Transbilayer Movement of Phosphatidylserine in Nonhuman Erythrocytes: Evidence That the Aminophospholipid Transporter Is a Ubiquitous Membrane Protein†

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ABSTRACT: A 31–32-kDa integral membrane protein has been previously identified in erythrocytes as the protein most likely to be responsible for the transbilayer movement of phosphatidylserine (PS) [Connor & Schroit (1988) *Biochemistry 27*, 848–851]. Using similar techniques, we have identified analogous proteins of identical molecular weights in bovine, equine, ovine, porcine, canine, caprine, and rhesus red blood cells. Similar to human red blood cells, all of the mammalian cells were able to specifically transport an exogenously supplied fluorescent PS analogue from their outer-to-inner membrane leaflet. In addition, transport could be reversibly inhibited with the sulfhydryl-specific inhibitor pyridyldithioethylamine (PDA). PDA-sensitive PS transport was also observed in nucleated human and murine cell lines. Analysis of isolated plasma membranes from ^{125}I-PDA-labeled cells revealed marked labeling of a 32 000-Da component. Attempts to inhibit PS transport by treating the cells with proteases, lectins, or antibody suggested that the 32-kDa polypeptide is an integral membrane protein that does not contain sites critical to its function at the cell surface.

The maintenance of a particular distribution of phospholipids between membrane bilayer leaflets appears to be a common property of eukaryotic cells. Whereas all phospholipids exhibit some degree of membrane asymmetry in nucleated cells (Etemadi, 1980; Op den Kamp, 1979), phosphatidylserine (PS)[1] is the only phospholipid which appears to be localized exclusively in the inner leaflet of both red blood cells (Verkleij et al., 1973; Gordesky et al., 1975) and resting platelets (Chap et al., 1977; Bevers et al., 1983). In red blood cells, this highly asymmetric organization of PS may be required for homeostasis; it has been suggested that increased exposure of PS in the cells' outer leaflet, which occurs, for example, in sickle cells (Chiu et al., 1979; Lubin et al., 1981), may contribute to intravascular occlusion (Franck et al., 1985) and their removal by phagocytic cells (Schwartz et al., 1985). Activation of platelets, on the other hand, results in a reorientation of PS from the inner to outer leaflet (Bevers et al., 1982, 1983) which serves an important physiologic function in hemostasis by its participation in the coagulation cascade (Bevers et al., 1982; Rosing et al., 1985).

Although the mechanism(s) responsible for maintaining membrane lipid asymmetry is (are) not known, recent studies using labeled lipid analogues have demonstrated in red blood cells (Seigneuret & Devaux, 1984; Tilley et al., 1986; Connor & Schroit, 1987), platelets (Sune et al., 1987), and nucleated cells (Martin & Pagano, 1987; Zachowski et al., 1987) that exogenously added PS analogues preferentially translocate from their site of insertion in the outer leaflet to the inner leaflet, thereby adopting an asymmetric distribution. This intrabilayer movement is protein mediated, since lipid transport is ATP dependent and temperature dependent and requires that membrane proteins be maintained in a reduced state (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Connor & Schroit, 1987, 1988).

Recent work from this laboratory has suggested that the protein responsible for the intrabilayer transport of PS is a 31–32-kDa membrane polypeptide. This identification has been determined independently by specifically labeling this protein with a photolabeled, radioiodinated transportable PS analogue (Schroit et al., 1987) and ^{125}I-labeled PDA (^{125}I-PDA), a potent inhibitor of aminophospholipid transport activity (Connor & Schroit, 1988). In this report, we examine the ability of nonhuman erythrocytes and nucleated cells to transport PS and investigate the similarity of these transporters to the human protein by immunologic analysis and ^{125}I-PDA labeling patterns. Our findings suggest that the transporter is a ubiquitous protein present in nonhuman erythrocytes and nucleated cells.

