IgE-receptor type I dependent tyrosine phosphorylation of phospholipid scramblase

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Running title: PLSCR tyrosine phosphorylation in FcεRI-stimulated mast cells
Summary

To identify new effectors of IgE receptor (FcεRI) signaling, we purified proteins from FcεRI-stimulated RBL-2H3 rat mast cells on anti-phosphotyrosine beads and generated mouse monoclonal antibodies (mAb) against these proteins. Two mAbs bound to a protein that was identified as a new isoform of Phospholipid Scramblase (PLSCR) after screening an RBL-2H3 cDNA expression library. This isoform differed from PLSCR1 by the absence of exon 3-encoded sequence and by an insert coding six Q-G-P-Y-P/A-G-P repeats. The PLSCR family of proteins is responsible for a redistribution of phospholipids across the plasma membrane. Although rat PLSCR is a 37-kDa protein, anti-phosphotyrosine immunoblots revealed the presence of 37-49 kDa phosphoproteins, in the material immunoprecipitated with either anti-PLSCR mAb, but not with unrelated monoclonal or polyclonal antibodies. Depletion of PLSCR resulted in the absence of these phosphoproteins. Additional experiments led to the identification of these phosphoproteins as phospho-PLSCR itself. Stimulation of RBL-2H3 cells upon FcεRI engagement resulted in a dramatic increase in PLSCR tyrosine phosphorylation. Comparison of the relative amounts of phospho-PLSCR and non-phosphorylated PLSCR demonstrated that only a tiny fraction was thus modified, indicating a finely targeted involvement of PLSCR in FcεRI signaling. Thus, this study reports the cloning of a new isoform of PLSCR, and the first observation that a member of the PLSCR family is a target for tyrosine kinases and is involved in signaling by an immune receptor. This opens new perspectives pertaining to the role of phospholipid scramblases and to the mechanisms involved in their regulation.
Introduction

Plasma membranes have an asymmetric lipid distribution. Whereas both plasma membrane leaflets are mainly composed of choline-containing phospholipids (phosphatidylcholine and sphingomyelin), the inner leaflet is enriched relative to the outer leaflet in primary amine-containing phospholipids (phosphatidylserine, PS\(^1\), and phosphatidylethanolamine). This asymmetry is actively maintained by two ATP-dependent enzymes: a phospholipid translocase that selectively transports aminophospholipids from the outer to the inner leaflet, and a floppase that moves all phospholipids in the opposite direction (1). The physiological importance of the maintenance of this energy-consuming unbalance in membrane composition is unveiled when this asymmetry is disrupted. When cells undergo apoptosis, translocase and floppase become inactivated and a phospholipid scramblase (PLSCR), an enzyme that moves all phospholipids bidirectionally, becomes activated (1). The consequence is a loss of phospholipid asymmetry with a resulting increase in PS expression in the outer leaflet of the plasma membrane. The apoptotic cell is then recognized and engulfed by phagocytes expressing receptors for PS that confer both recognition and phagocytic capabilities to these cells (2).

Yet, the role of phospholipid scrambling is not restricted to apoptosis. In blood platelets and vascular endothelial cells, activation induces an increase in the surface expression of PS that in turn activates coagulation enzymes (3). In at least one instance of bleeding disorder known as Scott syndrome, a defect in coagulation was related to a defect in scramblase activity in intact cells (4,5). Another role for phospholipid scrambling is suggested by the increased surface expression of PS after mast cell activation (6,7). These cells are involved in innate immunity: they are capable to phagocytose and kill pathogens, recruit leukocytes and lymphocytes through cytokine production, and present antigen to immunocompetent cells (8). These cells are also involved in allergic reactions: they express receptors with high affinity for IgE (FceRI) that, when aggregated by specific IgE and allergen, trigger mast cell degranulation with the release of vasoactive amines (such as histamine) and of proteases, and production of proinflammatory mediators (such as leukotrienes and prostaglandins) and of cytokines (9).

