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Purification and characterization of a major phosphatidylserine-binding phosphoprotein from human platelets

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We describe the isolation, lipid-binding properties and partial amino acid sequence of PS-p68, a novel 68 kDa phosphatidylserine-binding protein from human platelets. PS-p68 is an abundant constituent of platelets, accounting for 0.5–0.75 % of total cell protein. It was purified from platelet cytosol by affinity chromatography. Amino acid sequence analysis yielded no similarity to identified proteins. In contrast with most known phospholipid-binding proteins, PS-p68 does not bind Ca^{2+} and does not require Ca^{2+} for its binding of phosphatidylserine. Phosphatidylserine binding to PS-p68 was inhibited by phosphatidic acid and by alkylphospholipids. PS-p68 was isolated as a major phosphoprotein from ^{32}P-labelled platelets and was found to function as a protein kinase C substrate *in vitro*. However, treatment of intact platelets with phorbol 12-myristate 13-acetate, thrombin or carbacyclin did not increase PS-p68 phosphorylation. Platelets appear to be the only blood cells containing PS-p68, which was not detected in neutrophils, monocytes and lymphocytes.

INTRODUCTION

In the accompanying paper we describe a novel class of proteins that bind phosphatidylserine (PS). These proteins bind phospholipids in the absence of Ca^{2+} and also bind protein kinase C in the presence of PS [1]. PS-binding proteins of molecular masses 115 kDa and 100 kDa were found in neutrophils, monocytes, lymphocytes, platelets and erythrocytes. In platelets, however, the principal PS-binding species is a cytosolic protein of molecular mass 68 kDa. In the present paper we describe the purification, partial amino acid sequence and properties of this novel PS-binding protein, termed PS-p68.

MATERIALS AND METHODS

Cells

Platelets, neutrophils, monocytes and lymphocytes were isolated from buffy coats of citrate-anticoagulated donor blood (Swiss Red Cross Laboratory, Bern, Switzerland) as described in the accompanying paper [1].

Purification of PS-p68

Cytosol from 100 ml of platelets (7.5×10^9 cells/ml) was prepared by sonication (Branson Sonic Power, 4 °C for 2 min) in a buffer containing 20 mM-Hepes, 138 mM-NaCl, 3.3 mM-KCl, 1 mM-$MgCl_2$, 5 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride and 0.045 mM-leupeptin, pH 7.3, followed by ultracentrifugation (150000 *g* for 30 min). Cytosolic proteins were fractionated by $(NH_4)_2SO_4$ precipitation. The precipitate obtained at 20–40 % saturation was resuspended in 27 ml of a buffer containing 20 mM-Tris/HCl, 150 mM-NaCl, 4 M-urea, 1 mM-EDTA and 1 mM-EGTA, pH 7.7, and 6.2 ml portions were loaded on a 100 cm × 2 cm Ultrogel AcA-34 column (IBF Biotechnics, Villeneuve-la-Garenne, France). After SDS/PAGE and transfer to nitrocellulose, the fractions containing PS-p68 were identified by overlay with [^{14}C]PS or with anti-PS-p68 serum. Active fractions were pooled and chromatographed on a 12 cm × 1.6 cm Q-Sepharose column (Pharmacia, Uppsala, Sweden) with a gradient of 0.15–1.0 M-NaCl in a buffer containing 10 mM-Tris/HCl, 4 M-urea and 0.1 mM-EDTA, pH 7.7. PS-p68-containing fractions were again pooled, freed of urea by dialysis against buffer A (20 mM-Tris/HCl containing 200 mM-NaCl, pH 7.7) and loaded on to a PS–Sepharose affinity column. The column was then washed with 5 column volumes of buffer A, and PS-p68 was eluted with 3 M-urea in buffer A. The PS–Sepharose affinity column was prepared by coupling of PS vesicles to CNBr-activated Sepharose [2]. Briefly, 15 mg of phosphatidylserine (PS), 7.5 mg of phosphatidylethanolamine and 7.5 mg of phosphatidylcholine (PC) were mixed and dried under N_2. The mixture was sonicated in ice for 10 min in 30 ml of 0.1 M-$NaHCO_3$ containing 0.5 M-NaCl, pH 8.0, and then allowed to couple to CNBr-activated Sepharose at room temperature for 1 h. After blocking for 1 h with 0.1 M-Tris/HCl containing 0.5 M-NaCl, pH 8.0, a column was prepared (1–15 ml gel volume), washed three times with alternating 0.1 M-sodium acetate containing 0.5 M-NaCl, pH 5.0, and 0.1 M-Tris/HCl containing 0.5 M-NaCl, pH 8.0, and then equilibrated in buffer A.

