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Transbilayer Movement of Phosphatidylserine in Erythrocytes: Inhibition of Transport and Preferential Labeling of a 31 000-Dalton Protein by Sulfhydryl Reactive Reagents†

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ABSTRACT: A series of labeled thiolation reagents were synthesized on the basis of the parent structure pyridyldithioethylamine (PDA). These compounds specifically and reversibly inhibit the active intrabilayer transport of phosphatidylserine (PS) in human red blood cells. The binding of PDA to cells can be quantified since the thiol–disulfide exchange reaction yields a chromophore. In addition, the presence of a primary amine makes it amenable to derivatization with a variety of compounds. An iodinated derivative of PDA preferentially labeled a 31 000-dalton red blood cell peptide. The labeled component, which may represent the PS transporter, comigrated with integral membrane protein band 7.

The asymmetric distribution of phosphatidylserine (PS)[1] in the membrane of normal red blood cells (RBC) is unique since it is the only phospholipid that resides exclusively in the cells' inner leaflet (Verkleij et al., 1973; Gordesky et al., 1975). That maintenance of this asymmetry is an important component of homeostasis is suggested by the fact that the exposure of PS in the cells' outer leaflet has dramatic pathophysiological consequences. The translocation of endogenous PS from the inner to outer leaflet in sickled RBC, for example, or the exposure of exogenously inserted fluorescent PS analogues in RBC results in their recognition by cells of the reticuloendothelial system (RES) (Tanaka & Schroit, 1983; Schwartz et al., 1985; Schroit et al., 1985). These results have suggested that the appearance of PS on the cells' outer leaflet may play a specific role as a recognition moiety signaling RBC removal by the RES.

Although the translocation of PS seems to be an important factor of macrophage recognition in the maintenance of ho-

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[1] Abbreviations: [^{125}I]B/H, 3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionate (monoiodinated Bolton–Hunter reagent); 2-TP, 2-thiopyridone; DTT, dithiothreitol; DOPC, dioleoylphosphatidylcholine; HEPES–saline, 145 mM NaCl, 5 mM KCl, 20 mM HEPES, and 10 mM glucose; NBD-PS, 1-oleoyl-2-[[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]caproyl]phosphatidylserine; PDA, pyridyldithioethylamine; RBC, human red blood cells; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; Tnp, trinitrophenyl; DMSO, dimethyl sulfoxide; kDa, kilodalton(s).

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meostasis, the underlying mechanism responsible for the intrabilayer movement of PS in RBC is unknown. Previous work using labeled lipid analogues has demonstrated, in both RBC (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit, 1987) and nucleated cells (Zachowski et al., 1987; Martin & Pagano, 1987), that exogenously added PS analogues preferentially translocate from their initial site of insertion in the outer leaflet to the inner leaflet, thereby adopting an asymmetric distribution. This intrabilayer movement appears to be protein mediated, since it is has been shown to be ATP dependent and inhibitable by sulfhydryl-oxidizing reagents (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Connor & Schroit, 1987).

Since the translocation of PS is inhibitable by sulfhydryl-reactive compounds, we synthesized a series of reagents on the basis of the parent compound PDA (Johnson & Chenoweth, 1985) in an effort to identify the protein components responsible for maintaining a particular intrabilayer distribution of PS. These sulfhydryl-oxidizing reagents (1) reversibly inhibit the outside-to-inside translocation of a fluorescent PS analogue by disulfide exchange with free protein sulfhydryls, (2) can be quantified for binding since a chromophore is stoichiometrically released upon disulfide exchange, and (3) can be labeled because a primary amine is located at a noncritical site. Using an iodinated derivative of this probe, we have identified a 31-kDa protein that may be responsible for the specific translocation of PS in RBC. The labeled protein comigrates with integral membrane protein band 7 (Steck, 1974) and appears to be the same protein previously identified with a photoactivatable PS analogue (Schroit et al., 1987).