So far, four PLSCR (numbered 1 to 4) have been cloned in humans and three (numbered 1 to 3) in the mouse, demonstrating the existence of a family of PLSCR (10). Comparison of the amino
acid sequence of all the members of the PLSCR family shows that they are type-II membrane proteins with strong homology for the transmembrane domain and for most of the intracellular domain. This strongly suggests that these represent the portion of the molecule that accounts for scramblase activity. The extracellular domain is not conserved among members and extremely short or absent, suggesting it plays no, or little, role in scrambling activity. Interestingly, the intracellular amino-terminus is extremely variable in length and composition among members of the family. It is thus possible that it serves as a regulatory domain for the scrambling activity or that it serves additional functions unrelated to scrambling. In this regard, it should be noted that counterstructures for Src-homology (SH) 3 domains are present in this region of the molecule for most members of the family.

Original studies focusing on the regulation of PLSCR activity, demonstrated that it is dependent on calcium (11). Yet, most of these studies were performed with purified PLSCR reconstituted into liposomes (11-13) or into inside-out vesicles (13), i.e. away from other potential regulatory elements. Today many data from the litterature point to other regulatory mechanisms of PLSCR activation. PS exposure correlates with the level of PLSCR expressed in cells stimulated with a calcium ionophore (14) but not when the cells are stimulated by a more physiologic stimulus (15). The calcium concentration required for the onset of scramblase activity is higher when this enzyme is reconstituted into inside-out vesicles than into liposomes (13) suggesting that elements associated with the plasma membrane might negatively regulate the enzyme. In Scott syndrome, although phospholipid scrambling is impaired, the level of PLSCR1 in blood cells is normal, the nucleotide sequence of PLSCR1 shows no mutation and its activity after purification and reconstitution into liposomes is normal (16-18). Additional data suggest that regulatory elements rather than PLSCR itself are impaired in this syndrome (19,20). In mast cells, externalisation of PS induced by FceRI-mediated cell activation is transient (7), revealing the action of down-regulatory elements. Furthermore in these cells, the increase in free intracellular calcium after FceRI aggregation is at most of 1µM (21), far from the millimolar range required to activate purified PLSCR in vitro (13). More direct evidences for regulations that are not limited to calcium, have been recently reported. Thus, PLSCR is a target for protein kinase C (PKC) δ:
when phosphorylated by the latter, the former has an increased activity (22). As well, it has been reported that palmitoylation of PLSCR potentiates its activation by calcium (23).

Here, we have cloned a new member of the PLSCR family expressed in rat mast cells using specific mouse monoclonal antibodies (mAbs). We demonstrate that, upon aggregation of FceRI, PLSCR shows dramatic increase in tyrosine phosphorylation. Thus, PLSCR is a target for tyrosine kinase activity and is involved in signaling by an immune receptor. The significance of these observations is discussed.
Experimental procedures

Purification of phosphoproteins for mAb production.

Fifteen 150 cm² culture flasks containing ~5x10⁸ RBL-2H3 cells altogether were washed twice with Hank’s buffer saline solution (Life Technologies, Cergy Pontoise, France) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Saint Quentin Fallavier, France) and 50 mM Tris, pH 7.2 (HBT) and stimulated with 100 ng/ml of anti-rat FcεRIα mAb BC4 (a generous gift from Dr. R. Siraganian, NIH, Bethesda, MD) in HBT for 10 min. After 2 washes in ice-cold phosphate buffered saline solution (PBS) the cells were lysed in 2 ml per flask of lysis buffer (50 mM Tris, pH 7.2 containing 1% Triton X-100, 0.1% SDS, 50 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 250 KIU/ml aprotinin and 50 µg/ml leupeptin). After 10 min on ice, the cell lysate was recovered by scraping, and the post-nuclear supernatant was immunoprecipitated with 3 ml of a mixture of anti-phosphotyrosine mAb 4G10 coupled to agarose beads (Upstate Biotech., Lake Placid, NY) and PY20 coupled to agarose beads (Transduction Labs, Lexington, KY). After 4 hr on a rotating wheel at 4°C the beads were washed 6 times at 4°C with 10 ml lysis buffer, followed by 2 washes with 10 ml PBS, and 2 washes with 10 ml PBS containing 30 mM octyl-glucopyranoside (Sigma-Aldrich, Saint Quentin Fallavier, France) (PBS-O). The beads were then eluted twice by a 30-min incubation at 4°C on a rotating wheel in 3 ml PBS-O containing 40 mM phenylphosphate. The eluates were pooled and concentrated to 50-100 µl. After each purification, the quality of the material was controled in anti-phosphotyrosine immunoblots. The eluates were found to be specific since, in contrast to phenylphosphate elution, no protein was eluted by PBS-O alone as observed after silver staining. After elution, the beads were regenerated by two 30-min elutions with 1 M NaCl and conserved at 4°C in 0.2 M borate buffered saline solution, pH 8.