PS-binding and Ca^{2+}-binding assays

Samples of PS-p68 were subjected to SDS/Page on an 8 % polyacrylamide gel (Mini Protean II cell; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and transferred to nitrocellulose. Non-specific binding was prevented with 3 % (w/v) BSA in assay buffer [containing 50 mM-Tris/HCl, 0.2 M-NaCl, 0.1 % BSA, 1 mg of poly(ethylene glycol) 20000/ml and 0.02 % NaN_3, pH 7.7]. The nitrocellulose was then incubated overnight in 5 ml of assay buffer at 4 °C, or for 3 h at room temperature with 5 μg of [^{14}C]PS (1,2-dioleoyl L-3-phosphatidyl-L-[3-^{14}C]serine; Amersham International, Amersham, Bucks., U.K.) (3 μCi/mg of lipid) [3]. After removal of unbound [^{14}C]PS by repeated washing with assay buffer, PS-binding proteins were revealed by autoradiography on Hyperfilm-betamax (Amersham International). To study phospholipid binding under non-denaturing conditions, purified PS-p68 was incubated at room temperature with PS or PC vesicles (0.25 mg of lipid/ml) in a buffer containing 10 mM-Pipes/HCl, 100 mM-KCl, 3 mM-NaCl, 3.5 mM-$MgCl_2$ and 0.1 mM-EDTA, pH 7.3. After 20 min, the vesicles were centrifuged (225000 *g* for 20 min, Beckman TL-100

Abbreviations used: PS, phosphatidylserine; PC, phosphatidylcholine; PBP, phospholipid-binding protein.

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ultracentrifuge), and supernatant and pellet were analysed by SDS/PAGE. Ca^{2+} binding to proteins dot-blotted on to nitrocellulose membranes was measured by using the procedure of Maruyama *et al.* [4] with $^{45}Ca^{2+}$.

Protein sequencing

All reagents used were of the purest grade available. SDS was recrystallized twice [5]. The Tricine/SDS/polyacrylamide gel employed for the separation of CNBr-cleavage fragments was pre-electrophoresed [6]. Partially purified PS-p68 was subjected to SDS/PAGE on an 8% polyacrylamide gel and blotted in 50 mM-boric acid/20% (v/v) methanol/0.05% SDS, pH 9.0 on to Immobilon [poly(vinylidene difluoride)] membrane (Millipore, Bedford, MA, U.S.A.). Proteins were stained with Ponceau Red, and the lower band of the PS-p68 doublet was cut out and cleaved on the membrane by CNBr treatment [7]. The resulting fragments were eluted with 50 mM-Tris/HCl containing 2% SDS and 1% Triton X-100, pH 9.1, separated by Tricine/SDS/PAGE [8] and finally electroblotted on to Immobilon membrane. After staining with Coomassie Blue, areas containing the fragments were cut out and the individual peptides were sequenced on the membrane by automated Edman degradation/gas-phase chromatography (model 477A apparatus; Applied Biosystems 477A, Foster City, CA, U.S.A.) In some cases the fragments from the Immobilon membrane were subjected to pyridylethylation before being sequenced [9]. The sequencing was performed on duplicate samples independently at two different facilities.

Phosphorylation of platelets

A 10–20 ml portion of citrate-anticoagulated platelet-rich plasma was adjusted to pH 6.5 with 1 M-HCl. Apyrase (0.07 unit/ml; Sigma Chemical Co., St. Louis, MO, U.S.A.) and Iloprost (1 ng/ml; Schering A.G., Berlin, Germany) were added, and the volume was adjusted to 50 ml with washing buffer (containing 4.3 mM-K_2HPO_4, 4.3 mM-Na_2HPO_4, 24.4 mM-NaH_2PO_4, 113 mM-NaCl and 5.5 mM-glucose, pH 6.5). After centrifugation at 1200 *g* for 10 min, the sedimented platelets were resuspended at 10^9 cells/ml in a buffer containing 20 mM-Hepes, 140 mM-NaCl, 5.5 mM-glucose and 0.035 unit of apyrase/ml, pH 7.5. To allow relaxation [10], the cells were incubated at 37 °C for 1 h. [^{32}P]P_i (1 mCi/ml) was added and the cells were labelled at 37 °C for 1 h, and the cells were then layered on a 15% (w/v) sucrose cushion (1 ml of cells/ml of sucrose) and centrifuged at 14500 *g* for 3 min to remove free [^{32}P]P_i. The platelets were resuspended in the same buffer and incubated at 37 °C for 15–30 min, before exposure to the stimuli. Stimulation was terminated by freezing the samples in liquid N_2 after addition of proteinase inhibitors (2 mM-EDTA, 0.045 mM-leupeptin and 1 mM-phenylmethanesulphonyl fluoride) and, in some experiments, phosphatase inhibitors (0.1 mM-sodium orthovanadate and 10 mM-sodium pyrophosphate). After thawing, the platelets were lysed by sonication and centrifuged at 225000 *g* for 10 min at 4 °C (Beckman TL-100 ultracentrifuge). The supernatant was applied to the PS–Sepharose affinity column (1×10^9 platelets/200 μl of gel). After a washing with 3 ml of buffer A, PS-p68 was eluted with 3 M-urea. Proteins in the wash and eluate fractions were precipitated with 10% (w/v) trichloroacetic acid and analysed by SDS/PAGE. Alternatively, PS-p68 obtained from the affinity column was further purified by SDS/PAGE on an 8% polyacrylamide gel and blotting on to Immobilon membrane. Each band of the doublet migrating at 68 kDa was cut out and subjected to phospho amino acid analysis separately. Alternatively, stimulation was terminated by adding 1% SDS, and phosphoproteins were analysed by two-dimensional electrophoresis [11] followed by immunoblotting and autoradiography.

