Enzymatic measurement of phosphatidylserine in cultured cells

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Abstract Phosphatidylserine (PS) is a quantitatively minor membrane phospholipid involved in diverse cellular functions. In this study, we developed a new fluorometric method for measuring PS using combinations of specific enzymes and Amplex Red. The calibration curve for PS measurement was linear and hyperbolic at low (0–50 µM) and high (50–1000 µM) concentrations, respectively, and the detection limit was 5 µM (50 pmol in the reaction mixture). This assay quantified PS regardless of the chain length and the number of double bonds. We applied this new method to the determination of PS content in HEK293 cells, which was validated by a recovery study and comparison with TLC-phosphorus assay. We showed that the PS content was high in sparse cells. The overexpression of PS synthase 1 elevated not only the cellular PS content but also the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents, suggesting the conversion of PS into PE and the enhancement of PC production. This new assay for PS measurement is simple, specific, sensitive, and high throughput, and it will be useful to clarify the metabolism and biological functions of PS.—Morita, S-y., S. Shirakawa, Y. Kobayashi, K. Nakamura, R. Teraoka, S. Kitagawa, and T. Terada. Enzymatic measurement of phosphatidylserine in cultured cells. J. Lipid Res. 2012. 53: 325–330.

Supplementary key words Amplex Red • phosphatidylcholine • phosphatidylethanolamine • phosphatidylserine synthase 1

Phosphatidylserine (PS) is a quantitatively minor component of cell membranes, accounting for ~2–10% of total phospholipids, but it plays important roles in biological processes such as apoptosis, blood coagulation cascade, and cell signaling, in addition to a structural role in membranes (1). The PS synthesis in mammalian cells is catalyzed by PS synthase 1 (PSS1) and PS synthase 2 (PSS2), which are responsible for the exchange of L-serine with polar head groups of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), respectively (2, 3). On the other hand, in bacteria and yeast, PS is synthesized from CDP-diacylglycerol and L-serine (4, 5). Plants use both the base-exchange pathway and the CDP-diacylglycerol pathway to make PS (6). PS is also an important precursor of PE, which is produced by PS decarboxylase (PSD) in bacteria, yeast, plant, and mammalian cells (7).

In the plasma membrane of mammalian cells, the majority (>80%) of PS normally resides on the inner leaflet of the bilayer (1). During the early phase of apoptosis, PS becomes externalized to the outside of cells (8). The surface exposure of PS is one of the recognition signals by which apoptotic cells are removed by phagocytes. The overexpression of either PSS1 or PSS2 in Chinese hamster ovary (CHO) cells reduces the number of cells undergoing apoptosis in response to UV irradiation (9). When blood platelets are activated, PS is exposed on the platelet surface to trigger the clotting system (10). The proteolytic activity of the factor VIIa-tissue factor complex requires very high local concentrations of PS (11). In addition, PS becomes exposed on the outside of sperm during their maturation (12). Recently, Suzuki et al. reported that transmembrane protein 16F is an essential component for the Ca2+-dependent exposure of PS on the cell surface (13). PS activates several signaling proteins, including protein kinase C, neutral sphingomyelinase, cRaf1 protein kinase, and dynamin-1 (14–17). Hsp70 and Hsc70 display highly selective interaction with PS on membranes, followed by rapid incorporation into the lipid bilayer (18). Furthermore, PS directs proteins with moderately positive charge to the endocytic pathway (19). Histone H2B, a plasminogen receptor, tethers to the surface of cells by interacting with PS on differentiated or apoptotic monocytoid cells (20).

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Abbreviations: CHO, Chinese hamster ovary; LAAO, L-amino acid oxidase; LPS, lysophosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLD, phospholipase D; POPS, L-α-palmitoyl-oleoyl phosphatidylserine; PSD, phosphatidylserine decarboxylase; PSS1, phosphatidylserine synthase 1; PSS2, phosphatidylserine synthase 2.

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Materials and Methods

Materials

L-Amino acid oxidase (LAAO) from Crotalus adamanteus venom was obtained from Worthington (Lakewood, NJ). Phospholipase D (PLD) from Crotalus adamanteus venom was purchased from Worthington (Lakewood, NJ). Phospholipase D (PLD) from Streptomyces chromofuscus was purchased from Biomol International (Plymouth Meeting, PA). Peroxidase from horseradish roots was obtained from Oriental Yeast (Osaka, Japan). Amplex Red reagent was purchased from Molecular Probes (Eugene, OR). L-α-palmitoyl-oleoyl PS (POPS) sodium salt, PS sodium salt from soy, PS sodium salt from porcine brain, L-α-monooxyleol phosphatidylserine, L-α-palmitoyl-oleoyl PC, and L-α-palmitoyl-oleoyl PE were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals used were of the highest reagent grade.