Hybridoma production.

A BALB/c mouse was hyperimmununized with the phosphoproteins by several injections of the eluates into the footpads. A new batch of eluate was prepared immediately before each injection. The first injection was in 200 µl of a 1:1 mixture with complete Freund’s adjuvant, the second was a week later in a 1:1 mixture with incomplete Freund’s adjuvant, and the next 4 injections were at 1-
week intervals in PBS. One day after the last injection the mouse was sacrificed, the inguinal and popliteal lymph nodes excised, the cells were fused with Ag8.653 myeloma cells and the resulting hybridomas were selected as described (24). After selection, hybridomas were frozen and their culture supernatants conserved at 4°C in 0.1% NaN3 for screening.

Screening of the hybridomas.

Screening was completed by an immunoblotting approach. Purified phosphoproteins were resolved in 4-15% polyacrylamide 2-D minigels (BioRad, Ivry sur Seine, France), and transferred onto a polyvinylidenedifluoride membrane (Millipore, Bedford, MA). The membrane was incubated for 1 hr in 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (TTBS) containing 4 % BSA, inserted in the mini-Protean® II Multiscreen apparatus (BioRad, Ivry sur Seine, France) and each channel was filled with 600 µl of a 1:30 dilution of hybridoma supernatant in TTBS containing 0.5% BSA (TTBS-B). Fifteen supernatants were screened per membrane and each supernatant was tested with phosphoproteins resolved under reducing and non-reducing conditions. After 1 hr incubation, each channel was washed twice with 10 ml TTBS. The membranes were then incubated for 1 hr with a 1:30,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma-Aldrich, Saint Quentin Fallavier, France) in TTBS-BSA. After 3 washes in TTBS, proteins were visualized with the SuperSignal chemiluminescence kit from Pierce (Rockford, IL) and the membranes were exposed for autoradiography with X-OMAT films (Eastman Kodak, Rochester, NY).

Hybridomas corresponding to selected positive supernatants were thawed and subcloned, each set of subclones being screened likewise. Immunoglobulin G in cell culture supernatants or in ascitis fluid supernatants from selected positive subclones were purified on protein G-coupled beads and a fraction was coupled to CNBr-activated Sepharose 4B beads (both beads from Pharmacia, Uppsala, Sweden), according to the manufacturer’s recommendations. The mAbs used in this study (129.2, 17.3 and 9.18) were all IgG1.

Construction and screening of the library.
Total RNA was extracted from RBL-2H3 cells with TRIZOL™ (Life Technologies, Cergy Pontoise, France). mRNA was purified with the Messenger RNA Isolation kit from which 5 µg were used to construct a cDNA library in λZAP with the ZAP-cDNA™ Gigapack® III Gold Cloning kit, both kits from Stratagene (La Jolla, CA). All procedures were according to the corresponding manufacturer’s recommendations. Three million cDNA independent clones thus were obtained. Screening with purified mAb 129.2 of 4.5x10⁵ cDNA clones in λZAP was performed after infection of XL1 Blue-MRF’ bacteria, spreading onto agar plates, induction of protein synthesis by isopropyl β-D-thiogalactopyranoside and protein transfer by adsorption onto cellulose membrane. After extensive washings in TTBS containing 0.1% NaN₃, membranes were saturated by an overnight incubation in TTBS containing 4% BSA and blotted for 1 hr with 10 µg/ml mAb129.2 in TTBS-B. After washings of the membranes, the latter were incubated for 1 hr with a 1:30,000 dilution HRP-conjugated goat anti-mouse IgG antibodies in TTBS-B. After several washes, the positive clones were visualized with the SuperSignal kit from Pierce and exposed for autoradiography. Positive clones were plugged out from the agar plates and sequentially subjected to two additional rounds of screening to obtain complete isolation of the clones. The cDNA were excised in pBlueScript from the λZAP genome according to the manufacturer’s instructions. Sequencing of the two strands of the cDNA was performed by automated sequencing by Genome Express (Paris, France).

