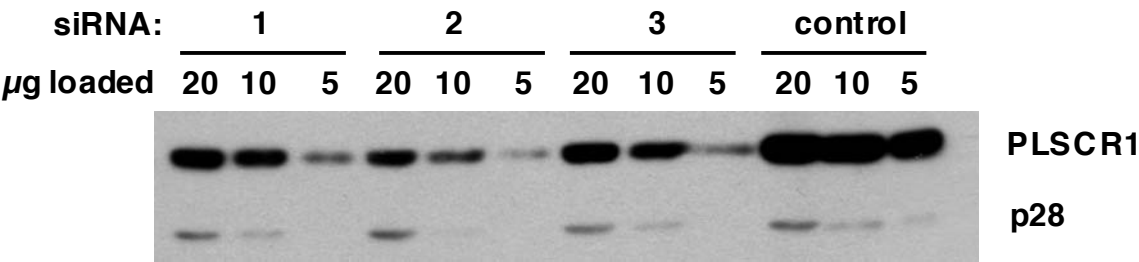
Supplemental Figure 2. Screening of cytokines produced by RBL-2H3 stimulated through FcεRI aggregation. IgE-sensitized control and PLSCR1 knocked-down RBL-3H3 cells were stimulated, or not stimulated, with 1µg/ml of DNP-HSA for 4 hours in culture medium. Supernatants were collected, centrifuged and the released cytokines were screened by probing a screening membrane from the Rat Cytokine Array according to the manufacturer's protocol (RayBiotech, TEBU, Le Perray en Yvelines, France).

Supplemental Figure 3. Expression of various signal effector molecules in shRNA-expressing cells. Twenty seven µg of proteins from cell lysates from control shRNA (lanes 1) or PLSCR1 shRNA2 (lanes 2) expressing cells were resolved by SDS-10% PAGE, transferred onto PVDF membranes and probed with the indicated antibodies.

Supplemental Figure 4. Unaltered ERK phosphorylation in PLSCR1 knocked-down RBL-2H3 cells. IgE-sensitized control and PLSCR1 knocked-down RBL-3H3 cells were stimulated with 1µg/ml of DNP-HSA for the time indicated. (A) Upper panels: Lysates from these cells were resolved by SDS-10% PAGE, and transferred onto PVDF membranes that were cut in two slices. The lower portion was probed with rabbit polyclonal anti-phospho-ERK antibody and the upper portion was probed with rabbit polyclonal anti-PLCγ1 for loading control. Lower panel: Quantification of ERK phosphorylation for the experiment shown in the upper panels was performed relative to the PLCγ1 bands in arbitrary units (A.U.) after normalisation with the unstimulated control cells. (B) Statistical analysis. For each time point, the value obtained in PLSCR1 knocked-down cells was normalized relative to the control. The student *t* test was used for statistical analysis. The data are the means +/- SD of four experiments.

Supplemental Figure 5. PLSCR1 is palmitoylated in mast cells. [³H]-palmitate labelled RBL-2H3 mast cells were lysed. The lysate was immunoprecipitated with anti-PLSCR1 monoclonal antibody 129.2. The immunoprecipitate was resolved by SDS-10% PAGE, and transferred onto a nitrocellulose membrane that was probed with 129.2 antibody (left pannel). Then, the corresponding lane was cut off the membrane and further cut every 2 mm, and the radioactivity incorporated in each slice was measured in a scintillation counter (right pannel). One out of three experiments.

Supplemental Figure 6. Tyrosine phosphorylation of the FcεRIβ chain is not altered in PLSCR1 knocked-down cells. IgE-sensitized RBL-2H3 cells expressing control shRNA or PLSCR1 shRNA were stimulated with antigen for the indicated time-length. After lysis of the cells, the FcεRIβ chain was immunoprecipitated and analyzed for tyrosine phosphorylation with the anti-phosphotyrosine (anti-PY) antibody 4G10. After stripping of the membrane immunoblotting with the anti-FcεRIβ chain monoclonal antibody 30.9 was performed to control for equal loading of the gel. One experiment representative of three.



	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
2	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
3	GM-CSF	IFN- γ	IL-1 α	IL-1 β	IL-4	IL-6	IL-10	LIX
4	GM-CSF	IFN- γ	IL-1 α	IL-1 β	IL-4	IL-6	IL-10	LIX
5	Leptin	MCP-1	MIP-3 α	β -NGF	TIMP-1	TNF- α	VEGF	BLANK
6	Leptin	MCP-1	MIP-3 α	β -NGF	TIMP-1	TNF- α	VEGF	BLANK
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

