# Identification of phosphatidylserine-binding proteins in human white blood cells

Marlene WOLF* and Marco BAGGIOLINI
Theodor Kocher Institut, Universität Bern, Postfach 99, CH-3000 Bern 9, Switzerland

Cytosol and membrane fractions from human neutrophils, monocytes, lymphocytes and platelets were separated by SDS/PAGE, blotted on to nitrocellulose and assayed for selective binding of phosphatidylserine (PS). Two PS-binding proteins with apparent molecular masses of 115 kDa and 100 kDa were identified in the cytosol of neutrophils, monocytes and lymphocytes. Corresponding bands along with other PS-binding proteins were detected in platelets in both cytosol and membrane fractions. These proteins were also found to bind protein kinase C (PKC) provided that PS was present. The 115 kDa and 100 kDa proteins (PS-p115/110) were partially purified from neutrophils and were used for the study of PS and PKC binding. The binding of PS did not require $Ca^{2+}$ or $Mg^{2+}$ and was inhibited by phosphatidic acid, by 1-alkyl-2-acetylphosphocholine and, to a lesser extent, by other lipids. The binding of PKC, however, was strictly PS- and $Ca^{2+}$-dependent and seems to occur secondarily to PS binding.

## INTRODUCTION

In a previous paper we described proteins from erythrocyte cytoskeletons and post-synaptic densities that bind phosphatidylserine (PS) and protein kinase C (PKC) [1]. The proteins from erythrocytes with molecular masses 115 kDa and 110 kDa were also substrates for PKC, and their binding to both PS and PKC was markedly inhibited after phosphorylation. These proteins were considered as possible regulators of the compartmentalization of PKC, which is known to be translocated from the cytosol to the plasma membrane in the course of activation [2–11]. It was also thought that such proteins could have anchoring function for the association of PKC with the cytoskeleton, where many of its substrates are localized [12–16]. The objectives of the present work were to examine other blood cells for PS- and PKC-binding proteins, and to characterize the binding properties of them. Proteins that bind PS and PKC having molecular masses 115 kDa and 100 kDa (PS-p115/110) were found in neutrophils, monocytes, lymphocytes and platelets. Although similar in size, these proteins differ from those found in erythrocytes, since they are localized exclusively in the cytosol, and do not appear to be substrates of PKC. Thus the present paper describes another class of phospholipid-binding proteins that appear to be widespread and that differ from the well-known phospholipid-binding proteins such as calpactin or synexin [17,18], which are $Ca^{2+}$-dependent and also have lower molecular masses.

## MATERIALS AND METHODS

### Cells

White cells were isolated from buffy coats of donor blood stored for up to 20 h at 4–10 °C (Swiss Red Cross Laboratory, Bern, Switzerland). After low-speed centrifugation (760 $g$ for 5 min) a platelet-rich supernatant was obtained from which the platelets were pelleted at 1200 $g$ for 10 min. The platelets were washed with a Hepes/saline buffer containing 20 mM-Hepes, 137 mM-NaCl, 3.3 mM-KCl, 1 mM-MgCl$_2$ and 1 mM-EGTA, pH 7.3, and resuspended at a concentration of 7.5 × 10$^9$ cells/ml. Neutrophils were isolated by centrifugation on Lympho-paque gradients [19]. Lymphocytes and monocytes were purified from the mononuclear cell layer of the Lympho-paque gradients by centrifugal elutriation [20].

### Cell extracts

Neutrophils (2 × 10$^8$/ml), monocytes (1 × 10$^8$/ml) and lymphocytes (1.5 × 10$^8$/ml) were suspended in phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na$_2$HPO$_4$/1.5 mM-KH$_2$PO$_4$ buffer, pH 7.4) containing 20 $\mu$g of leupeptin/ml and disrupted by N$_2$ cavitation (3MPa for 20 min in ice). The cavitated suspension was supplemented with 1 mM-EGTA and 1 mM-phenylmethanesulphonyl fluoride, and a post-nuclear supernatant was prepared by centrifugation at 200 $g$ for 5 min. Portions (3 ml) of this supernatant were layered on 6 ml of 39 % (v/v) Percoll in phosphate-buffered saline and centrifuged at 40000 $g$ for 15 min in a Sorvall RC-5 centrifuge with an SS-34 rotor. The sample layer containing the cytosol and the membrane fragments (the latter being recovered at the interface with the Percoll layer) were collected and centrifuged at 200000 $g$ for 15 min in a Beckman TL-100 ultracentrifuge with a TL 100.2 rotor. The membrane pellet was resuspended in 3 ml of phosphate-buffered saline. Platelets (suspended as indicated above) were disrupted by sonication. The homogenate was centrifuged at 200000 $g$ for 15 min (see above) to separate the cytosol from a membrane fraction, which was washed once and resuspended in the original volume of Hepes/saline buffer. Membranes from human erythrocytes were prepared by hypo-osmotic lysis in 10 mM-Tris/HCl buffer, pH 7.6, followed by centrifugation at 40000 $g$ for 15 min in a Sorvall SS-34 rotor and three washes with the same buffer.