Phosphorylation of PS-p68 by protein kinase C

The reaction mixture (final volume 80 μl) contained 3 μg of PS-p68 protein, 0.05 μg of protein kinase C (purified from human platelets [1]) and 0.012 mM-ATP (containing 1 μCi of [γ-^{32}P]ATP) in 50 mM-Tris/HCl containing 10 mM-$MgCl_2$, 0.25 mM-$CaCl_2$, 2 μg of leupeptin and 2 μg of PS, pH 7.7. The mixture was incubated for 15 min at 30 °C, and the reaction was terminated by the addition of 34 μl of SDS sample buffer. After SDS/PAGE and transfer to Immobilon membrane, phosphorylated protein was detected by autoradiography, cut out and subjected to phospho amino acid analysis.

Phospho amino acid analysis

The proteins were hydrolysed directly on the Immobilon membrane in 5.7 M-HCl for 1 h at 110 °C, and the membrane pieces were dried by vacuum centrifugation, wetted with 40 μl of methanol and eluted three times with 200 μl of 0.1 M-HCl in 30% (v/v) methanol [12]. The hydrolysate was dried by vacuum centrifugation, supplemented with phospho amino acid standards (Sigma Chemical Co.) and Fuchsin, and dissolved in 7.8% (v/v) acetic acid/2.5% (v/v) formic acid. The sample was analysed by two-dimensional electrophoresis on cellulose t.l.c. plates (Merck A.G., Darmstadt, Germany) [13], stained with ninhydrin and autoradiographed (Hyperfilm-MP; Amersham International).

Anti-PS-p68 serum

Antiserum to PS-p68 was generated by subcutaneous immunization of rabbits with 30 μg of purified protein in complete (first injection) or incomplete Freund's adjuvant (three additional injections). Affinity-purified antibody was prepared as described by Olmsted [14].

Protein quantification

Protein concentration was determined by the bicinchoninic acid method (BCA; Pierce Chemical Co., Rockford, IL, U.S.A.) in 96-well micro-titre plates according to the manufacturer's instructions.

RESULTS

Purification of PS-p68

The PS-binding protein of PS-p68 from human platelet cytosol was purified in a four-step procedure. In early experiments the purification of PS-p68 was monitored by [^{14}C]PS binding, and in later experiments by immunoblotting with anti-PS-p68 serum. The material precipitated from the cytosol by $(NH_4)_2SO_4$ (20–40% saturation) was chromatographed on an Ultrogel AcA-34 column. Active fractions were pooled and applied to a Q-Sepharose column, from which PS-p68 was eluted at about 0.26 M-NaCl. The final step of purification consisted of a phospholipid–Sepharose affinity column, from which PS-p68 was eluted with 3 M-urea.

On an SDS/PAGE gel PS-p68 could be identified as a closely spaced doublet migrating with a molecular mass of about 68 kDa (Fig. 1*a*). The relative amounts of the two components of the doublet varied, but in general the lower band was more prominent on Coomassie Blue staining. The PS-p68 doublet was the only platelet cytosol protein reacting on an immunoblot with the affinity-purified antibody directed against PS-p68 (Fig. 1*b*). Fig. 1(*c*), on the other hand, shows that a few other proteins in addition to PS-p68 were able to bind [^{14}C]PS under the conditions used.