Experimental Procedures

Materials and Routine Procedures. NBD-PC and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was synthesized from NBD-PC by phospholipase D catalyzed base exchange in the presence of L-serine (Comfurius & Zwaal, 1977) and purified by thin-layer chromatography. ^{125}I Bolton–Hunter reagent (specific activity ~2000 Ci/mmol) was purchased from New England Nuclear. Red blood cells were obtained by venipuncture into heparinized syringes and washed with HEPES–saline buffer. Human red blood cells

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[1] Abbreviations: ^{125}I-PDA, *N*-[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]pyridyldithioethylamine (Bolton–Hunter-labeled PDA); DTT, dithiothreitol; DOPC, dioleoylphosphatidylcholine; HEPES–saline, 145 mM NaCl, 5 mM KCl, 20 mM HEPES, and 10 mM glucose; NBD-PC, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; NBD-PS, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylserine; PDA, pyridyldithioethylamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TNBS, trinitrobenzenesulfonic acid; Tnp, trinitrophenyl.

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were from healthy volunteers. Bovine, equine, ovine, porcine, canine, caprine, and rhesus red blood cells were obtained from The University of Texas M. D. Anderson Science Park, Smithville, TX. The cells were labeled with ^{51}Cr, washed, and resuspended in HEPES–saline buffer. All red blood cells were used within 48 h of collection. Steady-state fluorescence was measured at 535 nm (λ_{ex} 468 nm) with a Farrand MK II spectrophotofluorometer. PDA and ^{125}I-PDA were synthesized as previously described (Connor & Schroit, 1988). Trypsin, protease (*Streptomyces griseus*), wheat germ agglutinin, pokeweed mitogen, red algae lectin (*Ptilota plumosa*), *Helix pomatia* lectin, and ricin were obtained from Sigma. Concanavalin A and Pronase (*Streptomyces griseus*) were from Calbiochem, proteinase K (mushroom) was from Merck, and phytohemagglutinin was from Difco. Tnp antibodies were produced in rabbits by repeated injections of trinitrophenylated bovine serum albumin.

Cells. K562 (human erythroleukemic cells), MEL (murine erythroleukemic cells), and A431 (human epithelioid carcinoma cells) were grown in RPMI media supplemented with glutamine, fetal bovine serum (5%), nonessential amino acids, and vitamins. Differentiation of the K562 and MEL cells (d-K562 and d-MEL) was initiated by the addition of 25 μM hemin (Sutherland et al., 1986) and 5 mM hexamethylenebisacetamide (Fibach et al., 1977), respectively. Differentiation was monitored by the cells' accumulation of hemoglobin and was considered complete after 5 days.

Preparation of Antibodies to the 32-kDa Polypeptide. Human red blood cells were labeled with trace amounts of ^{125}I-PDA to identify the 32-kDa polypeptide (Connor & Schroit, 1988). Ghosts were prepared by hypotonic lysis at 4 °C in 5 mM phosphate buffer, pH 8.0 (Steck, 1974), and subjected to SDS–PAGE, under nonreducing conditions, using a 12% separating gel, a 6% stacking gel, and the discontinuous buffer system of Laemmli (1970). The region containing the labeled protein was excised and dialyzed to remove SDS. The eluted protein was concentrated, emulsified in Freund's adjuvant, and injected (5–20 μg) into New Zealand White rabbits at multiple intradermal sites. The animals were boosted every 2–3 weeks over a period of 3–5 months.

Transbilayer Movement of PS. Red blood cells and nucleated cells were adjusted to a 1% hematocrit and to 5×10^6 cells/mL, respectively, and incubated in the presence or absence of 2 mM PDA for 30 min at 4 °C. The cells were then washed with HEPES–saline buffer, rapidly mixed with NBD-labeled lipids [100 ng of lipid (10 μL of EtOH)$^{-1}$ mL^{-1}], and incubated for 45 min with continuous shaking at 37 °C (red blood cells) or 4 °C (nucleated cells). After washing, NBD-lipid remaining in the cells' outer leaflet (not transported) was removed by "back-exchange" by incubation with 1 mg of DOPC "acceptor" vesicles for 45 min at 0 °C. Acceptor vesicles were removed by centrifugation, and the cells were solubilized with 2% Triton X-100. The fraction of NBD-lipid remaining in the cells after this back-exchange procedure was determined by fluorescence using cells not subjected to back-exchange as controls. Inhibition of PS transport by PDA was reversed by adding DTT (20 mM) to the cell suspensions during the 37 °C incubation (Connor & Schroit, 1988). All fluorescent measurements were normalized for variations in cell number (<10%) based on ^{51}Cr counting of the final detergent lysates.