### Purification of PKC from human platelets

Platelets (7.5 × 10$^9$/ml) were suspended in a buffer containing 20 mM-Tris/HCl, 2 mM-EDTA, 2.5 mM-EGTA, 10 mM-dithiothreitol, 0.5 mM-phenylmethanesulphonyl fluoride and 20 $\mu$g of leupeptin/ml, pH 7.7, and were disrupted by sonication for 2 min in ice. The sonicated suspension was centrifuged at 150000 $g$ for 30 min, and the supernatant containing soluble PKC was affinity-purified with inside-out vesicles from human erythrocytes [21]. The PKC-enriched eluate (5 ml) was adjusted to 1.5 M-NaCl and applied to a phenyl-Sepharose column (9 mm × 7 mm) equilibrated with a buffer containing 20 mM-

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Abbreviations used: PS, phosphatidylserine; PKC, protein kinase C; PDBu, 4$\beta$-phorbol 12,13-dibutyrate.
* To whom correspondence should be addressed.

Tris/HCl, 1.5 M-NaCl, 1 mM-EDTA and 1 mM-dithiothreitol, pH 7.7. PKC was eluted with 6 ml of the above buffer without NaCl, and was subjected again to affinity purification with inside-out vesicles. The enzyme obtained by this method was at least 90% pure and kept its activity for several weeks when stored at 4 °C.

## PKC antisera

Antibodies were raised in rabbits with four injections of 40 $\mu$g of purified PKC at 3-week intervals. Serum was collected 10 days after the last injection and was absorbed with human erythrocyte membranes to eliminate antibodies to the vesicles used in the purification of PKC. This antiserum was normally used at a dilution of 1:1000.

## Partial purification of PS-p115/100 from neutrophils

Batches (10 ml) of neutrophils ($2 \times 10^8$/ml) in phosphate-buffered saline containing 1 mM-EGTA and 1 mM-phenyl-methanesulphonyl fluoride were disrupted by sonication in ice. The sonicated suspension was centrifuged at 200 $g$ for 5 min, and the cytosol was isolated from the post-nuclear supernatant by centrifugation at 200000 $g$ for 30 min (Beckman TL-100 ultracentrifuge with a TL-100.2 rotor). A 5 ml sample of cytosol was diluted 1:2 with column buffer (10 mM-Tris/HCl containing 1 mM-EDTA, pH 7.7) and loaded on an HR 5/5 Mono Q column (Pharmacia f.p.l.c. chromatography system). The column was eluted with 30 ml of a linear NaCl gradient (0–0.6 M) in the above buffer. Fractions with PS- and PKC-binding activity were pooled and applied to a phenyl-Sepharose column (9 mm × 14 mm) equilibrated with a buffer containing 20 mM-Tris/HCl, 0.5 M-NaCl and 1 mM-EDTA, pH 7.7. The column was washed with the same buffer, followed by 5 ml of the buffer without NaCl, and PS-p115/100 was then eluted with a buffer containing 20 mM-Tris/HCl, 1 mM-EDTA and 50% (v/v) ethylene glycol, pH 7.7.