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 TLC. Diamide, dithiopyridine, iodoacetamide, *N*-ethylmaleimide, DTT, and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Succinimidyl 3-(4-hydroxyphenyl)propionate and *N*-hydroxysuccinimidylbiotin were obtained from Pierce Chemical Co. (Rockford, IL). [^{125}I]B/H reagent (sp act. ~2000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Bimane sold under the trade name Thiolyte MB was obtained from Calbiochem. RBC in heparinized saline were obtained by venipuncture, pelleted by centrifugation, and washed 3 times with 10 volumes of HEPES–saline buffer. The cells were labeled with ^{51}Cr, washed, and resuspended at 2×10^7 RBC/mL. Steady-state fluorescence of the NBD lipids was measured at 535 nm (λ_{ex} 468 nm with a 468-nm band-pass filter) with a Farrand MK II spectrofluorometer at room temperature using 10-nm slit widths.

Chemical Synthesis. (*A*) *PDA Synthesis.* PDA was synthesized by a modification of the procedures described by Carlsson et al. (1978) and Johnson and Chenoweth (1985). Briefly, a 2-fold mole excess of dithiopyridine (17 μmol) was reacted by thiol–disulfide exchange with cysteamine hydrochloride (8.5 μmol) in 15 mL of EtOH at 20 °C for several hours. Completion of the reaction was monitored by the production of 2-TP, which is released stoichiometrically. The product was purified by 2× crystallization from ethanol/diethyl ether (2:3) at −20 °C. The product was found to be >99% pure on the basis of TLC using disulfide-sensitive spray reagents (Glaser et al., 1970) and weight versus absorption (Grassetti & Murray, 1967) characteristics.

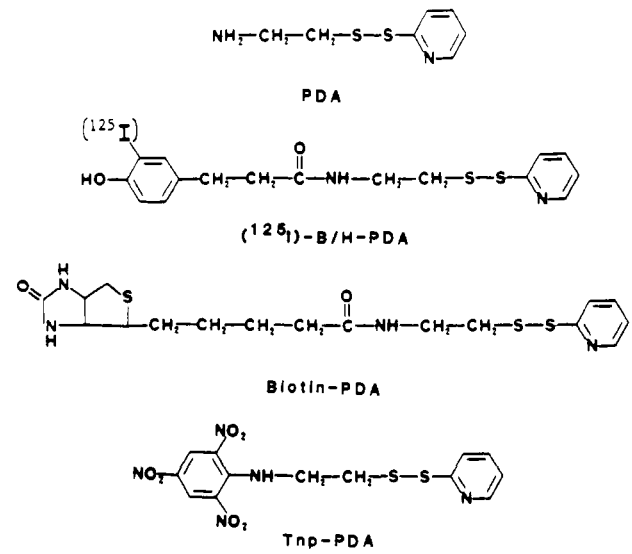

FIGURE 1: Structure of PDA and its labeled derivatives.

(*B*) *PDA Derivatives.* The structures of the various derivatives are shown in Figure 1. [^{125}I]B/H-PDA was prepared by mixing [^{125}I]Bolton–Hunter reagent with 10 μg of PDA in $CHCl_3$/MeOH/triethylamine (1:2:0.01) at 4 °C for 18 h. The product was purified by TLC in ethyl acetate/toluene (2:1). B/H-PDA (no radiolabel) was synthesized as described above with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate. Biotin–PDA was prepared by mixing *N*-hydroxysuccinimidylbiotin with PDA in dimethylformamide for 6 h at room temperature. The product was precipitated by the addition of water and crystallized from 2-propanol. Tnp-PDA was prepared by reacting TNBS with PDA in 0.5 M Na_2HCO_3. The orange product was extracted with diethyl ether and dried.