Alignments were with the Dialign 2.1 program (25) and edited with Boxshade 3.33c.

RBL-2H3 cell culture and stimulation, and peritoneal mast cell purification.

RBL-2H3 cells were maintained as published (26). Routine stimulation was as followed: 1x10⁶ cells per well were plated in 6-well plates overnight with or without a 1:1,000 dilution of an ascitic fluid supernatant of anti-DNP monoclonal IgE DNP-48 (a kind gift of Dr. R. Siraganian, NIH, Bethesda, MD). After two washes in HBT, the cells were stimulated with 1 µg/ml DNP-human serum albumin in HBT for 30 min at 37°C. Peritoneal mast cells were purified to 95% purity from 250-g male Sprague-Dawley rats (Janvier, Le Genest St Isle, France) according to ref. 27.
**Immunoprecipitations and immunoblot analyses.**

After stimulation and two washes in ice-cold PBS, the cells were lysed on ice in 200 µl of lysis buffer. After 10 min incubation on ice, the cell lysate was recovered by scraping, and the post-nuclear supernatant was immunoprecipitated for 2 hr at 4°C with 10 µl of the indicated anti-rat PLSCR mAb-coupled beads. After 6 washes of the beads with 1 ml ice-cold lysis buffer, the immunoprecipitated material was eluted by boiling in 50 µl Laemmli loading buffer. Half the eluates were analysed by immunoblotting following procedures already described (28) with mAb PY20 coupled to HRP (dilution 1:2,500; Transduction Labs, Lexington, KY) or with mAb 129.2, 17.3 or 9.18 at 1 µg/ml followed by goat anti-mouse Ig coupled to HRP. In some cases the membranes were stripped by 3 washes of 5 min in methanol before reprobing. Where indicated, the samples were not transferred and the gel was subjected to silver staining.

For reprecipitation experiments, immunoprecipitated proteins were first eluted by boiling for 5 min in 50 µl 50 mM Tris pH 7.2, containing 1% SDS. The eluate was quenched by addition of 950 µl lysis buffer and reprecipitated for 2 hr with 20 µl mAb 129.2-coupled beads. After 6 washes with 1 ml lysis buffer, the final elution was in 50 µl boiling Laemmli loading buffer.

**Immunofluorescence studies.**

Anti-PLSCR mAb 129.2 binds the intracellular portion of rat PLSCR. Therefore, cells had to be permeabilized in immunofluorescence studies carried out with this mAb. RBL-2H3 cells grown on slides were rinsed twice with ice-cold PBS and fixed by a 20-min incubation in cold 3% paraformaldehyde. After 2 washes of 10 min each in PBS, the cells were incubated for 10 min in 0.1 M glycine in PBS. Cells were then permeabilized by a 30-min incubation in 0.05% saponin in PBS containing 0.2% BSA at room temperature. Cells were labeled thereafter by addition of 50 µg/ml anti-rat PLSCR mAb 129.2 or control IgG1. One hour later, cells were washed 3 times in PBS/saponin/BSA before a 45-min incubation with FITC-labeled anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL). Cells were subsequently washed three times in PBS/BSA without saponin and incubated for 5 min with 2 µg/ml Texas Red-labeled wheat germ agglutinin (Molecular Probes, Eugene, OR). This latter incubation was performed to visualise...
membrane labeling. After 3 last washes in PBS/BSA, the slides were fixed and the cells were examined in a confocal laser microscope (LSM 510 Carl Zeiss, Jena, Germany).
Results and Discussion