## PS-binding and PKC-binding assays

Samples were subjected to SDS/PAGE [22] in 8% acrylamide gels (Mini Protean II slab cell; Bio-Rad Laboratories) and were then transferred to nitrocellulose. Non-specific binding was blocked with 3% (w/v) BSA in blotting buffer [containing 50 mM-Tris/HCl, 0.2 mM-NaCl, 0.1% BSA, 1 mg of poly-(ethylene glycol) 20000/ml and 0.02% NaN$_3$, pH 7.7], and the nitrocellulose was then incubated for 3 h at room temperature or overnight at 4 °C with 5 $\mu$g of [$^{14}$C]PS (3 $\mu$Ci/mg of lipid) and 150 ng of PKC per ml of blotting buffer, as described previously [1]. After elimination of unbound material by washing with blotting buffer, PS-p115/100 was detected with anti-PKC serum followed by goat anti-(rabbit IgG) antibody coupled to alkaline phosphatase, with 5-bromo-4-chloroindol-3-yl phosphate as substrate [23]. In addition, bound [$^{14}$C]PS was located by autoradiography on X-ray films (Hyperfilm-beta-max; Amersham International) by a 24 h exposure. To ascertain that native protein is also able to bind PS, vesicles, were prepared by sonication and incubated at a concentration of 0.25 mg/ml with PS-p115/100 in a buffer containing 20 mM-Tris/HCl, 0.2 M-NaCl and 0.1 mM-EGTA, pH 7.7. After 1 h incubation on ice, the mixture was centrifuged at 225000 $g$ for 20 min (Beckman TL-100 ultracentrifuge). Supernatant and resuspended pellet were analysed by SDS/PAGE and Coomassie Blue staining for protein composition and by overlay with PS and PKC for PS-p115/100 localization.

## Biochemical assays

PKC activity was determined by Ca$^{2+}$- and PS-dependent autophosphorylation and by [$^3$H]PDBu-binding [10]. Protein
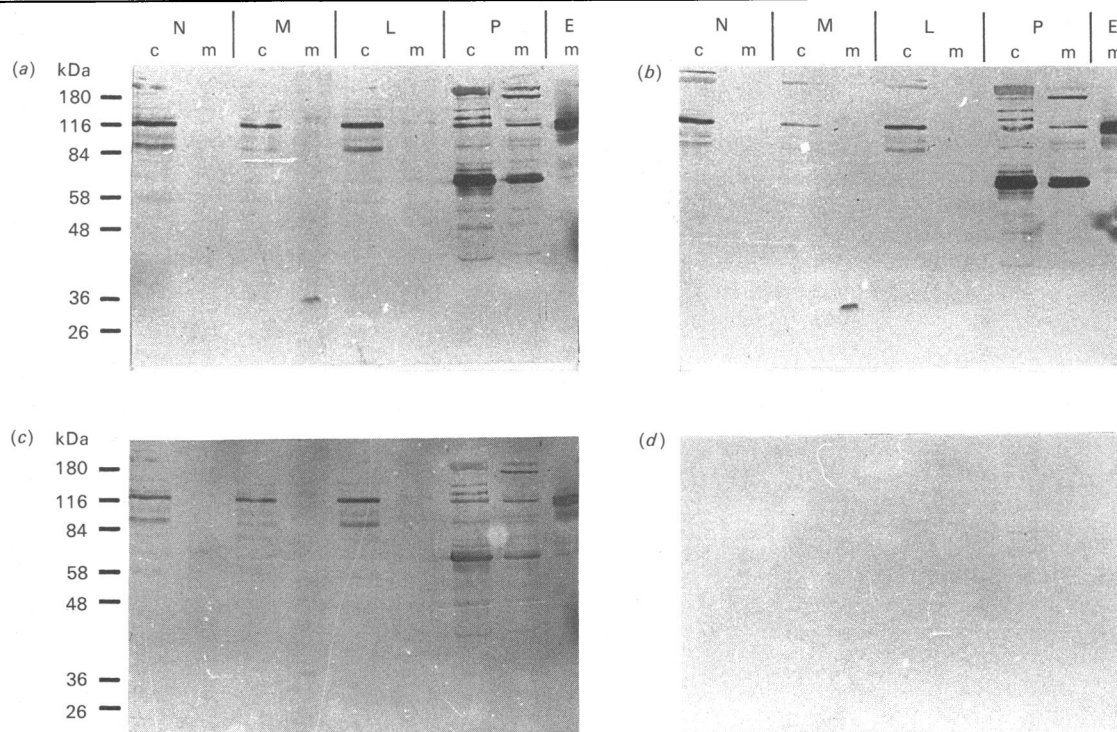


**Fig. 1. Binding proteins for PS and PKC in human blood cells**

Proteins from cytosol and membranes were separated by SDS/PAGE, then transferred to nitrocellulose, and the binding of PS and PKC was examined. The blots were incubated with [$^{14}$C]PS and with PKC (panels $a$ and $c$) or with [$^{14}$C]PS only (panels $b$ and $d$). PS binding was detected by autoradiography (panels $a$ and $b$) and PKC binding was determined with an anti-PKC antibody (panels $c$ and $d$). Samples of cytosol (c) and membranes (m) corresponding to $2 \times 10^6$ neutrophils (N), $1.5 \times 10^6$ monocytes (M), $2 \times 10^6$ lymphocytes (L) and $2.5 \times 10^7$ platelets (P) were run, together with an erythrocyte membrane sample (E) corresponding to 16 $\mu$g of protein.

was assayed by the method of Bradford [24], with BSA as standard.