PS-p68 is a relatively abundant protein in platelets. By comparing immunoblots of known amounts of purified PS-p68 with those of platelet lysates, we calculated that 10^7 platelets

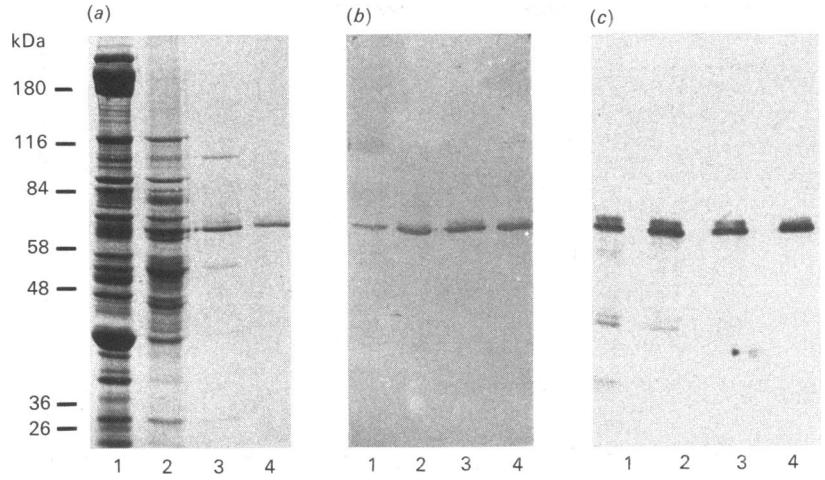

Fig. 1. Purification of PS-p68

Fractions from different stages of purification were analysed by SDS/PAGE and (*a*) by Coomassie Blue staining, (*b*) by immunoblots with affinity-purified anti-PS-p68 antibody and (*c*) by overlay with [^{14}C]PS on nitrocellulose. The applied fractions are: lanes 1, 16 μg of platelet cytosol; lanes 2, 4 μg of pooled AcA-34 column fractions; lanes 3, 0.7 μg of pooled Q-Sepharose column fractions; lanes 4, 0.5 μg of purified PS-p68. For [^{14}C]PS overlay (*c*) one-tenth of the above amounts of protein was used in each lane. The autoradiogram shown was exposed for 24 h.

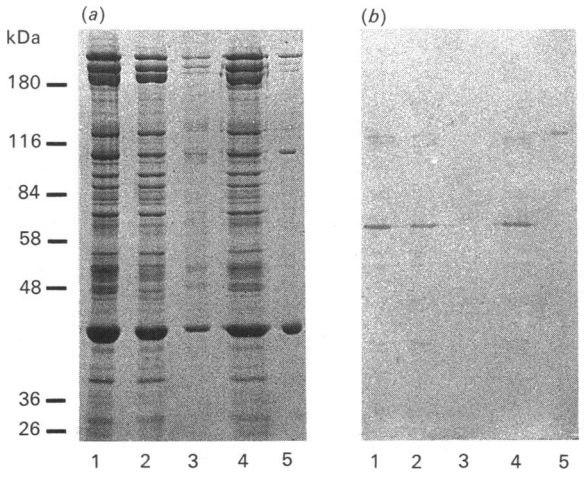

Fig. 2. Subcellular distribution of PS-p68 in platelets

Platelets (10^9/ml) were sonicated and cytosol was isolated. The particulate fraction was washed twice in 20 mM-Tris/HCl containing 0.15 M-NaCl, pH 7.7, and solubilized in SDS sample buffer. Triton-X-100-insoluble residues were isolated as pellets from platelets treated with 1% Triton X-100 in 50 mM-Tris/HCl containing 0.15 M-NaCl and 5 mM-EGTA, pH 7.7, at 4 °C and centrifugation at 14000 *g*. The equivalent of 2×10^7 platelets was used for SDS/PAGE. Lanes 1, platelet lysate; lanes 2, cytosol; lanes 3, particulate fraction; lanes 4, Triton-X-100-soluble residues; lanes 5, Triton-X-100-insoluble residues. (*a*) Coomassie Blue stain; (*b*) immunoblot with anti-PS-p68 antibody.

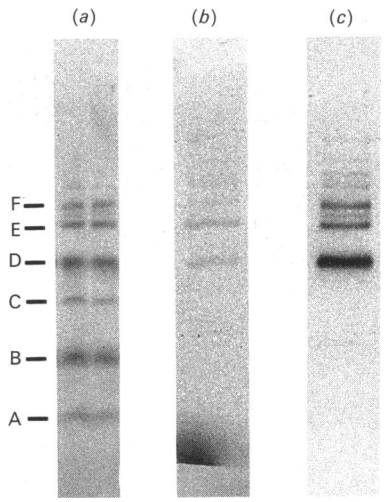

Fig. 3. CNBr-cleavage fragments of PS-p68

PS-p68 was electroblotted from an SDS/PAGE gel on to an Immobilon membrane and subjected to CNBr cleavage. The fragments were separated by Tricine/SDS/PAGE. (*a*) Coomassie Blue stain; (*b*) autoradiogram after overlay with [^{14}C]PS; (*c*) autoradiogram of the fragments from PS-p68 phosphorylated by protein kinase C.

contain about 0.10–0.15 μg of PS-p68, which corresponds to 0.5–0.75% of the total platelet protein.