Polypeptide Labeling with ^{125}I-PDA and Western Blot Immunostaining. Cells were labeled with ^{125}I-PDA for 30 min at 0 °C. Ghosts were prepared from red blood cells by hypotonic lysis, and plasma membrane preparations were prepared from the nucleated cells as described by Maeda et al. (1983). The final membrane preparations were solubilized in SDS buffer and subjected to SDS–PAGE using the same conditions described above. The gels were stained with Coomassie R-250, dried, and autoradiographed with Kodak XAR-5 X-ray film.

Polypeptides from duplicate gels were transferred to nitrocellulose paper using the Polyblot transfer system (American Bionetics; Haywood, CA) and probed with the rabbit 32-kDa antibodies. Briefly, the nitrocellulose was incubated under constant shaking with the 32-kDa antisera for 18 h at 37 °C. After being washed, bound rabbit antibodies were detected by using a horseradish peroxidase color development system (Sigma).

Fluorescence Micrographs. Cells pretreated with PDA (2 mM for 30 min at 4 °C) were incubated with 0.5 μg of NBD-PS (10 μL of EtOH)$^{-1}$ (mL of cells)$^{-1}$ at 4 °C for 30 min. The cells were washed, equilibrated at room temperature for 5 min, and photographed with a Nikon UFX fluorescence microscope equipped with a 40× objective. Images were recorded on Kodak Tri-X film and processed at 1600 ASA with Diafine developer.

Effect of Proteases and Lectins on the Translocation of NBD-PS. Human red blood cells were preincubated with the indicated proteases or lectins for 1 h. The cells were then washed and tested for their ability to translocate NBD-lipids as described above. In addition, red blood cells were incubated with ^{125}I-PDA either before or after protease treatment, and the labeling pattern was analyzed by SDS–PAGE as described above.

Table I: Transport of NBD-PS in Nonhuman Erythrocytes and Its Inhibition by PDA[a]

cells	NBD-PS transport (%)			NBD-PC (%)
	−PDA	+PDA	DTT	
bovine	98 ± 6	15 ± 4	96 ± 8	14 ± 5
equine	74 ± 8	17 ± 6	84 ± 3	10 ± 5
ovine	97 ± 2	33 ± 5	97 ± 16	12 ± 4
swine	97 ± 3	28 ± 4	100 ± 7	11 ± 5
canine	95 ± 8	31 ± 3	99 ± 4	13 ± 6
caprine	97 ± 4	27 ± 6	96 ± 11	10 ± 6
rhesus	91 ± 7	46 ± 3	85 ± 1	8 ± 3
murine	99 ± 12	23 ± 2	97 ± 10	15 ± 3
human	95 ± 5	17 ± 4	97 ± 5	15 ± 6

[a] Red blood cells were incubated in the absence or presence of PDA (2 mM) for 30 min on ice. The cells were then washed and incubated with NBD-PS in the absence or presence of DTT (20 mM) for 45 min at 37 °C. The fraction of NBD-PS transported to the cells' inner leaflet was determined by back-exchange as described under Experimental Procedures.

Results

Translocation of NBD-PS in Cells. When red blood cells were incubated with NBD-PS, >95% of the lipid became cell-associated within several minutes, irrespective of incubation temperature (results not shown). Extraction and chromatographic analysis of the fluorescent lipids after 1 h of incubation at 37 °C did not reveal any fluorescent products other than the exogenously added lipid, indicating that the probes were not metabolized during the course of these studies.

Table I shows that all the mammalian red blood cells studied efficiently transported NBD-PS, but not NBD-PC, from the outer to inner bilayer leaflet. Similar to results with human red blood cells (Connor & Schroit, 1988), the outside-to-inside transport of NBD-PS was significantly inhibited by pretreatment of the cells with the disulfide-exchange reagent PDA. As expected, PDA-mediated inhibition of NBD-PS

Table II: Transport of NBD-PS in Nucleated Cells and Its Inhibition by PDA[a]

cell line	NBD-PS transport (%)[b]	
	−PDA	+PDA
K562	98	37
d-K562	75	9
MEL	95	44
d-MEL	78	33
A431	69	32

[a] Cells were incubated in the absence or presence of PDA (2 mM) for 30 min on ice. The cells were then washed and incubated with NBD-PS for 45 min at 4 °C. The fraction of NBD-PS transported to the cells' inner leaflet was determined as described under Experimental Procedures. [b] Percentage of cell-associated lipid transported to the inner leaflet.