## Materials

Leupeptin, 4β-phorbol 12,13-dibutyrate (PDBu), phosphatidylserine (PS), phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylinositol and prestained molecular-mass markers for SDS/PAGE were from Sigma Chemical Co., St. Louis, MO, U.S.A.; platelet-activating factor, lyso-platelet-activating factor, 1-oleoyl-2-acetylglycerol and 1-O-hexadecyl-2-O-methylglycerol were from Bachem A.G., Bubendorf, Switzerland; goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate and 5-bromo-4-chloroindol-3-yl phosphate were from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Lympho-paque was from Hyegaard and Co., Oslo, Norway; Percoll and phenyl-Sepharose were from Pharmacia, Uppsala, Sweden; nitrocellulose membrane was from Schleicher und Schüll, Feldbach, Switzerland; BSA and poly(ethylene glycol) 20000 were from Fluka A.G., Buchs, Switzerland; 1,2-dioleoyl L-3-phosphatidyl-L-[3-$^{14}$C]serine ([$^{14}$C]PS) (39 $\mu$Ci/mg) was from Amersham International, Amersham, Bucks., U.K.; 4β-[20-$^3$H(n)]phorbol 12,13-dibutyrate ([$^3$H]PDBu) (10.2 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.

## RESULTS

### PS- and PKC-binding proteins

The binding of PS and PKC to white-blood-cell and erythrocyte proteins was compared. Erythrocyte membranes contain two prominent binding proteins of molecular masses 115 kDa and 110 kDa (Fig. 1 and [1]). No such proteins were found in erythrocyte cytosol (results not shown). As shown in Fig. 1, binding proteins with similar molecular masses (115 kDa and 100 kDa) as well as a larger one (200 kDa) were found in neutrophils, monocytes and lymphocytes. In the white blood cells, however, the binding proteins were detected in the cytosol rather than in the membrane fraction. In platelet preparations the binding pattern was more complex: four well-defined bands, a major one of molecular mass 68 kDa and three additional ones of molecular mass 200 kDa, 160 kDa and 115 kDa were present in both the cytosol and the membrane fraction. Other bands in the molecular-mass ranges 115–200 kDa and 40–60 kDa were found in the cytosol. As shown in Fig. 1, the PS-binding patterns were identical in the presence and in the absence of PKC. By contrast, PKC binding was observed only when PS was present, suggesting that PS is the primary ligand, and that PKC binding is mediated by PS.

Since the binding proteins of molecular masses 115 kDa and 100 kDa (PS-p115/100) were common to all cells studied, we selected these two species for further work, using neutrophils as the source. In the accompanying paper [25] we describe the purification and characterization of the major PS-binding protein from platelets, PS-p68.

### Partial purification of PS-p115/100 from human neutrophils

Neutrophil cytosol was loaded on a Mono Q f.p.l.c. column, which was eluted with a salt concentration gradient. As shown in Fig. 2, PS-p115/100 was eluted at about 0.3 M-NaCl. The distribution of [$^3$H]PDBu-binding activity, which was determined as a measure of PKC distribution, showed a peak in fractions 12–14, and was therefore almost completely resolved from PS-p115/100 (fractions 15–17), suggesting that PKC is not associated with these proteins in resting neutrophils, or that an association with them precludes the binding of [$^3$H]PDBu.