Enzymatic measurement of PS

Measurement was performed using a three-reagent system. Reagent S1 contained 600 units/ml PLD, 20 unit/ml LAAO, 50 mM NaCl and 50 mM Tris-HCl (pH 7.4). Reagent S2 contained 6.25 unit/ml peroxidase, 187.5 µM Amplex Red, 0.125% Triton X-100, 50 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Amplex Red Stop Reagent was purchased from Molecular Probes. PS standard solutions were dissolved in 1% Triton X-100 aqueous solution. Sample (10 µl) was added to Reagent S1 (10 µl) and incubated at 25°C for 240 min. After the incubation, Reagent S2 (80 µl) was added. After 15 min of incubation at room temperature, Amplex Red Stop Reagent (20 µl) was added. The fluorescence intensity was measured using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Rockford, IL). The excitation and emission wavelength filters were set at 544 and 590 nm, respectively.

Recombinant plasmid construction

The human PSS1 gene (GenBank: D14694) was obtained from Kazusa DNA Research Institute (Chiba, Japan). Using PCR, an oligonucleotide encoding a myc (EQKLISEEDL)-tagged epitope was appended to the 5’ end of PSS1. These PCR products were ligated into the Afl II and Bam HI sites of the pIRESngeo3 mammalian expression vector (Clontech, Mountain View, CA) to generate the plasmids pIRESngeo3/myc-PSS1. pIRESngeo3 contains the internal ribosome entry site, which permits the translation of two open reading frames from one mRNA. This expression system facilitates the establishment of pools of stably transfected cell lines whereby nearly all cells surviving in selective media express the gene of interest, as the neomycin phosphotransferase gene is expressed under the control of the same promoter (26).

Cell culture

HEK293 cells were grown in MEM supplemented with 10% heat-inactivated FBS in a humidified incubator (5% CO2) at 37°C.

Establishment of stable transfectants of myc-PSS1

HEK293 cells were transfected with pIRESngeo3/myc-PSS1 using Lipofectamine Reagent and PLUS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were selected with 1.2 mg/ml G418 disulfate, and a large number of G418-resistant clones were pooled in one dish.

Expression of PSS1

The expression of PSS1 was examined by Western blotting. Cells were lysed with PBS containing 1% Triton X-100 and protease inhibitors (100 µg/ml p-APMSF, 10 µg/ml leupeptin, and 2 µg/ml aprotinin). Cell lysate proteins were separated by SDS-PAGE on a 10% polyacrylamide gel calibrated with Precision Plus Protein WesternC Standards (Bio-Rad Laboratories, Hercules, CA). These proteins were transferred to PVDF membranes and immunoblotted with the monoclonal anti-Myc antibody MC045 (1:1000 dilution; Nacalai Tesque, Kyoto, Japan), polyclonal anti-PSS1 antibody V-19 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or monoclonal anti-β-actin antibody AC-15 (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Protein-antibody complexes were detected by enhanced chemiluminescence using horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000 dilution; Invitrogen) or donkey anti-goat IgG (1:5000 dilution; Promega, Madison, WI) and exposed to X-ray films.

Measurement of PS, PC, and PE contents in cells

Cells were subcultured in 10 cm dishes at various cell densities in MEM supplemented with 10% FBS. After incubation for 48 h, the cells were washed with fresh medium and incubated with MEM containing 0.02% BSA for 18 h at 37°C. After incubation, the cells were chilled on ice, washed, and scraped with cold PBS. The cells were sonicated, and the cell protein concentration was measured using a BCA protein assay kit (Thermo Scientific). Cells were lysed with PBS containing 1% Triton X-100 and protease inhibitors (100 µg/ml p-APMSF, 10 µg/ml leupeptin, and 2 µg/ml aprotinin). Cell lysate proteins were separated by SDS-PAGE on a 10% polyacrylamide gel calibrated with Precision Plus Protein WesternC Standards (Bio-Rad Laboratories, Hercules, CA). These proteins were transferred to PVDF membranes and immunoblotted with the monoclonal anti-Myc antibody MC045 (1:1000 dilution; Nacalai Tesque, Kyoto, Japan), polyclonal anti-PSS1 antibody V-19 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or monoclonal anti-β-actin antibody AC-15 (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Protein-antibody complexes were detected by enhanced chemiluminescence using horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000 dilution; Invitrogen) or donkey anti-goat IgG (1:5000 dilution; Promega, Madison, WI) and exposed to X-ray films.