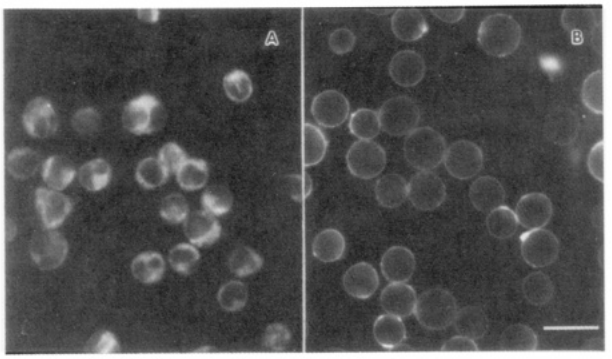

FIGURE 1: Inhibition of NBD-PS transport by PDA. MEL cells were incubated at 0 °C (A) or pretreated with 2 mM PDA (B) for 30 min. The cells were then incubated with NBD-PS for 5 min at 0 °C, washed, allowed to warm to room temperature for 5–10 min, and photographed. Bar is 20 μm.

transport could be abrogated by the addition of DTT.

Similar to the results obtained with red blood cells, the translocation of fluorescent PS from the outer-to-inner leaflet in nucleated cells was PDA-sensitive.[2] This can be seen from the results presented in Table II, which show that the lipid could not be removed by back-exchange from A431, K562, d-K562, MEL, or d-MEL cells. However, when internalization was blocked by PDA, most of the NBD-lipid could be removed from the cell surface, indicating substantial inhibition of lipid transport. These findings are consistent with the fluorescent microscopic observations shown in Figure 1. Prominent intracellular staining was observed when MEL cells were incubated with NBD-PS at 0 °C and warmed to room temperature for several minutes (Figure 1A), indicating internalization of the lipid. On the other hand, typical peripheral ring fluorescence (indicative of cell surface labeling) was observed if the cells were treated with PDA (Figure 1B), suggesting that the lipid was restricted to the cells' outer leaflet.

Labeling of the 32-kDa Polypeptide with ^{125}I-PDA. SDS–PAGE analysis of red blood cells incubated with ^{125}I-PDA at 0 °C revealed prominent labeling of the 32-kDa polypeptide in all the mammalian red blood cells (Figure 2). Although the 32-kDa polypeptide was the most prominently labeled band, treatment of canine red blood cells also revealed several unidentified heavily labeled bands.

Nucleated cells were also labeled with ^{125}I-PDA at 0 °C. SDS–PAGE analysis of isolated plasma membranes from

[2] Back-exchange of nucleated cells treated with NBD-PC at 0 °C resulted in removal of >80% of cell-associated lipid. Ambiguous results were, however, obtained at increasing temperatures since NBD-PC is internalized into nucleated cells by endocytosis (Struck & Pagano, 1980; Sleight & Pagano, 1984) and not by transmembrane movement.