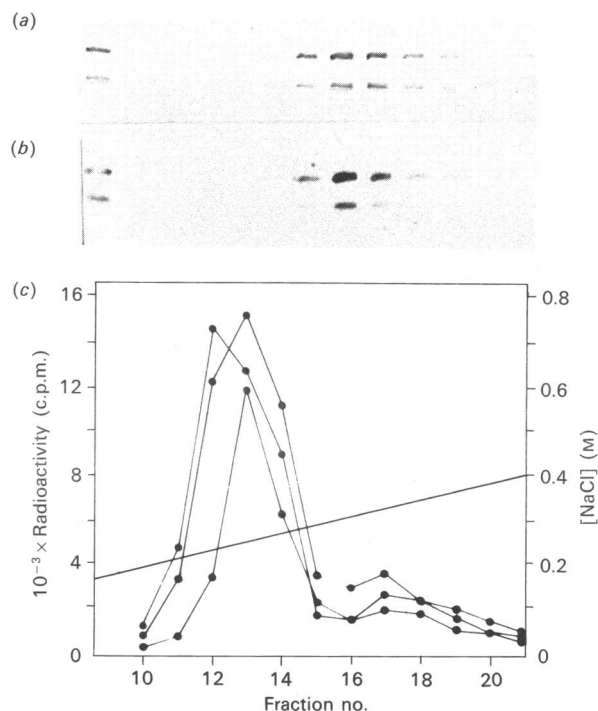Fractions 15–18 from the Mono Q column were pooled and

**Fig. 2. Mono Q anion-exchange chromatography of PS-p115/100 from human neutrophils**

Cytosol from $10^9$ neutrophils, homogenized in 5 ml of phosphate-buffered saline was diluted with the same volume of 10 mM-Tris/HCl containing 1 mM-EDTA, pH 7.7, and was subjected to chromatography on a Mono Q column as described in the Materials and methods section. Fractions 11–21 were assayed for PKC-binding activity in the presence of PS either with PKC and an anti-PKC antibody (a) or with $^{32}$P-radiolabelled PKC (b). The samples were cytosol (first lane) and the Mono Q fractions 11–21. Panel (c) shows [$^3$H]PDBu binding to each column fraction as a measure of endogenous PKC, obtained in three independent experiments.
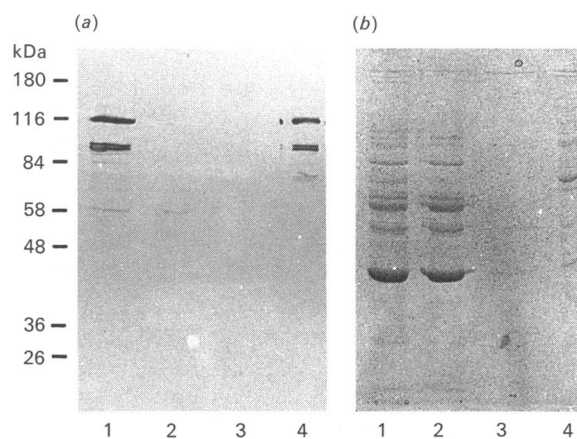
**Fig. 3. Fractionation of PS-p115/100 on phenyl-Sepharose**

Fractions 15–18 from the Mono Q anion-exchange chromatography were pooled and loaded directly on a phenyl-Sepharose column. The column was washed with 20 mM-Tris/HCl containing 0.5 M-NaCl and 1 mM-EDTA, pH 7.7, and the proteins were then eluted first with 2 ml of the same buffer containing no NaCl, followed by 2 ml of this buffer containing 50 % ethylene glycol. A 20 $\mu$l sample of each fraction was analysed for PS-p115/100: (a) immunochemical detection of bound PKC in the presence of PS; (b) Coomassie Blue stain. Lane 1, pooled fractions 15–18 from the Mono Q column; lane 2, protein that was not retained by the phenyl-Sepharose; lane 3, protein eluted with 20 mM-Tris/HCl, pH 7.7, containing 1 mM-EDTA; lane 4, protein eluted with buffer as in lane 3, containing 50 % ethylene glycol.
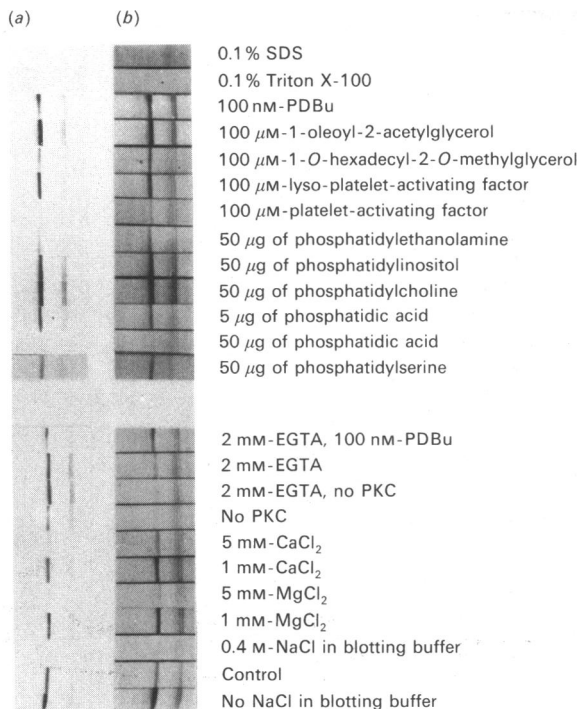
(a)    (b)