As a means to identify new protein targets for tyrosine kinases in FcεRI-stimulated mast cells, the bulk of tyrosine-phosphorylated proteins from FcεRI stimulated RBL-2H3 mast cells was purified with anti-phosphotyrosine-coupled beads and specifically eluted with phenyl phosphate. MAbs were raised against these proteins and, after screening of 750 hybridoma clones, ten groups totalling 105 positive clones were identified, each group recognizing a different phosphoprotein of defined $M_r$. MAbs directed against a protein that migrated at ~40 kDa in 4-15% polyacrylamide gradient gels were first selected. One of these, mAb 129.2, was used to screen an RBL-2H3 cDNA expression library constructed in λZAP. Seven independent positive cDNA clones were isolated from 4.5x10^5 clones. One of these positive clones, clone 312, was fully sequenced. It was 1.6 kb in length that included the complete 3'-untranslated region, the complete coding sequence of a 335-amino acid protein and part of the 5'-untranslated region\(^1\) (Fig. 1a). A rat EST sequence\(^3\) was found in the GenBank nucleotide data bank that was identical to the stretch extending from nt 1056 to nt 1436 in clone 312 (data not shown). Matching the translated sequence from clone 312 against the translated GenBank nucleotide data bank showed also that it was most homologue to human\(^4\) (68 % identity) and mouse\(^5\) (84 % identity) PLSCR1 (Fig. 1b). Yet, two major differences are apparent. The human gene for PLSCR1 is composed of 9 exons, the third one coding for the sequence extending from N5 to Q31 (10). This sequence is absent in rat PLSCR (Fig. 1b). Rat PLSCR also contains an additional sequence made of six Q-G-P-Y-P/A-G-P repeats (Fig. 1b). Although this sequence is reminiscent of repeats present in the sequence of collagens its function remains so far unknown. Thus, it appears that the rat PLSCR that we have cloned is related to, but distinct from, PLSCR1. Analysis of the six remaining positive clones by partial sequencing and/or restriction digest indicated that all were also full-length cDNA clones of the same gene (data not shown). The deduced amino acid sequence analyzed with the TMpred program at the ISREC server, was consistent with a type-II transmembrane protein with a short extracellular domain (Fig. 1b). It contains an intracellular calcium-binding domain (29), multiple cysteines potential targets for palmitoylation (23), and three P-(X)4-P-(X)2-P domains that could associate with SH3 domains (Fig. 1b). A PKC target consensus sequence (R-X-X-T-X-
R) is conserved at T178. These combined features are found in the sequence of many members of the PLSCR family (10,30).

Immunoprecipitation and immunoblotting of rat PLSCR showed a single band of $M_r$ 37,000 (Fig. 2a) corresponding to the size expected from the deduced amino-acid sequence. In some instances, a doublet was observed that could be due to degradation by proteases. Identical results were obtained from purified rat peritoneal mast cells, further confirming that rat PLSCR is indeed expressed in rat mast cells (Fig. 2a). Immunofluorescence analysis showed that, in contrast to control IgG1, anti-rat PLSCR mAb 129.2 labeled RBL-2H3 cells with a pattern that was consistent with a membrane location of the protein (Fig. 2b, panel D). This was further confirmed by colocalization experiments carried out with wheat germ agglutinin, a lectin that binds extracellular domains of glycosylated membrane proteins (Fig. 2b, panels E and F).

Since the anti-rat PLSCR antibodies had been generated by immunization with proteins purified with anti-phosphotyrosine antibodies, we wished to know whether this protein is phosphorylated in RBL-2H3 cells. To that effect, immunoprecipitations were performed with mAb 129.2 and the eluted phosphoproteins were detected by immunoblotting with anti-phosphotyrosine mAb PY20. Heterogeneous phosphoproteins of 37-49 kDa were observed that did not comigrate with PLSCR as observed in anti-rat PLSCR immunoblots of the precipitates (Fig. 3). These phosphoproteins were also precipitated with another anti-rat PLSCR mAb from the same group, mAb 17.3, but not with mAb 9.18 that recognizes a 130-kDa phosphoprotein (Fig. 3) and not with numerous other mAb or polyclonal mouse IgG of irrelevant specificities (data not shown). In addition, when the cell lysate was first depleted of its PLSCR content by immunoprecipitation with mAb 129.2 followed by a second immunoprecipitation procedure with either mAb 17.3 (Fig. 3) or 129.2 (data not shown), the phosphoproteins were not detected anymore. Thus, these proteins seemed to associate directly or indirectly with rat PLSCR.