Subcellular distribution of PS-p68

As a phospholipid-binding protein, PS-p68 could be expected to be associated with membranes. Subcellular fractionation of platelets, however, showed that it is localized exclusively in the soluble fraction (Fig. 2). To assess whether any PS-p68 is associated with the cytoskeleton, platelets were lysed with 1% Triton X-100 and the cytoskeleton was isolated as the insoluble fraction after centrifugation at 14000 *g* for 5 min. The immunoblot in Fig. 2 shows that PS-p68 is not associated with the cytoskeleton.

Partial amino acid sequence of PS-p68

The Ca^{2+}-independent binding of phospholipids to a 68 kDa protein from platelets has not been described before. In order to determine if we are dealing with a novel protein or with an hitherto unknown function of a known one, we compared several partial amino acid sequences of the lower-molecular-mass component of the PS-p68 doublet with those of known proteins. It was not possible to sequence intact PS-p68, suggesting that its *N*-terminus is blocked. For this reason sequencing was performed on CNBr-cleavage fragments obtained on the Immobilon membrane. Fig. 3(*a*) shows a Coomassie Blue stain of the fragments, from which the molecular masses, indicated in Table 1, were derived. An overlay with [^{14}C]PS showed that the PS-binding site is localized in fragment D (22 kDa). Furthermore, PS-p68 was found to be a protein kinase C substrate (see also Fig. 6*c*) and the phosphorylation site is also localized in fragment D (Figs. 3*b* and

Table 1. Amino acid sequence of PS-p68 CNBr-cleavage fragments

Peptides C and F are blocked, and the *N*-terminal end of peptide E is identical with that of peptide A. Amino acid residues in parentheses represent tentative assignments and question marks represent unassigned residues.

Peptide	Molecular mass (kDa)	Amino acid sequence
A	6.5	EQRQISLEGSVKGIQNDLTKLSKYQAST?N
B	11.0	NKLGTKIVSVE(R)REKI(K)?SL
C	18.0	
D	23.0	D(R)Q?AQVKRLENN?AQLLRRN?FKV
E	29.5	
F	33.0	

3*c*). Fragments A–F were subjected to amino acid sequencing (Table 1). Judged by the identity of sequences obtained, fragment E is only partially cleaved and includes fragment A at its *N*-terminus. Fragments C and F appeared to be blocked and may thus correspond to the *N*-terminus of PS-p68. A computer search of the SWISSPRO database yielded no protein containing identical or significantly similar sequences, suggesting that PS-p68 is a novel protein.

Characterization of PS binding

PS-p68 was electrophoretically transferred to nitrocelluloses and incubated with [^{14}C]PS under various conditions. PS binding was assessed by autoradiography of the nitrocellulose strips (Fig. 4*a*). $CaCl_2$ and $MgCl_2$ were not required for binding and were inhibitory at 5 mM. PS binding was also inhibited by two negatively charged phospholipids, phosphatidic acid and phosphatidylinositol, and by the alkylphospholipids platelet-activating factor, lyso-platelet-activating factor and 1-*O*-hexadecyl-2-*O*-methylglycerol. Protein kinase C added during or after incubation with PS bound to the complex of PS and PS-p68 by a mechanism similar to that described in the accompanying paper for PS-p115/100 [1].

To obtain assurance that PS-p68 also binds to phospholipids under physiological conditions, the native protein was incubated with PS vesicles or PC vesicles in a buffer that mimics intracellular ionic concentrations. As shown in Fig. 4(*b*), PS-p68 associated with PS vesicles but not PC vesicles in a Ca^{2+}-independent manner.

The conditions for the binding of PS-p68 to phospholipids were further studied by using PS–Sepharose affinity chromatography. Cytosol of ^{32}P-labelled platelets was supplemented with 3 mM-$CaCl_2$ and loaded on to the PS–Sepharose affinity column, which was then eluted with a buffer containing 2 mM-EDTA. Fig. 5 (lane 3) demonstrates that a number of proteins, but no PS-p68, could be released from the column under Ca^{2+}-chelating conditions. Elution of PS-p68 required addition of 3 M-urea to the buffer (Fig. 5, lane 4). The autoradiogram in Fig. 5(*b*) indicates that PS-p68 is the only phosphoprotein from unstimulated platelets that binds to the PS–Sepharose affinity column. Moreover, the fact that PS binding is Ca^{2+}-independent is consistent with the observation that $^{45}Ca^{2+}$ did not bind to PS-p68 on a dot-blot assay (results not shown).