Inhibition of NBD-PS Translocation. ^{51}Cr-Labeled RBC (2×10^7/mL) were incubated with the various sulfhydryl reagents in HEPES–saline for 30 min at 4 °C. The cells were then washed, rapidly mixed with NBD-PS [100 ng (10 μL of EtOH)$^{-1}$ (mL of RBC)$^{-1}$], and incubated at 37 °C for 1 h with constant shaking. After washing (to remove unincorporated NBD lipid), the RBC were incubated with 1 mg of DOPC "acceptor" SUV for 30 min at 20 °C to remove any NBD lipid remaining in the cells' outer leaflet. The SUV were then removed by centrifugation and the RBC solubilized with 2% Triton X-100 (final concentration). The fraction of NBD lipid remaining in the cells after this "back-exchange" procedure was determined by measuring the residual NBD fluorescence. Reversal of the inhibition of NBD-PS intrabilayer transport was accomplished by adding DTT or glutathione to the NBD-PS-containing RBC suspension. All fluorescent measurements were normalized for variations in RBC number (<10%) to 2×10^7 RBC on the basis of ^{51}Cr counting of the final detergent lysates.

PDA Binding and Inhibition. RBC were incubated with increasing concentrations of PDA for 30 min at 4 °C. The cells were pelleted, and an aliquot of the supernatant was analyzed for the presence of 2-TP by measuring absorbance at 343 nm (Grassetti & Murray, 1967). The washed RBC were then analyzed for their ability to transport exogenously inserted NBD-PS from the outer to inner leaflet as described above.

Interaction of [^{125}I]B/H-PDA with RBC. RBC were incubated with [^{125}I]B/H-PDA [13 μCi (2×10^8 RBC)$^{-1}$ mL^{-1}] for 30 min at 4 °C. The cells were then washed, and the fraction of cell bound [^{125}I]B/H-PDA was determined by scintillation counting. Ghosts were prepared from these RBC

Table I: Effects of Sulfhydryl-Oxidizing Reagents on the Transbilayer Movement of NBD-PS[a]

inhibitor	PS translocation (%)		
	nonreduced	DTT	glutathione
control (no treatment)	81 ± 6	79 ± 7	78 ± 4
diamide (5 mM)	16 ± 4	75 ± 7	22 ± 2
N-ethylmaleimide (2 mM)	14 ± 2	20 ± 6	18 ± 3
iodoacetamide (10 mM)	38 ± 4	36 ± 5	39 ± 3
iodoacetamide (10 mM at 37 °C)	19 ± 3	19 ± 5	25 ± 5
bimane (5 mM)[b]	21 ± 4	42 ± 3	24 ± 4
PDA (1 mM)	15 ± 2	72 ± 3	23 ± 2
B/H-PDA (1 mM)	24 ± 2	82 ± 4	28 ± 3
biotin-PDA (1 mM)[b]	13 ± 2	74 ± 3	16 ± 3
Tnp-PDA (0.1 mM)[b]	39	75	
dithiopyridine (1 mM)[b]	14 ± 1	71 ± 3	20 ± 3

[a] RBC (2×10^7/mL) were incubated with the indicated inhibitors for 30 min at 4 °C. The cells were then washed and incubated with NBD-PS [100 ng (10 μL of EtOH)$^{-1}$ (mL of RBC)$^{-1}$] in the absence or presence of DTT (20 mM) or glutathione (20 mM) at 37 °C. The cells were then washed, and the fraction of NBD-PS remaining in the cells was determined as described under Experimental Procedures. [b] Bimane was added to the cells from a stock solution in CH_3CN. Tnp-PDA and dithiopyridine were added to the cells from concentrated stock solutions in EtOH, and biotin-PDA was added from concentrated stock solutions in DMSO. All final solvent concentrations were <1%.

by lysis with 5 mM phosphate buffer (pH 8.0), followed by centrifugation at 30000*g*. The fraction of [^{125}I]B/H-PDA associated with the ghosts was determined from the radiation remaining with the ghost pellet. To determine the fraction of [^{125}I]B/H-PDA associated with globin, the hemoglobin-containing ghost supernatant (first spin) was precipitated with acidified acetone (Gordesky et al., 1975). To identify which proteins became labeled with [^{125}I]B/H-PDA, ghosts were subjected to SDS–polyacrylamide gel electrophoresis under nonreducing conditions using a 12% separating and 6% stacking gel with the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie R-250, dried, and autoradiographed with Kodak XAR-5 X-ray film.