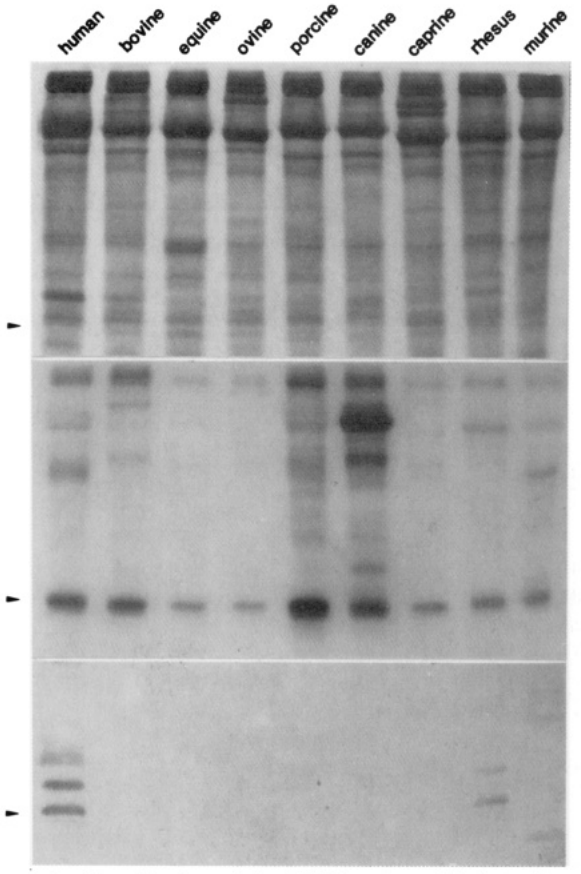

FIGURE 2: Identification of the 32-kDa polypeptide in nonhuman erythrocytes. Red blood cells were treated with ^{125}I-PDA as described under Experimental Procedures. Ghosts were solubilized in SDS without reducing agents, and ~10^8 red blood cell equivalents were applied to each lane. The gels were fixed, stained with Coomassie blue (top), and autoradiographed (middle). For Western blot immunostaining (bottom), duplicate gels were transferred to nitrocellulose, incubated with rabbit 32-kDa antibodies, and developed with goat anti-rabbit Ig conjugated to horseradish peroxidase using 5-chloro-1-naphthol as a substrate. Arrows indicate the position of the 32-kDa polypeptide.

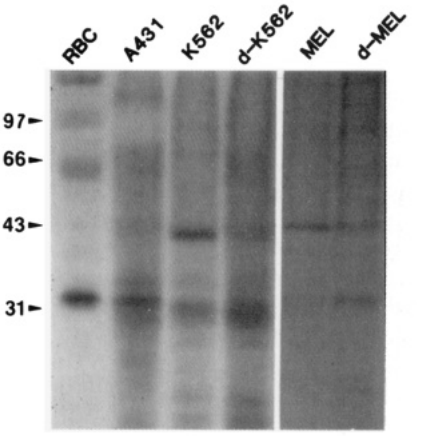

FIGURE 3: Autoradiography of ^{125}I-PDA-treated nucleated cells. Cells were labeled with ^{125}I-PDA for 30 min at 0 °C. The cells were washed, and plasma membranes were prepared as described under Experimental Procedures. Aliquots were solubilized in SDS without reducing agents and analyzed by SDS–PAGE.

A431, K562, d-K562, MEL, and d-MEL cells also revealed labeling of proteins in the 31–32-kDa region (Figure 3). However, in addition to demonstrating the 32-kDa polypeptide, undifferentiated cells also consistently showed prominent labeling of a ca. 42-kDa polypeptide which appeared to decrease

Table III: Effects of Various Proteases and Lectins on the Transbilayer Movement of NBD-PS[a]

treatment[b]	concn	transport (%) NBD-PS	NBD-PC
enzymes			
neuraminidase	0.5 mg/mL	87	14
trypsin	0.5 mg/mL	92	13
protease	0.5 mg/mL	79	17
pronase	0.5 mg/mL	82	14
proteinase K	0.5 mg/mL	86	18
lectins[c]			
wheat germ agglutinin	1.9 μg/mL	83	15
concanavalin A	1.0 mg/mL	78	14
pokeweed mitogen	7.6 ng/mL	86	16
phytohemagglutinin	122.0 ng/mL	89	17
Ptilota plumosa	0.5 mg/mL	78	18
Helix pomatia	0.5 mg/mL	75	23
ricin	61.0 ng/mL	88	17
control		88	13

[a] Human red blood cells were incubated with the indicated reagents for 60 min. The cells were then washed and incubated with NBD-PS or NBD-PC for 45 min at 37 °C. The fraction of NBD-lipid transported to the cells' inner leaflet was determined as described under Experimental Procedures. [b] Incubations with the proteolytic enzymes were done at 37 °C. Incubations with the lectins were done at room temperature. [c] Lectin concentrations were chosen on the basis of their ability to hemagglutinate red blood cells and were used at two double dilutions lower than their hemagglutination titers.