Phosphorylation of PS-p68

Platelets were labelled with [^{32}P]P_i and then stimulated with thrombin, carbacyclin (a stable form of prostacyclin that raises cyclic AMP concentrations in platelets) or phorbol 12-myristate 13-acetate. The reaction was stopped by freezing the cells in liquid N_2. Cytosol was prepared, loaded on to the PS–Sepharose

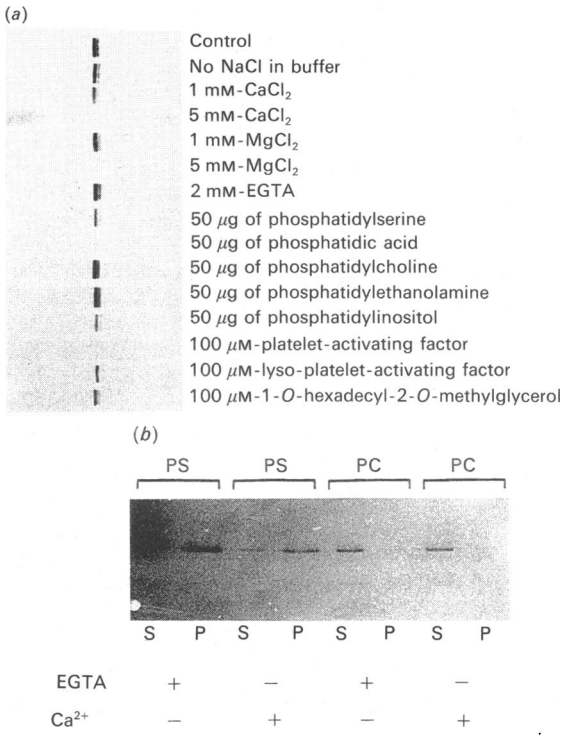

Fig. 4. Characterization of PS binding

(*a*) PS-p68 was blotted on to nitrocellulose and incubated with [^{14}C]PS in assay buffer with the changes indicated in the Figure. (*b*) Purified PS-p68 was incubated in a buffer containing 10 mM-Pipes/HCl, 100 mM-KCl, 3 mM-NaCl, 3.5 mM-$MgCl_2$ and 0.1 mM-EDTA, pH 7.3, with PS or PC and 0.2 mM-$CaCl_2$ or 0.1 mM-EGTA (as indicated). After centrifugation at 225000 *g*, supernatant (S) and pellet (P) were analysed by SDS/PAGE, and proteins were stained with Coomassie Blue.

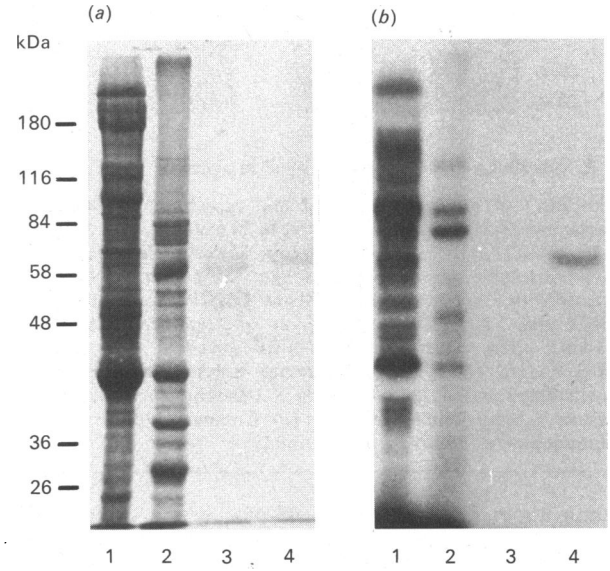

Fig. 5. Ca^{2+}-independent binding of PS-p68 to the PS–Sepharose affinity column

Cytosol was isolated from ^{32}P-labelled platelets (10^9 cells), made 3 mM in $CaCl_2$ and then loaded on to the PS–Sepharose affinity column (0.2 ml). Samples from the PS–Sepharose affinity column were analysed by SDS/PAGE. Lanes 1, proteins that did not bind to the PS–Sepharose affinity column; lanes 2, proteins eluted in the presence of 50 mM-Tris/HCl containing 0.2 M-NaCl and 1 mM-$CaCl_2$, pH 7.7; lanes 3, proteins eluted in the presence of 50 mM-Tris/HCl containing 0.2 M-NaCl and 2 mM-EDTA, pH 7.7; lanes 4, PS-p68 eluted with 3 M-urea in the buffer. (*a*) Coomassie Blue stain; (*b*) autoradiogram.