Results and Discussion

The ability of PS (Daleke & Huestis, 1985; Tilley et al., 1986) and its spin-labeled (Seigneuret & Devaux, 1984; Seigneuret et al., 1984) or fluorescent analogues (Connor & Schroit, 1987) to translocate from the outer to inner leaflet of RBC has been shown to be depend on maintenance of membrane protein sulfhydryls in the reduced state. Thus, treatment of cells with such nonreversible alkylating agents as diamide, *N*-ethylmaleimide, iodoacetamide, or bimane blocks PS transport (see Table I). These data strongly suggest that the protein responsible for the translocation of PS (a PS-specific flipase) bears a sulfhydryl critical to its function.

Because of this sulfhydryl, we reasoned that it might be possible to block the transport of PS with reagents that initiate thiol–disulfide exchange. Thus, in theory it should be possible to selectively inhibit transport yet reverse its effect upon reduction of the disulfide bridge. PDA was the reagent of choice since it is highly water soluble, contains a free amine amenable to labeling with a variety of reagents, and stoichiometrically releases 2-TP upon disulfide exchange, thus allowing binding of the probe to be quantified. Figure 2 shows the effect of increasing PDA concentrations on the binding of the probe to the cell and its effect on the inhibition of PS transport. Maximum inhibition of PS translocation occurred at ~1 mM PDA, which corresponds to the saturation point of PDA binding (assessed by the amount of 2-TP released).

Table I shows the effect of alkylating agents and several PDA derivatives on the inhibition of PS transport. The data

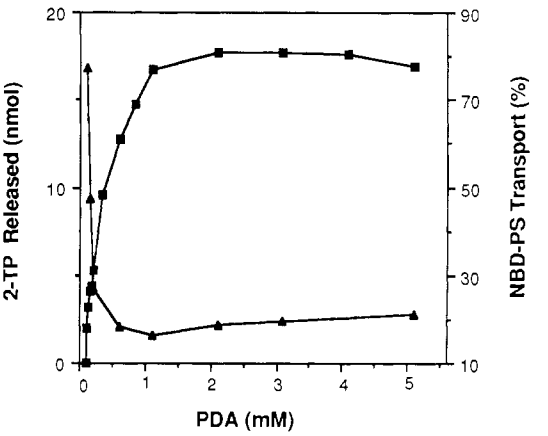

FIGURE 2: Correlation between PDA binding to RBC and inhibition of NBD-PS transport. RBC (2×10^7 RBC/mL) were incubated with increasing concentrations of PDA at 4 °C for 30 min. The cells were then pelleted, and aliquots of the supernatant were measured for 2-TP release at 343 nm (■). The cells were then washed, and their ability to transport NBD-PS from the outer to inner leaflet (▲) was determined as described under Experimental Procedures.

Table II: Binding of [^{125}I]B/H-PDA to RBC[a]

RBC fraction	[^{125}I]B/H-PDA (% bound)
intact RBC	37.0
ghosts	3.7
ghost supernatant	33.0
globin	1.8

[a] [^{125}I]B/H-PDA was incubated with RBC for 30 min at 4 °C [13 μCi (2×10^8 RBC)$^{-1}$ mL^{-1}]. The cells were then spun, and aliquots of the pellet and supernatants were counted for ^{125}I to determine the percentage of probe incorporation. The cells were then lysed with 5 mM phosphate buffer, pH 8.0, and the fraction of radiation in the ghost pellet and supernatant was determined. The amount of radiation present in the globin was determined after its precipitation from the ghost supernatant with acidified acetone.