Table IV: Effects of Antibody Treatment on the Transbilayer Movement of NBD-PS[a]

treatment	NBD-PS transport (%)
control	80
TNBS alone[b]	84
TNBS + anti-Tnp	76
TNBS + anti-Tnp + goat anti-rabbit Ig	83
anti 32-kDa[c]	82
anti 32-kDa + goat anti-rabbit Ig	80

[a] Human red blood cells were incubated with the indicated antibody preparations for 30 min on ice, washed, and then incubated with the second antibody on ice or incubated with NBD-PS for 45 min at 37 °C. The fraction of NBD-PS transported to the cells' inner leaflet was determined as described under Experimental Procedures. [b] Red blood cells were treated with 10 mM TNBS for 30 min on ice at pH 8.0. The cells were then washed and treated with a subhemagglutinating dilution (1/250) of Tnp antibodies. [c] Treatment of red blood cells with hemagglutinating or subhemagglutinating dilutions of anti-32-kDa did not alter the fraction of NBD-PS transported to the inner leaflet.

in intensity upon differentiation.

Reactivity of Rabbit 32-kDa Antibodies with Membrane Proteins. Rabbit antibodies to eluted human 32-kDa polypeptide produced visible hemagglutination only in human red blood cells (see footnote of Table IV). Analysis of these antibodies by Western blot immunostaining (Figure 2) indicated strong reactivity against the human 32-kDa polypeptide and cross-reacted with rhesus red blood cells. It should be noted that some antibody preparations also reacted with band 6 (glycero-6-phosphate dehydrogenase) probably because of contamination of the immunogen with this protein. No reactivity could be demonstrated against any proteins from the other mammalian red blood cells (Figure 2) or purified plasma membrane preparations from any of the nucleated cells (not shown).

Effect of Proteases and Lectins on NBD-PS Transport. Red blood cells were treated with a series of enzymes and lectins to determine whether the PS transporter has epitopes at the cell surface that are critical for its activity. Consistent with the observations on the transport of PS in fibroblasts (Martin & Pagano, 1987), proteolytic enzymes had no effect on the

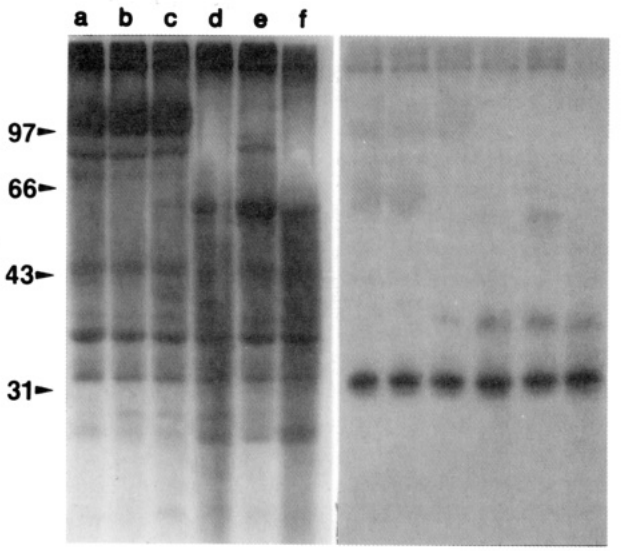

FIGURE 4: Effect of enzyme treatment of the electrophoretic pattern of the ^{125}I-PDA-labeled 32-kDa polypeptide. Red blood cells prelabeled with ^{125}I-PDA were incubated with the indicated enzymes using the protocol described in Table III. The cells were then washed, ghosts were prepared, and protein distribution was analyzed by SDS–PAGE. Coomassie blue stained gel (left), autoradiograph (right). Untreated (a), neuraminidase (b), trypsin (c), protease (d), Pronase (e), and proteinase K (f).

transport of NBD-PS in red blood cells (Table III). Similarly, treatment of the cells with various lectins was without effect. To determine whether proteolytic enzymes might partially cleave the 32-kDa polypeptide, cells were labeled with ^{125}I-PDA at 0 °C, washed, treated with the proteolytic enzymes for 1 h at 37 °C, and finally analyzed by SDS–PAGE (Figure 4). Consistent with the observations of the inability of proteolytic enzyme treatment to inhibit the transport of NBD-PS, enzyme treatment did not alter the molecular mass of the 32-kDa polypeptide (identical results were obtained if the cells were first enzyme-treated and subsequently labeled with ^{125}I-PDA) although band 3 was completely degraded by protease, Pronase, and proteinase K. These results strongly suggest that the PS-specific transporter is an integral membrane component that does not have any moieties critical to its function that are accessible at the cell surface.

