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Colocalization of Rh polypeptides and the aminophospholipid transporter in dilauroylphosphatidylcholine-induced erythrocyte vesicles

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Abstract

Cytoskeleton-free vesicles released from human red blood cells (RBC) transport exogenously supplied aminophospholipid analogues from the vesicle's outer to inner leaflet at rates comparable to those of normal RBC (Beleznay et al. (1993) Biochemistry 32, 3146–3152). Because polypeptides associated with the Rh blood group system have been implicated in the transbilayer movement of phosphatidylserine (PS), we investigated the relationship and co-localization of the aminophospholipid translocase and Rh in dilauroylphosphatidylcholine-induced RBC vesicles. The transbilayer movement of fluorescent (NBD-PS) and photoactivatable (^{125}I-N_3-PS) PS in RBC vesicles was ATP- and temperature-dependent. Inhibition of PS transport by sulfhydryl reagents could be accomplished by direct vesicle treatment or by treating RBC before vesiculation. In the case of diamide- and pyridyldithioethylamine-mediated inhibition, NBD-PS transport could be restored by reduction with dithiothreitol, indicating that the movement of the PS transporter into the emerging vesicle was independent of the oxidative status of membrane sulfhydryls. The presence of Rh polypeptides in the vesicles was verified by direct immunoprecipitation of isotopically-labeled Rh and semi-quantified by antibody adsorption assays. Similar to the movement of the PS transporter, localization of Rh polypeptides in the vesicle membrane was independent of the red cell's oxidative status. These results show that the PS translocase and Rh-related proteins colocalize in RBC vesicles suggesting that these proteins may be members of a multicomponent complex that plays a role in lipid movement and the generation of membrane lipid asymmetry.

Keywords: Phospholipid; Lipid transporter; Vesicle

1. Introduction

The phospholipids of the human erythrocyte (RBC) are distributed asymmetrically across the cell's membrane bilayer. The choline-containing lipids predominate in the cell's outer leaflet, whereas the aminophospholipids, phosphatidylethanolamine and phosphatidylserine (PS), preferentially or exclusively localize in the cell's inner leaflet. This distribution is, at least in part, controlled by the activity of an ATP-dependent aminophospholipid-specific transporter that shuttles lipids between membrane leaflets at different rates [1,2]. This activity, combined with other lipid–membrane interactions, generates and maintains unequal lipid distributions across the bilayer membrane [3,4].

Red cells can be induced to vesiculate by various techniques, including ATP depletion [5], calcium loading [6], ageing [7,8], and the insertion of amphipaths into the cell's outer leaflet [9–11]. Irrespective of the method used, however, the vesicle's protein and lipid composition always resembles that of the red cell, with the exception of essentially complete exclusion of cytoskeletal components [12]. There are, however, differences in the relative amounts of specific proteins that remain with the remnant red cell and that localize in the vesicle membrane. Diffusion-restricted, cytoskeletal-linked membrane components, for example, do not segregate into the vesicle [13], whereas freely diffusible components preferentially segregate into the emerging vesicle. These include the mobile fraction of band 3 [13], glycophorin, complement receptor [14], and GPI-anchored proteins such as acetylcholine esterase [15], DAF [15], and CD59 [16].

Abbreviations: BSA, bovine serum albumin; DLPC, dilauroylphosphatidylcholine; DTT, dithiothreitol; NBD-, 1-oleoyl-2-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproyl)-; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PDA, pyridyldithioethylamine; PS, phosphatidylserine; RBC, human red blood cells.

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Although the lipid transporter has been biochemically and functionally well characterized, the protein responsible for the cell's transport activity remains unidentified. Several studies suggest the involvement of 32 kDa Rh-associated band 7 proteins [17–19], a 120 kDa Mg-ATPase [20,21], or a multimeric complex of the Mg-ATPase and an Rh-related polypeptide [22]. Because DMPC-induced vesicles transport PS [23] and Ca^{2+}/EDTA/heat-induced vesicles have been reported to react with Rh(D) antibodies [24], this system provides a model for investigating the relationship between Rh and PS transport. In this study, we assessed the concomitant release of Rh polypeptides and the PS transporter into DLPC-induced vesicles [10]. We found that both Rh polypeptides and PS transport activity colocalize in the emerging vesicle membrane.