show that inhibition of transport induced by the alkylating agents was, for the most part, irreversible. In contrast, PDA and its derivatives, which bind to protein thiols by disulfide exchange, were reversible upon reduction by DTT. The inability to reverse inhibition by glutathione suggests that the sulfhydryl critical to the proteins' transport activity is located within the bilayer and not as the membrane surface. It should be noted that this result was not due to the inability of glutathione to reduce disulfides. Pretreatment of PDA and its labeled derivatives with glutathione (and DTT) resulted in quantitative release of 2-TP and abolished all inhibitory activity.

To assess the cellular distribution of the PDA derivatives, RBC were incubated with isotopic amounts of [^{125}I]B/H-PDA (Table II). About 10% of the cell-associated probe was actually bound to the ghost membranes while <5% was bound to globin. The remaining probe was found in the globin-precipitated supernatants. This fraction probably represents reagent reduced by intracellular glutathione and thus entrapped in the cytosol. Pretreatment of the cells with 1 mM PDA inhibited the binding of [^{125}I]B/H-PDA to intact RBC by 70%.

To determine which proteins might be involved in the translocation of PS, RBC were labeled with [^{125}I]B/H-PDA and the membrane proteins were separated by SDS gel electrophoresis under nonreducing conditions. Autoradiographic analysis of the separated proteins revealed that [^{125}I]B/H-PDA preferentially bound a protein with an apparent molecular mass of 30–32 kDa (Figure 3) that comigrated with band 7 (Steck, 1974). Interestingly, a 30–31-kDa protein, which also co-

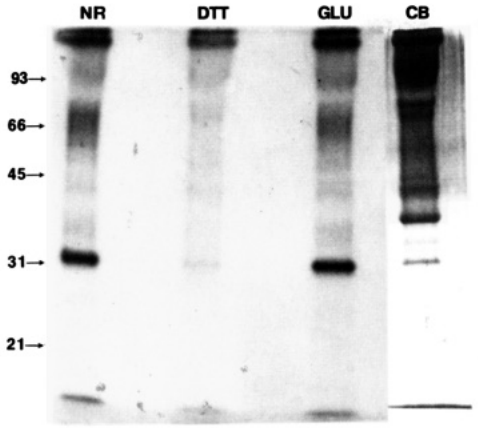

FIGURE 3: Electrophoretic distribution of [^{125}I]B/H-PDA in RBC. RBC were treated with [^{125}I]B/H-PDA as described under Experimental Procedures. After washing, the cells were treated with DTT or glutathione (20 mM) for 30 min at 20 °C. The reducing agents were removed by washing, and ghosts were prepared as described. The ghosts were solubilized in SDS without reducing agents, and $\sim 10^8$ RBC equivalents were applied to each lane. The gels were fixed, stained with Coomassie blue (CB), and autoradiographed. NR, nonreduced; DTT, dithiothreitol; GLU, glutathione.

migrated with band 7, has been previously identified through the use of a photoactivatable PS analogue (Schroit et al., 1987), suggesting that both techniques label the same protein. Although we do not have direct evidence that both independent techniques identify the same protein, we find it intriguing that a translocating substrate (a photoactivatable PS analogue) and an inhibitor of the transport activity (PDA) seem to label the same RBC protein.

Since the PDA derivatives appear to preferentially bind a protein that might be responsible for PS translocation, it should be possible to employ these reagents in an isolation protocol. Table I shows that both biotin–PDA and Tnp-PDA reversibly inhibit NBD-PS translocation. Thus, it should be possible to isolate the 30–32-kDa protein preferentially bound by PDA by employing appropriate avidin/antibody isolation protocols. This approach has the advantage of enabling recovery of proteins under extremely mild conditions without the attached probes since specific recovery can be obtained simply by the addition of DTT. Experiments using these techniques are currently under way in this laboratory.

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