Effects of Antibody on NBD-PS Transport. Red blood cells treated with 32-kDa antibodies did not demonstrate reduced capacity to transport NBD-PS (Table IV). To determine whether antibody cross-linking of other surface epitopes might inhibit PS transport, primary amines present on the cell surface were derivatized with TNBS. After being washed, the cells were incubated with a subhemagglutinating dilution of Tnp antibodies, and the cells' ability to transport PS was assessed. The results, shown in Table IV, indicate that even extensive cross-linking of cell surface proteins did not inhibit the translocation of NBD-PS from the outer to inner leaflet.

Discussion

It is well established that phospholipids are asymmetrically distributed between bilayer leaflets in eukaryotic cell membranes. Particularly striking is the apparent complete asymmetric distribution exhibited by phosphatidylserine. Although the mechanism responsible for maintaining this asymmetry is not known, recent evidence obtained by using synthetic phospholipid analogues has demonstrated that aminophospholipids exogenously inserted into the cells' outer leaflet are rapidly transported to the inner leaflet by a stereospecific (Martin & Pagano, 1987) and ATP-dependent protein-me-

diated mechanism (Seigneuret & Devaux, 1984; Tilley et al., 1986; Connor & Schroit, 1987).

Recent work from this laboratory has indicated that the protein most likely to be responsible for the intrabilayer transport of PS is a 31–32-kDa membrane polypeptide. This polypeptide has been identified by its specific labeling with a photolabeled PS analogue (Schroit et al., 1987) and with PDA, a potent inhibitor of the aminophospholipid translocase (Connor & Schroit, 1988).

In this report, we studied the translocation of NBD-PS in a variety of mammalian red blood cells and nucleated cells. We showed that, similar to human red blood cells, all of the cells examined transported exogenously inserted NBD-PS to their inner leaflet by a process that could be inhibited with the disulfide exchange reagent PDA. Attempts to identify the transporter by its preferential labeling with ^{125}I-PDA revealed that all the mammalian red blood cells expressed the 32-kDa polypeptide. On the other hand, ^{125}I-PDA treatment of nucleated cells resulted in preferential labeling of a 32-kDa polypeptide only if the cells were differentiated. Although the 32-kDa polypeptide could be detected in the nondifferentiated cells, both K562 and MEL cells also demonstrated strong labeling of a 42-kDa component. Whether this protein is involved in PS transport or is a precursor of the 32-kDa polypeptide seen in the differentiated cells or both is presently unknown.

The identification of proteins similar to the human transporter in nonhuman erythrocytes and in nucleated cells implies that these proteins may play a fundamental role in membrane physiology. Unfortunately, antibody raised against the human protein cross-reacted only with rhesus, precluding identification of possible homology between the proteins of different species and different cell types. The lack of demonstrable cross-reactivity, on the other hand, might indicate that portions of the protein may not be highly conserved across species other than among primates.

Although the ability of 32-kDa antibodies to agglutinate human red blood cells indicates that an antigenic portion of the polypeptide is at the cell surface, digestion of red blood cells with neuraminidase or proteases failed to cleave any detectable or functional sites of the protein. This can be seen by the inability of the enzyme treatments to alter the molecular mass of ^{125}I-PDA-labeled polypeptide (Figure 4) or to induce alterations in the cells' ability to transport NBD-PS (Table II). In addition, treatment of the cells with lectins had no effect. These results suggest that critical portions of the molecule are firmly embedded in the bilayer, and if glycosolated, cross-linking has no effect on its activity. Indeed, attempts to extract ^{125}I-PDA-labeled protein from human red blood cells under conditions that remove peripheral proteins (Steck & Yu, 1973) indicated that the 32-kDa polypeptide is an integral membrane protein (results not shown). Furthermore, the inability of antibody to inhibit transport might indicate that the protein cannot be extensively cross-linked, possibly because it is immobile in the plane of the membrane. This might be due to its association with cytoskeletal components, possibly spectrin, which has been shown to be involved in the maintenance of PS asymmetry (Haest & Deuticke, 1976; Haest et al., 1978).