2. Materials and methods

2.1. Materials and routine procedures

DLPC and NBD-PC were purchased from Avanti Polar Lipids (Birmingham, AL). Carrier-free ^{125}I-N_3-PC was synthesized from 1-oleoyl-2-aminocaproyl-PC and azido-^{125}I(iodo)phenylproprionate as previously described [25]. NBD-PS and ^{125}I-N_3-PS were prepared from NBD-PC and ^{125}I-N_3-PC by phospholipase D-catalyzed base exchange in the presence of L-serine as previously described [26]. PDA and ^{125}I-PDA were synthesized as previously described [18]. Diamide, NEM, and vanadate were obtained from Sigma. RBC were obtained from healthy volunteers by venipuncture into heparinized syringes. In some experiments, RBC were labeled with ^{51}Cr (~ 1 mCi) for 30 min at 37° C; ^{125}I-PDA (~ 0.1 mCi) for 20 min at 0° C; or Na^{125}I (~ 1 mCi) in the presence of four iodobeads (Pierce) for 20 min at 0° C. RBC ghost and vesicle proteins were analyzed by SDS-PAGE using 11% separating and 6% stacking gels with the discontinuous buffer system of Laemmli [27]. The gels were stained, dried and autoradiographed with Kodak XAR-5 film or imaged on a Molecular Dynamics PhosphorImager. Steady-state fluorescence of NBD-lipids was measured at 535 nm (λ_{ex} 468 nm) with a Farrand MKII spectrophotometer at room temperature using 10 nm slit widths. Radiation was monitored by scintillation counting using a Packard AutoGamma spectrometer.

2.2. Vesiculation

Small unilamellar vesicles were prepared by sonication of DLPC (1.5 mM) in Hepes-saline buffer (144 mM NaCl, 10 mM Hepes, 10 mM glucose, pH 7.3) followed by centrifugation at 30 000 × g to remove metal fragments and large vesicles. Freshly drawn RBC were washed four times by centrifugation (2000 × g for 5 min) in Hepes-saline. The buffy coat was removed by aspiration. In most experiments cellular ATP levels were increased by incubating the cells in buffer A (100 mM NaH_2PO_4, 33 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM glucose, 10 mM pyruvate, 10 mM inosine, and 2 mM adenosine, pH 7.4) for 2 h at 37° C as described [23]. The RBC were then washed, resuspended in Hepes-saline to a 10% hematocrit and incubated with DLPC (38 μM final concentration) at 4° C on an orbital shaker for the indicated period of time. Remnant RBC were removed by centrifugation at 850 × g for 5 min. This was repeated with the vesicle-containing supernatant to ensure complete RBC removal. Vesicles in the 850 × g supernatant were concentrated by centrifugation at 30 000 × g for 10 min. In early experiments, vesicles were purified by gel filtration chromatography on Sephacryl S-1000 as described by Beleznay et al. [23]. Other experiments indicated that this step was unnecessary and that the vesicles could be washed in Hepes-saline with identical results. Vesicle formation was monitored by scintillation counting of vesicles released from ^{125}I-PDA- and ^{51}Cr-labeled RBC or by acetylcholinesterase activity.

2.3. Electron microscopy

Purified RBC vesicles were pelleted and fixed with 2% paraformaldehyde/3% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4° C and post-fixed with 1% OsO_4. Samples for transmission electron microscopy (TEM) were stained with 1% uranylacetate, dehydrated in graded EtOH and embedded in Spurr resin. For scanning electron microscopy (SEM), the samples were air-dried and sputtered with Pt/Pd following procedures developed for the preservation of lipid vesicles [28].