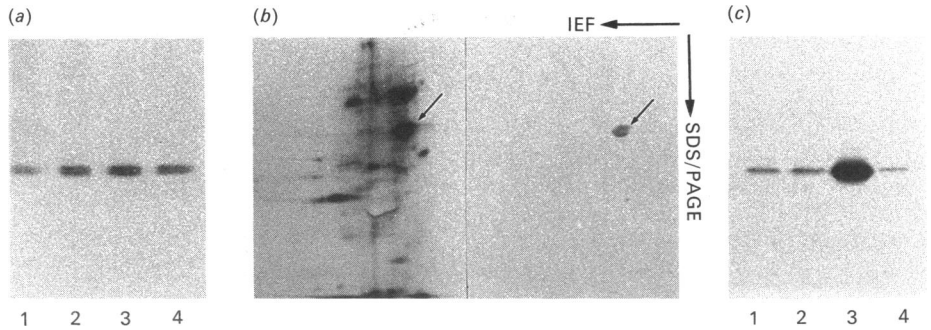

Fig. 6. Phosphorylation of PS-p68

(*a*) ^{32}P-labelled platelets were incubated with different agonists, and PS-p68 was isolated by PS–Sepharose affinity chromatography, analysed by SDS/PAGE and subjected to autoradiography. The agonists were: lane 1, thrombin (1 unit/ml, 1 min); lane 2, carbacyclin (1.5 μM, 2 min); lane 3, phorbol 12-myristate 13-acetate (100 nM, 7 min); lane 4, control. (*b*) Autoradiogram (left) and immunoblot (right) of a two-dimensional PAGE [SDS/PAGE and isoelectric focusing (IEF)] gel, of platelet proteins labelled with ^{32}P. The arrow denotes PS-p68. (*c*) Phosphorylation of purified PS-p68 by protein kinase C. Lane 1, no PS; lane 2, no $CaCl_2$; lane 3, complete reaction mixture; lane 4, no PS and no $CaCl_2$. An autoradiogram after SDS/PAGE is shown.

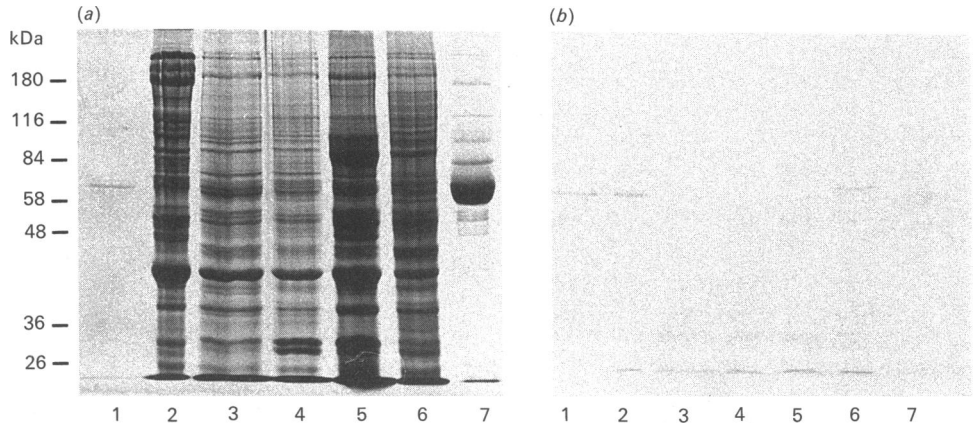

Fig. 7. Distribution of PS-p68 in blood cells

Cell lysates were fractionated by SDS/PAGE and stained for proteins by Coomassie Blue (*a*) or transferred to nitrocellulose and assayed with an affinity-purified anti-PS-p68 antibody (*b*). Lanes 1, purified protein; lanes 2, 2×10^7 platelets; lanes 3, 1×10^6 monocytes; lanes 4, 1×10^6 lymphocytes; lanes 5, 1×10^6 neutrophils; lanes 6, 5×10^5 human leukaemic (HL-60) cells; lanes 7, 10 μg of plasma protein.