To determine whether FcεRI-mediated cell activation affected the phosphorylation of these phosphoproteins and of PLSCR, RBL-2H3 cells were stimulated with IgE and antigen for 30 min. The material recovered from anti-rat PLSCR immunoprecipitates was analyzed in anti-phosphotyrosine immunoblots. A dramatic increase in the tyrosine phosphorylation of the phosphoproteins was clearly observed (Fig. 4). This increase was observed in mAb 17.3 (Fig. 4)
as well as in mAb 129.2 (data not shown) immunoprecipitates. Yet, no clear-cut result was obtained regarding the tyrosine phosphorylation of PLSCR since, here again, the phosphoproteins did not comigrate with the latter.

Thus, to that point, we had not been able to determine whether rat PLSCR was actually phosphorylated on tyrosine. To solve this question, we first immunoprecipitated PLSCR with its supposedly associated phosphoproteins, dissociated the complex by boiling for 5 min in 1% SDS and reprecipitated PLSCR from the eluted material. Analysis of the tyrosine phosphorylated proteins showed a pattern identical to that of the phosphoproteins recovered without prior dissociation of the complex (Fig. 5a). It became apparent therefore, that the phosphoproteins were not precipitated through their association with rat PLSCR but through direct binding of anti-rat PLSCR mAb. Thus, the phosphoproteins could be PLSCR itself.

To definitely determine whether the phosphoproteins were in fact phospho-PLSCR, tyrosine phosphorylated proteins were purified from a large number of stimulated RBL-2H3 cells, concentrated and analyzed in anti-rat PLSCR immunoblots. An identical band of heterogeneous proteins of 37-49 kDa was observed in these samples and in anti-phosphotyrosine immunoblots of anti-rat PLSCR immunoprecipitates (Fig. 5b). As a control, mAb 9.18 immunoblots of anti-phosphotyrosine immunoprecipitates showed the expected 130-kDa phosphoprotein but not the 37-49 kDa proteins (Fig. 5b). Therefore, the phosphoproteins are in fact phospho-PLSCR.

Rat PLSCR was readily detected in silver staining of immunoprecipitates from 2x10^6 cells whereas phospho-PLSCR was not (Fig. 5c) demonstrating that only a very small fraction of this protein is phosphorylated on tyrosine. This explains the lack of detection of phospho-PLSCR in mAb 129.2 (or 17.3) immunoblotting of anti-rat PLSCR immunoprecipitates. This suggests that only finely targeted PLSCR are involved in FceRI signaling. The difference between the $M_r$ of non-phosphorylated PLSCR and the $M_r$ of phospho-PLSCR could be due to different causes. It is well known that phosphorylation of proteins can result in retarded migration in gels. Thus, tyrosine phosphorylation of rat PLSCR could result in an heterogeneous and retarded apparent $M_r$. This could be observed all the more if rat PLSCR is additionally phosphorylated on threonine by protein kinase Cδ (22). Of interest, PKCδ is involved in FceRI signaling (31,32). Another
reason for the retarded \( M_r \) observed could be the potential multiple palmitoylations of rat PLSCR on the numerous clusters of cysteines that are present in the molecule (ref. 23 and Fig.1b). Additional studies will be necessary to determine the degree of palmitoylation of PLSCR in rat mast cells. Thus, it is likely that combined post-transcriptional modifications account for the large and heterogeneous shift in the \( M_r \) of a fraction of PLSCR after Fc\(\varepsilon\)RI engagement.