2.4. Translocation of lipid analogues

Lipid transport in RBC and in vesicles was monitored by back-exchange with BSA. Briefly, RBC ($2 \cdot 10^8$/ml) or vesicles (from 1 ml packed RBC resuspended to 1 ml) were resuspended in Hepes-saline, mixed with NBD-lipids in ethanol (1 μg lipid/10 μl of EtOH/ml) and incubated at 37° C. Aliquots (100 μl) were removed at the indicated times and mixed with 1 ml of 1% BSA to remove lipid that was not transported to the cells' or vesicles' inner leaflet. The suspension was centrifuged for 5 min at 2000 × g (RBC) or 20 000 × g (vesicles) and the pellets were solubilized in 1% Triton X-100. The fraction of lipid transported was determined by fluorescence measurements of the amount of total vesicle-associated lipid (fluorescence of vesicles centrifuged through Hepes-saline) and fluorescence remaining in the vesicles after BSA back-exchange.

Transport of azide-labeled lipids was carried out in the same manner except that the experiments were done under a red safelight and 5% BSA was used for back-exchange.

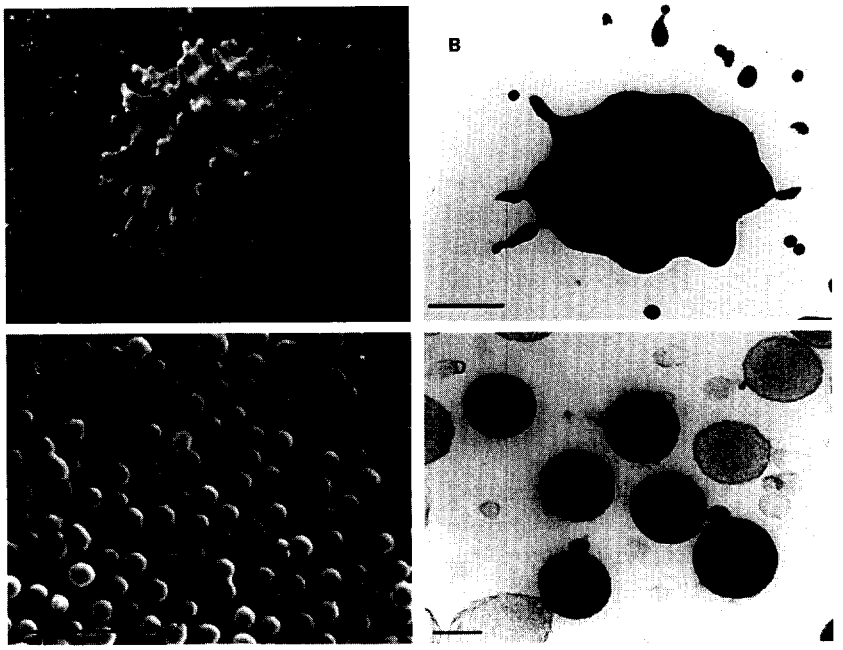

Fig. 1. Scanning and transmission electron microscopy of DLPC-induced vesiculation. Scanning (A) and transmission (B) EM of vesiculating red cells after 20 min incubation at 4° C in the presence of 38 μM DLPC. Scanning (C) and transmission (D) EM of purified DLPC-induced RBC vesicles. Bar = 1 μm except in (D) where bar = 0.1 μm.

2.5. Azide-lipid labeling

Washed RBC ($2 \cdot 10^8$ cells/ml) were incubated with ^{125}I-N_3-PC or ^{125}I-N_3-PS for 20 min at 37° C and washed with Hepes-saline. In some experiments, labeled cells were vesiculated with DLPC and the purified RBC vesicles were irradiated for 3 min (12 cm from an Osram HBO 100W/2 super-pressure mercury lamp). In other experiments, the labeled cells were irradiated, washed, and then vesiculated.