affinity column and eluted with 3 M-urea. The autoradiogram in Fig. 6(*a*) shows that PS-p68 exists as a phosphoprotein in intact platelets. Only minor changes in the degree of phosphorylation were observed upon exposure of the platelets to thrombin, carbacyclin or phorbol 12-myristate 13-acetate, the apparent decrease following stimulation with thrombin being due to a consistent decrease in the PS-p68 yield under these conditions. On the other hand, thrombin and phorbol 12-myristate 13-acetate induced a marked increase of ^{32}P incorporation into p47, the major protein kinase C substrate in platelets (results not shown). The degree of phosphorylation of PS-p68 was not affected by the addition of phosphatase inhibitors (10 mM-sodium pyrophosphate and 0.1 mM-sodium orthovanadate) before freezing the cells. Two-dimensional SDS/PAGE of ^{32}P-labelled platelets showed that PS-p68, which was identified as a doublet by immunoblotting, is a major phosphoprotein (Fig. 6*b*). In a cell-free assay, PS-p68 was readily phosphorylated by protein kinase C (Fig. 6*c*). Phospho amino acid analysis revealed that both components of the p68 doublet were phosphate-labelled on serine residues when PS-p68 was phosphorylated in intact platelets or by protein kinase C after purification (results not shown).

Distribution of PS-p68 in blood cells

Using an affinity-purified antibody to PS-p68, we examined neutrophils, monocytes, lymphocytes, erythrocytes (results not shown), blood plasma and the leukaemic cell line HL-60 for this protein. Western blotting after SDS/PAGE of cell lysates demonstrates that PS-p68 is unique for platelets, since none of the other cells showed immunoreactivity (Fig. 7). The lysate of HL-60 cells, however, revealed an immunoreactive band of somewhat larger size than PS-p68.

DISCUSSION

We purified and partially characterized PS-p68, a novel phospholipid-binding protein (PBP) that is an abundant constituent of human platelet cytosol. PBPs have been implicated in a wide range of cellular functions (as reviewed in ref. [15]), including exocytosis (synexin and calelectrin), interactions of cytoskeletal elements with plasma membrane (calpactins, endonexin and calelectrin) and modulation of membrane-associated enzymes or receptors (calpactins). At least six distinct PBPs belong to the annexin family of proteins, which share a common repeated subunit structure [16,17]. Within this family two major size classes have been described: proteins in the 35–40 kDa range and proteins in the 68–70 kDa range. Although similar in size to the 67 kDa calelectrin, PS-p68 does not bind to cytoskeletal structures. Unlike calpactins, however, PS-p68 does not inhibit phospholipase A_2 (results not shown).

Most PBPs have affinity for negatively charged phospholipids without strict selectivity for single phospholipid head-groups [15]. The binding of PS-p68 to phosphatidic acid and platelet-activating factor therefore does not necessarily imply a lipid-regulatory role for this protein. In view of the antagonistic effects of platelet-activating factor and the possible involvement of phosphatidic acid in signal transduction, further studies are needed to explore the role of PS-p68 in platelet function. Such studies may also help to explain why a protein that binds so strongly to PS is recovered in the cytosol rather than in association with membranes.

The interaction of known PBPs with phospholipids requires Ca^{2+} in the micromolar range or greater [15,18], and many of these proteins have been shown to bind Ca^{2+} with an affinity that is approximately in the same range. Although PS-p68 resembles other PBPs in its ability to bind phospholipids, it does not bind Ca^{2+} or require micromolar Ca^{2+} for its PS-binding activity. Thus the Ca^{2+} requirement of PBPs described to date may not be an essential feature of these proteins.

Certain PBPs are tyrosine kinase substrates [15] (calpactin I is a substrate of $pp60^{src}$, calpactin II is a substrate of the epidermal-growth-factor receptor). Unlike calpactins, PS-p68 is phosphorylated exclusively on serine residues and is not recognized by anti-phosphotyrosine antibodies. Although in these preliminary experiments we did not observe stimulus-dependent changes in PS-p68 phosphorylation, more detailed time-course studies with a wider range of stimuli will be necessary to determine how the phosphorylation of PS-p68 is controlled. Many proteins are phosphorylated in resting platelets [19], and PS-p68, which was identified as a major phosphoprotein on two-dimensional PAGE, could belong to this category. PS-p68 most probably corresponds to the cytosolic phosphoprotein termed p80 in a study of hydrophilic and hydrophobic platelet proteins using Triton X-114 phase separation [11]. The role of phosphorylation and dephosphorylation has not been clarified so far. *In vitro*, PS-p68 was readily phosphorylated by protein kinase C, but incorporation of ^{32}P was not increased in platelets that were stimulated with phorbol 12-myristate 13-acetate, suggesting that PS-p68 may not be accessible to protein kinase C in intact cells either through masking or binding by other proteins.

The properties of PS-p68 differ from those of other PBPs reported to date, and the available sequence data (70 of the estimated total of 570 amino acid residues) establish that PS-p68 is distinct from known and completely sequenced proteins. The role of this and other PBPs in cellular function requires further investigation.

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