The role of tyrosine phosphorylation of PLSCR could be related to the known ability of this family of proteins to promote a redistribution of phospholipids across the plasma membrane (11). Indeed, activation of mast cells through Fc\(\varepsilon\)RI leads to transient externalisation of PS that correlates with degranulation (6,7). Although it is known that externalisation of PS is not dependent on tyrosine kinases in erythrocytes stimulated by calcium ionophores (20), this dependence could be true in mast cells stimulated through Fc\(\varepsilon\)RI. Indeed, it is also well known that tyrosine kinases are important for IgE-dependent mast cell degranulation (33). Alternatively, PLSCR tyrosine phosphorylation could be involved in the down-regulation of Fc\(\varepsilon\)RI-induced phospholipid scrambling, since the latter is transient in mast cells (7). Another possible role for PLSCR phosphorylation could be to make this protein serve as an adaptor for other effector molecules. It contains three proline-rich domains that could associate with SH3 domains on other proteins, and its location could be used by the Fc\(\varepsilon\)RI-generated signal to target SH2 domain-containing effector molecules at the plasma membrane. All these possibilities are under active investigation with the use of mutant and chimeric proteins. In summary, this study reports the cloning of a new member of the PLSCR family, and the first observation that a member of this family is a target for tyrosine kinases and is involved in signaling by an immune receptor. This opens new perspectives pertaining to the role of phospholipid scramblases and to the mechanisms involved in their regulation.

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Footnotes

1 Abbreviations used in this paper:
BSA, bovine serum albumin; FcεRI, IgE receptor type I; HBT, Hank’s buffer saline solution supplemented with BSA and Tris; HRP, horseradish peroxidase; mAb, monoclonal antibody; PBS, phosphate-buffered saline solution; PBS-O, PBS containing octyl glucopyranoside; PLSCR, phospholipid scramblase; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; SH, Src-Homology domain; PKC, protein kinase C; TTBS, Tris-buffered saline solution containing Tween; TTBS-B, TTBS containing BSA.

2 The nucleotide sequence for the rat PLSCR has been deposited in the GenBank database under GenBank Accession Number AY024347

3 GenBank Accession Number AA924029

4 GenBank Accession Number AF098642 and GenBank Accession Number AB006746

5 GenBank Accession Number AF159593
Figure legends

**Figure 1. Sequencing of rat PLSCR.** a) Nucleotide and deduced amino acid sequence of clone 312. b) Comparison between rat PLSCR, and human and mouse PLSCR1 amino acid sequences. Identical residues are boxed in black, conservative substitutions are boxed in gray. The potential transmembrane domain identified with the TMpred program at the ISREC server is underlined with a thick line. The calcium binding domain is underlined with a dotted line, the three proline-rich domains (P-(X)4-P-(X)2-P) with a thin line, the three cysteine-rich regions with a broken line and the threonine residue that is a consensus target for PKC is shown with an arrowhead. The exon 3-encoded region of human (and possibly mouse) PLSCR1 that is missing in rat PLSCR is indicated by a double underline.

**Figure 2. Expression of PLSCR by rat mast cells.** a) Immunoblot analysis in RBL-2H3 cells and rat peritoneal mast cells. PLSCR immunoprecipitated with mAb 129.2 from 5x10⁵ RBL-2H3 cells (as indicated) or 2.5x10⁵ purified peritoneal rat mast cells (PMC) was analysed in mAb 129.2 immunoblots from 10% polyacrylamide gel. b) Immunofluorescence analysis. RBL-2H3 cells were labeled as described in the Experimental Procedures section. The labeling was with control mouse IgG1 (panel B) or anti-rat PLSCR mAb 129.2 (panel D) and FITC-labeled secondary antibody, or with Texas Red-labeled wheat germ agglutinin (panel E). Panel F shows merging of panels D and E. Panels A and C show the light transmission microscopy corresponding to panels B and D-F, respectively.