Further characterization and positive identification of the 32-kDa protein in other cells will be greatly facilitated by the availability of better antibody preparations. It is noteworthy, however, that all the properties of the protein described here are consistent with recent characterizations of Rh polypeptides (Gahmberg, 1983; Gahmberg & Karhi, 1984; Ridgwell et al., 1984; Agre et al., 1987; Saboori et al., 1988), in particular their presence in nonhuman erythrocytes (Saboori et al., 1989). Whether the PS-specific lipid transporter and Rh polypeptides are synonymous remains to be established.

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Spin-Label ESR Studies on the Interaction of Bovine Spinal Cord Myelin Basic Protein with Dimyristoylphosphatidylglycerol Dispersions†

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ABSTRACT: Electron spin resonance (ESR) spectroscopy and chemical binding assays were used to study the interaction of bovine spinal cord myelin basic protein (MBP) with dimyristoylphosphatidylglycerol (DMPG) membranes. Increasing binding of MBP to DMPG bilayers resulted in an increasing motional restriction of PG spin-labeled at the C-5 atom position in the acyl chain, up to a maximum degree of association of 1 MBP molecule per 36 lipid molecules. ESR spectra of PG spin-labels labeled at other positions in the *sn*-2 chain showed a similar motional restriction, while still preserving the chain flexibility gradient characteristic of fluid lipid bilayers. In addition, labels at the C-12 and C-14 atom positions gave two-component spectra, suggesting a partial hydrophobic penetration of the MBP into the bilayer. Spectral subtractions were used to quantitate the membrane penetration in terms of the stoichiometry of the lipid–protein complexes. Approximately 50% of the spin-labeled lipid chains were directly affected at saturation protein binding. The salt and pH dependence of the ESR spectra and of the protein binding demonstrated that electrostatic interaction of the basic residues of the MBP with the PG headgroups is necessary for an effective association of the MBP with phospholipid bilayers. Binding of the protein, and concomitant perturbation of the lipid chain mobility, was reduced as the ionic strength increased, until at salt concentrations above 1 M NaCl the protein was no longer bound. The binding and ESR spectral perturbation also decreased as the protein charge was reduced by pH titration to above the p*I* of the protein at approximately pH 10. The obligatory electrostatic requirement was further evidenced by the strongly reduced binding of MBP to dimyristoylphosphatidylcholine bilayers compared with that to DMPG bilayers.

The basic protein from the myelin sheath (MBP)[1] is an extrinsic membrane protein which is located in the interbilayer spaces of the multilamellar structures formed by the oligodendroglial plasma membranes (Kies et al., 1958; Laatsch et al., 1962). This protein represents 30% of the total protein present in central nervous system myelin [see Braun (1984)] and is the factor responsible for the induction of experimental allergic encephalomyelitis (Eylar et al., 1970). The molecular mechanism of this pathological activity is not precisely known, but the location of the protein on the cytoplasmic side of the myelin membrane and its binding to the membrane surface suggest that the MBP stabilizes the multilamellar compact structure by joining the apposed surfaces of the myelin plasma membranes.

MBP binds strongly to acidic lipids (Palmer & Dawson, 1969). The molecular weight of the protein is 18.4K, and, together with the proteolipid protein of molecular weight 25K, it constitutes approximately 80% by weight of the total protein in myelin [see Boggs and Moscarello (1978a) and Boggs et al. (1982a)]. Several physicochemical investigations on the structure of the MBP have shown that the protein is devoid of tertiary structure in aqueous solutions (Eylar & Thompson, 1969; Chao & Einstein, 1970; Palmer & Dawson, 1969) but assumes highly ordered conformations in organic solvent mixtures (Liebes et al., 1975; Stone et al., 1985) and upon

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[1] Abbreviations: DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; ESR, electron spin resonance; MBP, bovine spinal cord myelin basic protein; *n*-PGSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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