0.1 % SDS
0.1 % Triton X-100
100 nM-PDBu
100 μM-1-oleoyl-2-acetylglycerol
100 μM-1-O-hexadecyl-2-O-methylglycerol
100 μM-lyso-platelet-activating factor
100 μM-platelet-activating factor
50 μg of phosphatidylethanolamine
50 μg of phosphatidylinositol
50 μg of phosphatidylcholine
5 μg of phosphatidic acid
50 μg of phosphatidic acid
50 μg of phosphatidylserine

2 mM-EGTA, 100 nM-PDBu
2 mM-EGTA
2 mM-EGTA, no PKC
No PKC
5 mM-CaCl$_2$
1 mM-CaCl$_2$
5 mM-MgCl$_2$
1 mM-MgCl$_2$
0.4 M-NaCl in blotting buffer
Control
No NaCl in blotting buffer

**Fig. 4. Characterization of PS and PKC binding**

Samples of PS-p115/100 purified by Mono Q and phenyl-Sepharose chromatography were transferred electrophoretically to nitrocellulose membranes. Nitrocellulose strips were incubated overnight in 1 ml of blotting buffer containing 120 ng of PKC and 5 μg of [¹⁴C]PS (3 μCi/mg) and the indicated additions or omissions. Different ionic conditions and the influence of lipids and detergents on PS and PKC binding were studied. Panel (a) represents an autoradiography for [¹⁴C]PS binding, and panel (b) depicts PKC binding, detected with an anti-PKC antibody.

further fractionated on phenyl-Sepharose. This column completely retained PS-p115/100, which could only be eluted upon addition of 50% ethylene glycol to the buffer (Fig. 3a). By contrast, most of the other proteins were not retained. Electrophoresis of the proteins containing PS-p115/100 revealed a number of bands in addition to those of molecular masses 115 kDa and 100 kDa, which were not prominent on these gels, suggesting that PS-p115/100 represents only a minor component of the cytosol (Fig. 3b).

**PS binding and PKC binding**

Partially purified PS-p115/100 transferred to nitrocellulose strips was incubated with [¹⁴C]PS and purified PKC under various conditions, and the binding was assessed radiometrically and immunochemically (Fig. 4). The binding of PS was inhibited by hyper-osmotic concentrations of NaCl or KCl (above 0.2 M). CaCl$_2$ and MgCl$_2$ were not required for PS binding. No effects were observed up to 1 mM, but both salts inhibited binding at 5 mM. By contrast, PKC binding (in the presence of PS) required Ca$^{2+}$ and was inhibited by 1 mM-EGTA. The effect of EGTA, however, was overcome by the addition of 0.1 μM-PDBu. The selectivity of PS binding was studied in competition experiments with other lipids. At 10-fold the PS concentration, no inhibition was observed with phosphatidylcholine, phosphatidylinositol and the PKC ligands 1-oleoyl-2-acetylglycerol and PDBu, but strong inhibition was obtained with phosphatidic acid and platelet-activating factor. Smaller but significant effects were obtained with phosphatidylethanolamine, lyso-platelet-activating factor and 1-O-hexadecyl-2-O-methylglycerol. PS

binding could be displaced by other lipids, indicating that the PS-binding site of the binding proteins is not absolutely selective. PKC binding, however, required more stringent conditions, PS could not be replaced by other lipids, and Ca$^{2+}$ was required. Furthermore, as for the translocation of PKC to membranes, the requirement for Ca$^{2+}$ could be overcome by the addition of phorbol esters. The binding of PS and PKC was sensitive to detergents and was fully prevented by 0.1% Triton X-100 or 0.1% SDS. This effect is probably due to a disturbance of the interaction between PS and the binding proteins, possibly through a disruption of the PS vesicles by the detergents. PKC itself is not particularly sensitive to non-ionic detergents, since its catalytic activity is not affected by 0.1% Triton X-100 [26].