2.6. Immunoprecipitation

RBC of known Rh phenotype (R_1R_1, [DCe/DCe]; rr, [cde/cde]) were prelabeled with ^{125}I-PDA or $Na^{125}I$ and then vesiculated with DLPC as described above. In the case of ^{125}I-N_3-PS labeling, RBC bearing cde/cde polypeptides were labeled and vesiculated in the dark. The resulting vesicles were then irradiated as described above. Membrane vesicles obtained from 1 ml of packed RBC were resuspended in 400 μl of phosphate-buffered saline

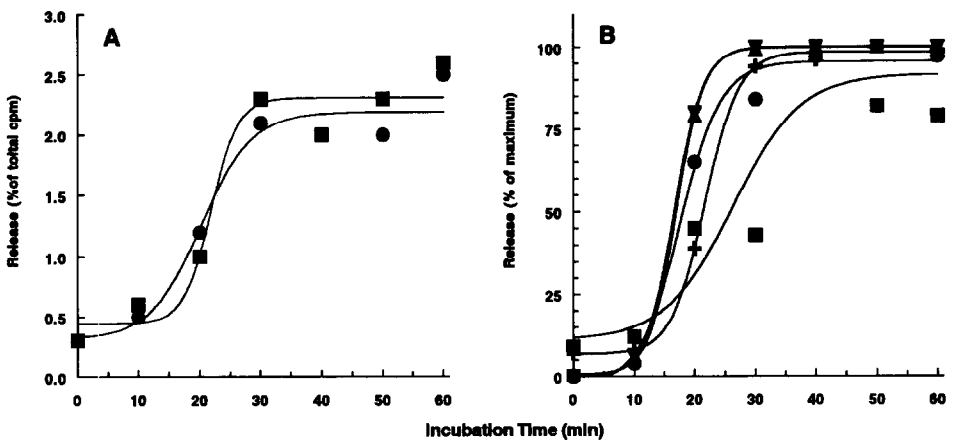

Fig. 2. Release of DLPC-induced membrane vesicles from RBC. (A) Isotopically-labeled RBC (10% packed) were incubated at 4° C with 38 μM sonicated DLPC. At the indicated intervals, the fraction of radiation present in 850 × *g* supernatants was determined by scintillation counting. (■), ^{51}Cr; (●), ^{125}I-PDA. (B) Release of acetylcholinesterase activity from RBC incubated with 38 μM DLPC as described for panel A except that the cells were pretreated with the indicated inhibitors as described in Materials and methods. The release of acetylcholinesterase, expressed as the percent of maximum release, represents approx. 35% of the total cell-associated acetylcholinesterase activity. (+), control cells; (●), PDA; (▼), vanadate; (■), diamide; (▲), NEM.

(PBS) and incubated overnight at 4° C with 10-25 μg of monoclonal antibody. Excess antibody was removed by washing, and the vesicles were solubilized in 300 μl of PBS containing 3% Triton X-100 for 1 h at 4° C. Insoluble material was removed by centrifugation at 20000 × *g* for 30 min. Immune complexes in the supernatant were then isolated with protein-A Sepharose (Sigma) as described previously [25]. The Sepharose-bound immunoprecipitated protein was released by heating in 1% SDS and analyzed by autoradiography after SDS-PAGE.

2.7. Hemagglutination

The direct hemagglutination titer of Rh antibodies and vesicle-adsorbed Rh antibodies were tested on papain-treated heterozygous (R_1r) RBC. RBC vesicles were prepared from the red cells of homozygous Rh(D) (R_1R_1) and homozygous Rh(c) (rr) donors. These vesicles were purified as described above and incubated with polyclonal anti-D or anti-c antibodies for 1 h at room temperature followed by an additional 30 min at 0° C. The vesicle-antibody mixture was centrifuged at 20000 × *g* for 15 min at 4° C. The antibody-containing supernatant was removed and titered using standard protocols.