Statistical analysis

The statistical significance of differences between mean values was analyzed using the nonpaired t-test. Multiple comparisons were performed using the Bonferroni test following ANOVA. Differences were considered significant at P < 0.05. Unless indicated otherwise, results are given as mean ± SE.
RESULTS AND DISCUSSION

PS measurement

We developed a new method for the enzymatic measurement of PS. The three steps for the enzymatic measurement of PS are illustrated in Fig. 1: i) PS is hydrolyzed to serine and PA by PLD; ii) serine is oxidized by LAAO, which generates \( \text{H}_2\text{O}_2 \), ammonia, and 2-oxo-3-hydroxypropionic acid; iii) finally, in the presence of peroxidase, \( \text{H}_2\text{O}_2 \) reacts with Amplex Red to produce highly fluorescent resorufin, which can be measured. This method requires only a 10 µl sample volume in a 96-well format.

To validate this novel assay for PS measurement, a calibration reaction was performed using POPS standard solutions. At high concentrations of PS, the curve was less linear and fit a hyperbolic regression equation \( (r = 0.9998) \) (Fig. 2A). As shown in Fig. 2B, the standard curve for PS measurement was linear at low concentrations \( (r = 0.9987) \), and the detection limit was as low as 5 µM (50 pmol in the reaction mixture). The fluorescence change in response to POPS was equal to that in response to soy PS or brain PS containing mixed acyl chains (Fig. 2C). Therefore, the chain length or the number of double bonds does not affect this PS measurement. PS and lysophosphatidylserine (LPS) increased the fluorescence to the same extent when normalized to moles (Fig. 2C), indicating that PLD also hydrolyzes LPS to release serine. Thus, this measurement cannot distinguish between PS and LPS. On the other hand, PE or PC was not detected by this assay (Fig. 2C).

Measurement of PS in cultured cells

Cultured cells contain considerable amounts of amino acids, which confuse the PS measurement. The method of Bligh and Dyer has been widely used for lipid extraction, followed by quantification of PS using radiolabeled serine and/or TLC \( (25, 29, 30) \). Accordingly, to remove the contaminating amino acids that react with LAAO, lipid extraction from cells is necessary for the enzymatic measurement of PS.

The LPS content in cellular membrane is generally much lower than the PS content and not detectable \( (31, 32) \). Additionally, the extraction of lysophospholipids using the method of Bligh and Dyer requires acidic conditions \( (33) \). Hence, the LPS concentration may be low enough to be negligible in the cellular lipid extract prepared by the normal method of Bligh and Dyer even though the enzymatic assay measures the total concentration of PS and LPS.

\[
\text{PS} + \text{H}_2\text{O} \xrightarrow{\text{PLD}} \text{PA} + \text{Serine} \\
\text{Serine} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{LAAO}} \text{H}_2\text{O}_2 + \text{NH}_3 + 2\text{-Oxo-3-hydroxypropionic Acid} \\
\text{H}_2\text{O}_2 + \text{Amplex Red} \xrightarrow{\text{Peroxidase}} \text{Resorufin}
\]

To test the accuracy of the method, we conducted a recovery test, in which known quantities of POPS were added to the cellular lipid extract \( (\text{Table 1}) \). The mean percentage recovery of PS was 98.6%, indicating no interference of hydrophobic compounds extracted from the cells.

To validate our novel PS assay, we compared it with the TLC-phosphorus assay. Thirty samples of HEK293 cells were analyzed by both the present method and the TLC-phosphorus method. We found that the two approaches correlated well \( (r = 0.9803, y = -0.8007 + 0.9999x) \) (Fig. 3).

Effect of cell density on PS content in HEK293 cells

It has been difficult to measure the PS content in sparse cell cultures, and the relationship between cell density and...
PS content has not yet been characterized. Using the enzymatic measurement of PS, we quantified the PS content in HEK293 cells at various cell densities. As shown in Fig. 4, the PS content was constant between the medium and highest cell densities (45.2 and 120.3 µg protein/cm², respectively), whereas the PS content at the lowest cell density (22.9 µg protein/cm²) was significantly greater than the PS content at all other cell densities. We previously reported that an increase in the density of HEK293 cells is accompanied by a decrease in the PA content, whereas the PC and PE contents increase with increasing cell density (24, 25). These findings suggest that the membrane phospholipid metabolism, including the synthesis and degradation of PS, is controlled by the signaling from cell-cell adhesion and/or cellular maturation.

Effect of PSS1 overexpression on PS content in HEK293 cells

To test whether the new assay can detect the change of cellular PS content, we established a HEK293 cell line stably expressing myc-PSS1 (HEK/myc-PSS1). A myc-tag was fused to the N-terminus of PSS1 for immunodetection using anti-myc antibody. It has been shown that the tagging of PSS1 does not affect the enzymatic activity or the subcellular targeting of PSS1 (3, 34). Fig. 5 shows that myc-PSS1 was detected by immunoblotting with anti-myc antibody. The expression level of myc-PSS1 detected using polyclonal anti-PSS1 antibody was strikingly higher than that of endogenous PSS1 in HEK293 cells. PSS1 protein exhibited a low apparent molecular mass of 37 kDa on SDS-PAGE compared with the size predicted from the amino acid sequence (55.5 kDa), which may be attributable to the extremely high content of hydrophobic amino acids in PSS1 (34, 35).