The above results demonstrated PS binding to proteins transferred to nitrocellulose after SDS/PAGE. To verify that these proteins can also bind PS under native conditions, column fractions after Mono Q f.p.l.c. separation of neutrophil cytosol were incubated with PS vesicles in the presence of 0.1 mM-EGTA and then analysed for vesicle-bound PS-p115/100. As indicated in Fig. 5(a) for column fraction 16, most proteins did not bind to the PS vesicles, yet PS-p115/100 was entirely recovered in the pellet. Moreover, when the individual fractions from the Mono Q column were incubated with PS vesicles (Fig. 5b), PS-p115/100 was detected in the same fractions as described for Fig. 2. Likewise, Fig. 5 shows that our method is suitable for measuring PS-binding proteins by blotting after SDS/PAGE. We also attempted to phosphorylate PS-p115/100 with PKC while bound to the PS vesicles, but found that these proteins are not substrates for PKC (results not shown).

PS binding to the blotted proteins was found to be saturable. Under the experimental conditions adopted, saturation was
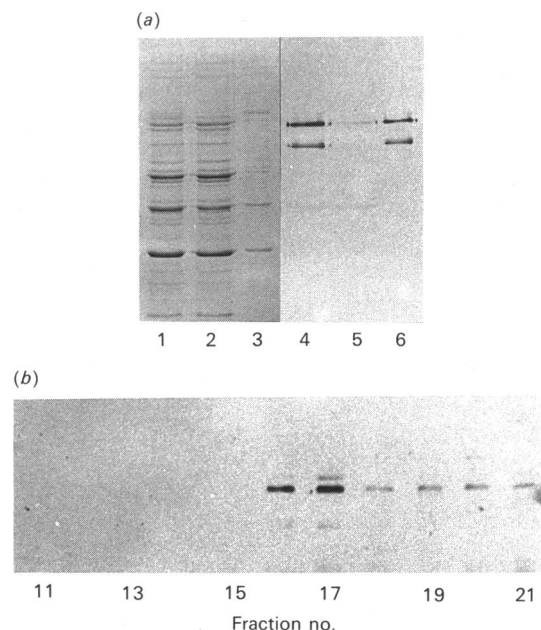
(a)

1    2    3    4    5    6

(b)

11    13    15    17    19    21
Fraction no.

**Fig. 5. Binding of PS-p115/100 to PS vesicles**

(a) A 240 μl portion of fraction 16 of the Mono Q column separation (Fig. 2) was incubated with PS vesicles as described in the Materials and methods section. Supernatant and resuspended pellet were analysed by SDS/PAGE, stained with Coomassie Blue (lanes 1–3) or tested for PS and PKC binding after transfer to nitrocellulose (lanes 4–6). Lanes 1 and 4 depict samples before centrifugation, lanes 2 and 5 samples from the supernatant and lanes 3 and 6 samples from the pellet fraction. (b) Mono Q column fractions were incubated with PS vesicles. The PS pellets from fractions 11–21 were resuspended and analysed for PS and PKC binding as described for (a).
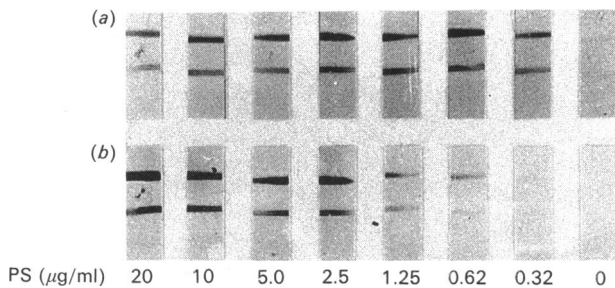
Fig. 6. PS-concentration-dependence of PS and PKC binding

Nitrocellulose strips containing PS-p115/100 (as in Fig. 4) were incubated with decreasing concentrations of [$^{14}$C]PS and with 120 ng of PKC. PKC binding and PS binding are shown in panels (a) and (b) respectively.

reached at 10 $\mu$g of PS/ml. As shown in Fig. 6, however, maximum PKC binding was obtained at subsaturating concentrations of PS, ranging between 0.6 and 2.5 $\mu$g/ml. In these experiments about twice as much PS was bound to the 115 kDa as to the 100 kDa band, but further purification is needed to get more information about the protein ratio of the two bands.