3. Results

3.1. Release of DLPC-induced membrane vesicles

Because aminophospholipid translocase-mediated PS movement across the red cell membrane is critically dependent on cellular ATP levels, RBC were vesiculated at 4° C to minimize nucleotide consumption. Freshly drawn RBC were labeled with ^{51}Cr or ^{125}I-PDA and incubated with DLPC (38 μM) for 1 h at 4° C. Similar to vesiculation obtained with DMPC at 30–37° C [9], RBC incubated with DLPC at 4° C underwent dramatic discocyte-to-echinocyte shape changes which most likely resulted in the release of vesicles from the tips of the membrane projections (Fig. 1A and B). The time-course of vesiculation was assessed by measuring the amount of isotopically labeled proteins (Fig. 2A) and acetylcholinesterase (Fig. 2B) released from the cells into the supernatant. RBC vesicles first appeared in the supernatant after an initial lag period of about 10 min and was complete within 45 min. Morphological examination of vesicles obtained after removal of remnant RBC by centrifugation at 850 × *g* revealed relatively uniform hemoglobin-containing and empty spherical structures with an average diameter of 0.15 μm (Fig. 1C and D). The empty spheres are most likely unsealed vesi-

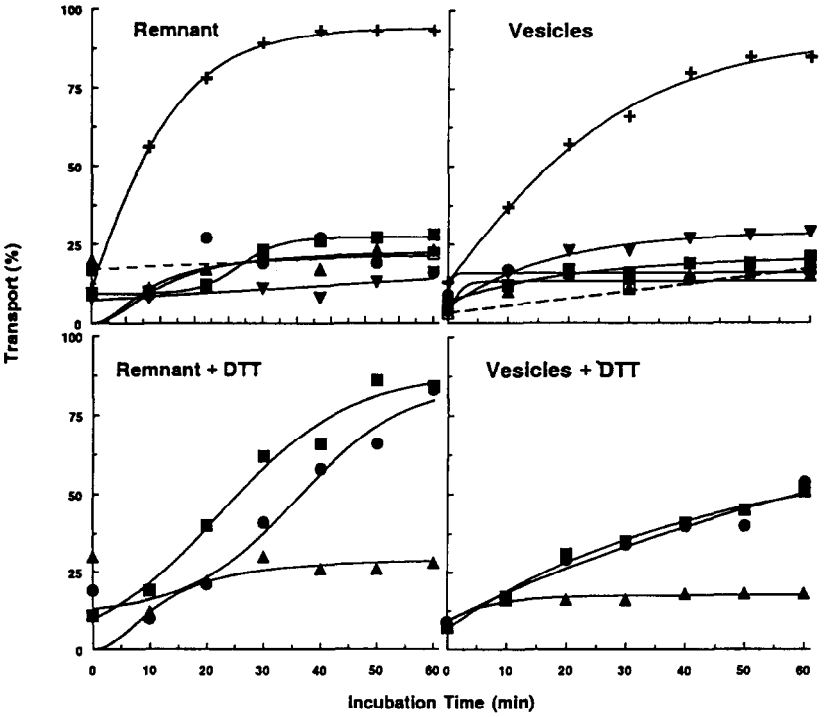

Fig. 3. Transport of NBD-PS in remnant cells and in vesicles generated from inhibitor-treated RBC. RBC were pretreated with the indicated inhibitors as described in Materials and methods and then vesiculated with DLPC. The suspension was centrifuged at 850 × *g* and the vesicle containing supernatant was separated from the remnant cell pellet. The remnant cells and vesicles were then washed and their ability to transport NBD-PS (solid lines) and NBD-PC (dashed lines) was determined in the absence or presence of 10 mM DTT. (+), control cells; (●), PDA; (▼), vanadate; (■), diamide; (▲), NEM.

cles, the fraction of which varied between 20–40% of the total vesicle population. Because these vesicles do not contain ATP, their presence is without affect on the uptake studies shown below.

To determine if known inhibitors of aminophospholipid transport affect the ability of the cells to vesiculate, vesicle release from RBC pretreated with PDA, NEM, diamide, and vanadate was assessed. Fig. 2B shows essentially identical kinetics and similar quantities of membrane released from control and inhibitor-treated cells. This indicates that DLPC-induced vesiculation proceeded in a manner that was independent of ATP and the oxidative status of membrane proteins.