**Figure 3. Phosphoproteins in anti-rat PLSCR immunoprecipitates.** Lanes 1 and 2: proteins from non-stimulated RBL-2H3 cell lysates were first immunoprecipitated with control mAb 9.18 anti-130 kDa protein (C) or 129.2 (129). Lane 3 and 4: the effluents were submitted to a second round of immunoprecipitation with mAb 17.3 as indicated. Eluates from both rounds of immunoprecipitation were analysed in anti-phosphotyrosine (anti-PY) and, after stripping, anti-rat PLSCR (129.2) immunoblotting. Lane 3: immunoprecipitate from the effluent of the sample analysed in lane 1. Lane 4: immunoprecipitate from the effluent of the sample analysed in lane 2.
The arrow shows migration of the phosphoproteins in anti-rat PLSCR immunoprecipitates. The arrowhead shows the position of rat PLSCR. Gel: 12% polyacrylamide.

**Figure 4.** *Increased tyrosine phosphorylation of the phosphoproteins after FceRI engagement.* RBL-2H3 cells were stimulated (+) or not (−) with IgE and antigen for 30 min. Proteins from the cell lysates were immunoprecipitated with anti-rat PLSCR mAb 17.3 (IP 17.3) and immunoblotted with anti-phosphotyrosine antibody (anti-PY) and, after stripping of the membrane, with anti-rat PLSCR mAb 129.2 as indicated. The arrow shows migration of the phosphoproteins in anti-rat PLSCR immunoprecipitates. The arrowhead shows the position of rat PLSCR. Exposure time of the anti-PY immunoblot is shorter than in the experiment depicted in Fig. 3 to emphasize the dramatic difference between samples from stimulated and non-stimulated cells. Gel: 12% polyacrylamide.

**Figure 5.** *Identification of the phosphoproteins as phospho-PLSCR.* a) Reprecipitation experiments. RBL-2H3 cells were stimulated (+) or not (−) with IgE and antigen for 30 min. Proteins from the cell lysates were either immunoprecipitated with anti-rat PLSCR mAb 129.2 (IP 129), or immunoprecipitated with anti-rat PLSCR mAb 129.2, eluted by boiling in 1% SDS for 5 min and reprecipitated with mAb 129.2 as indicated. Samples were immunoblotted with anti-phosphotyrosine antibody (anti-PY) and, after stripping of the membrane, with anti-rat PLSCR mAb 17.3 as indicated. The arrow shows migration of the phosphoproteins in anti-rat PLSCR immunoprecipitates. The arrowhead shows the position of rat PLSCR. Gel: 7.5% polyacrylamide.

b) Anti-rat PLSCR immunoblotting of anti-phosphotyrosine immunoprecipitates. Proteins from 5x10⁵ RBL-2H3 cells were immunoprecipitated (IP) with mAb 129.2 as indicated and eluted by boiling in Laemmli loading buffer. Proteins from 2x10⁷ RBL-2H3 were immunoprecipitated with anti-phosphotyrosine mAb PY20 (PY), eluted with 40 mM phenylphosphate and concentrated before boiling in Laemmli loading buffer. Eluates were immunoblotted (Blot) with anti-phosphotyrosine antibody (PY), 129.2 or 9.18 as indicated. The arrow shows the migration of
phospho-PLSCR. The arrowhead shows the position of the 130-kDa phosphoprotein recognized by mAb 9.18. Note that additional proteins are detected in both 129.2 and 9.18 immunoblots that are due to binding of the secondary antibody. Gel: 7.5% polyacrylamide. c) Silver staining of anti-rat PLSCR precipitates. Rat PLSCR was immunoprecipitated from the lysates of 2x10^6 RBL-2H3 cells stimulated or not through FceRI, and resolved in 7.5% polyacrylamide gel before silver staining of the gel. Migration of PLSCR is indicated by an arrow and Ig heavy chain by an arrowhead.
Fig 1b
Fig. 2a
Fig. 3
Fig. 4

| kDa | 175 | 83 | 68 | 47.5 | 32.5 | 27 | 16 |
|-----|-----|----|----|------|------|----|----|

stimulation - + IP 17.3 - +

blot anti-PY 129.2
Fig. 5a
Fig. 5b
stimulation

-  +

kDa

175
83
68
47.5
32.5

Fig. 5c
IgE-receptor type I dependent tyrosine phosphorylation of phospholipid scramblase
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