## DISCUSSION

Our study shows the existence of proteins from human white blood cells that bind PS independently of Ca$^{2+}$, but that in the presence of Ca$^{2+}$ and PS also bind PKC. Two major forms of molecular masses 115 kDa and 100 kDa were identified in neutrophils, monocytes and lymphocytes and were found to be exclusively localized in the cytosol. Platelets contained additional proteins with similar binding properties but different size, which were present in both soluble and particulate fractions. These results extend the previous observations with human erythrocytes and rat brain [1] and suggest that this family of PS-binding proteins may be widespread.

Partial purification from neutrophils suggests that the 115 kDa and 100 kDa proteins are closely related (since they behaved similarly upon Mono Q and phenyl-Sepharose chromatography) or may even constitute subunits of the same functional entity. Experiments with the partially purified proteins showed that PS was absolutely required for the binding of PKC. PS could thus represent the common ligand for the binding proteins and PKC and exert a cross-linking function. It is further conceivable that PS-p115/100 and PKC bind to different sites on PS, or that they bind to different PS molecules of the same PS vesicle. It cannot be excluded, however, that PKC binding is conditioned by the binding of PS without functional relation between the kinase and the PS-binding proteins. On the other hand, the binding of PS could change the conformation of PS-p115/100 and/or of PKC and make these proteins competent to interact with each other directly. At least for PKC such a conformational change can be expected, since it has been reported that the presence of PS and Ca$^{2+}$ exposes a hydrophobic site on the kinase molecule that is apparently involved in its activation [27].

Our results provide evidence that PS associates in a different way with PS-p115/100 and PKC. The binding to PS-p115/110 was Ca$^{2+}$-independent whereas that to PKC required Ca$^{2+}$. Higher PS concentrations were necessary to saturate PS binding to PS-p115/100 than to give maximal PKC binding, and high PS concentrations ($>2.5$ $\mu$g/ml) inhibited PKC binding. A number of lipids (in order of potency phosphatidic acid, platelet-activating factor, 1-O-hexadecyl-2-O-methylglycerol and phosphatidylethanolamine) were found to compete for PS binding. However, they could not substitute for PS in mediating PKC

binding to PS-p115/100. A different lipid requirement was observed for the activation of PKC in vitro, where PS could be partly replaced by phosphatidylinositol, phosphatidic acid and, to a lesser extent, phosphatidylethanolamine [26,28]. Likewise, denaturation during SDS/PAGE did not affect the interaction of PS with the binding protein, but prevented PS binding to PKC, since no PS bound to electrophoretically transferred PKC [1]. By contrast, PS was found to associate with PKC on a non-denaturing dot-blot, suggesting that the PS-binding site of the kinase is damaged by denaturation.

In terms of size and PS-binding properties, the white-blood-cell proteins described here resemble the PS- and PKC-binding proteins previously identified in human erythrocytes and rat brain homogenates [1]. The two species of molecular masses 115 kDa and 110 kDa from erythrocytes were found to be associated with the cytoskeleton. They are both PKC substrates, and their binding of PS and PKC is probably subject to regulation, as it was decreased upon phosphorylation. The size, subcellular distribution and phosphorylation by PKC indicate that these proteins most probably correspond to adducin, a cytoskeleton-associated protein recently detected in erythrocytes [29–31]. By contrast, there is no evidence that the proteins from neutrophils described in the present paper are substrates for PKC, since no phosphorylation was detected when PS-p115/100 was incubated with PKC.

The observed Ca$^{2+}$-independence of PS binding suggests that this process is not regulated by stimulus-dependent changes in the concentration of cytosolic free Ca$^{2+}$. Experiments with the native (i.e. not nitrocellulose-bound) polypeptides confirmed the Ca$^{2+}$-independence: PS-p115/100 was able to associate with PS vesicles in the presence of EGTA and no added Ca$^{2+}$ (Fig. 5). This property distinguishes PS-p115/100 from reported phospholipid-binding proteins such as calpactin [17], synexin [18] and chromobindins [32,33], which all require micromolar Ca$^{2+}$ concentrations for association with phospholipids or membranes [34,35].

PS-binding proteins would be expected to function in association with membranes that are rich in PS. The apparently exclusive cytosolic location of such proteins in white blood cells was therefore surprising. We have no indications for a possible function of these proteins in the cytosol, or for a way by which they may associate with membranes.

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