By using enzymatic assays, we determined the contents of PS, PC, and PE in HEK293 and HEK/myc-PSS1 cells at similar cell densities (73.1 ± 2.4 and 68.4 ± 2.0 µg protein/cm², respectively). As shown in Fig. 6A, HEK/myc-PSS1 cells exhibited a 1.3-fold increase in the PS content compared with HEK293 cells. In addition, the PC and PE contents in HEK/myc-PSS1 cells were significantly higher than those in HEK293 cells (Fig. 6B, C). We also assessed the effect of myc-PSS1 expression on the PS/PC, PE/PC, and PS/PE ratios. The PS/PC and PE/PC ratios were slightly but significantly increased in HEK/myc-PSS1 cells (Fig. 6D, E). On the other hand, the PS/PE ratio was similar between the two cell lines (Fig. 6F).

Table 1. Recovery of PS added to the cellular lipid extract

| Added (µM) | Measured (µM) | Expected (µM) | Recovery (%) |
|------------|---------------|---------------|--------------|
| 0          | 49.9          |               |              |
| 25         | 74.4          | 74.9          | 99.3         |
| 50         | 94.2          | 96.3          | 97.8         |
| 125        | 189.4         | 188.3         | 100.6        |
| 250        | 303.1         | 313.3         | 96.8         |

The POPS standard solution was added to the lipid extract from HEK293 cells. The concentrations of PS were measured by the enzymatic assay.

CHO-K1 cells transfected with Chinese hamster PSS1 gene show only slight increases in the PS and PE contents (30). The levels of PS, PE, and PC are not significantly increased in McArdle 7777 rat hepatoma cells overexpressing mouse PSS1 (29). The PS synthesis in CHO-K1 cells is remarkably inhibited by exogenous PS, which may occur through the feedback inhibition of PSS1 and PSS2 by PS (30, 36). PE is generated in mammalian cells by the CDP-ethanolamine pathway and the PS decarboxylation pathway (1). The medium used for the culture of animal cells usually lacks ethanolamine, and cultured cells produce PE largely through the PS decarboxylation pathway (1, 37). In the present study, PSS1 overexpression led to an increase in the cellular PE content (Fig 6C), which may be ascribed to the conversion of excess PS into PE by PSD. The overexpression of human PSS1 induced only a modest increase in the PS content (Fig. 6A), presumably due to the feedback control of PSS1 and the PS decarboxylation. Notably, the overexpression of PSS1 had no effect on the PS/PE ratio (Fig. 6F), suggesting the cells tightly maintain the PS/PE ratio through the synthesis and decarboxylation of PS. HEK293 cells synthesize PC via the CDP-choline pathway and do not express PE N-methyltransferase (25). CTP:phosphocholine cytidylyltransferase, a rate-limiting enzyme, catalyzes the formation of CTP:phosphocholine, which is necessary for the synthesis of PE. These findings suggest that the membrane phospholipid metabolism, including the synthesis and degradation of PS, is controlled by the signaling from cell-cell adhesion and/or cellular maturation.
The enzyme in the CDP-choline pathway, is activated by reversible binding to cell membrane lipids. PS and PE promote the binding and activation of this enzyme (38, 39). It is conceivable that the PC production was enhanced by increased PS and PE in HEK/myc-PSS1 cells, which resulted in the suppression of the changes in PS/PC and PE/PC ratios. Furthermore, it is possible that PSS1 overexpression affected de novo L-serine synthesis via phosphorylated pathway and/or exogenous L-serine uptake into the cells.

Thus, the enzymatic methods may be useful to clarify the regulation of phospholipid composition.

In conclusion, we developed and validated a novel enzymatic assay for measuring PS in cultured cells. This convenient method has better specificity and requires a smaller quantity of samples than previously reported methods, and it allows many samples to be processed in parallel. In addition, all enzymes and substrates are available commercially. The usefulness of this assay was demonstrated using HEK293 cells overexpressing PSS1. In principle, this method will be applicable to PS measurement in subcellular organelles or in animal tissues and fluids. This enzymatic measurement may be helpful to study the biological functions of PS and its related enzymes, including PSS1, PSS2, and PSD. In the near future, the high-throughput enzymatic methods for measuring every phospholipid will be developed and replace the conventional method.

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