3.2. Transport of labeled PS analogues in RBC vesicles and remnant cells

Fig. 3 shows that remnant RBC (Fig. 3A) and RBC vesicles (Fig. 3B) transported NBD-labeled PS but not PC. Similar to normal RBC, vesicles and remnant cells obtained from RBC pretreated with various inhibitors of aminophospholipid transport did not transport PS. It can be seen that PDA, diamide, NEM, and vanadate abrogated transport in the remnant cells and in the vesicles (Fig. 3A and B), and that inhibition of PS transport by the disulfide exchange reagents was reversible upon treatment with DTT (Fig. 3C and D). These data suggest that the movement of the aminophospholipid transporter into the budding vesicle was unaffected by oxidation of membrane sulfhydryls. Transport inhibition by vanadate in remnant cells and in vesicles required higher concentrations (3 mM) than that of normal cells (0.1 mM). This was probably due to increased ATP levels in the remnant cells that were preincubated with buffer A (5–8-fold higher than normal levels) and to the poor uptake of solutes into the vesicle membrane.

Inhibition of PS transport in the vesicles was not dependent on pretreatment of the donor RBC with inhibitors. Fig. 4 shows that vesicles derived from untreated RBC were sensitive to the same inhibitors and, in the case of PDA and diamide, inhibition was reversible with DTT. Essentially identical results were obtained in experiments employing ^{125}I-N_3-PS (results not shown). These data indicate that all of the components required for aminophospholipid-specific transport partitioned from the red cell membrane into the budding vesicle membrane.

3.3. Protein analysis of RBC vesicle proteins

SDS-PAGE analysis of Coomassie blue-stained ghost membranes and DLPC-induced vesicles derived from the same cells showed that the major cytoskeletal proteins were absent in the vesicles, and that band 3 and 32 kDa band 7 proteins were the two major membrane proteins released under these conditions (Fig. 5B). Semi-quantitative scanning densitometry of the Coomassie-stained gels

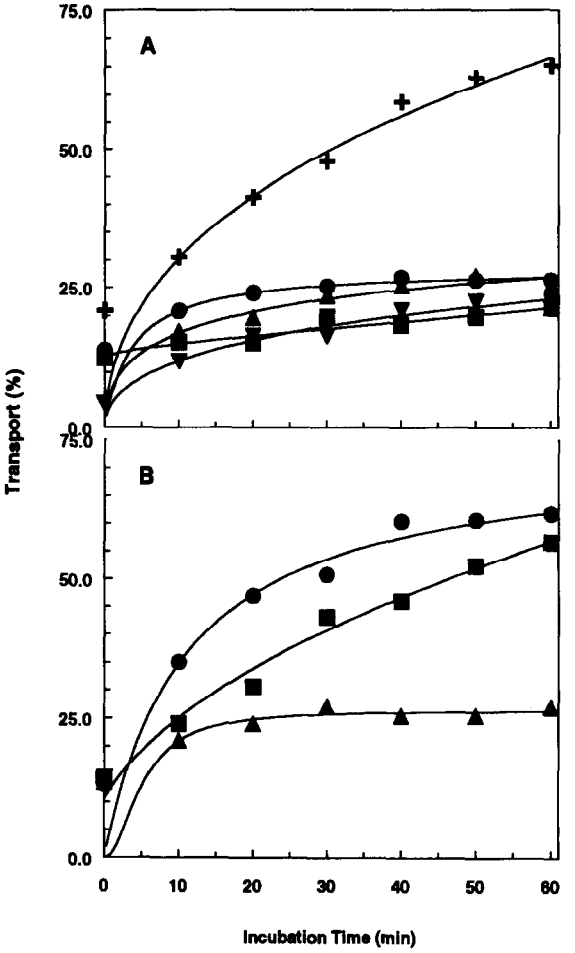

Fig. 4. Effect of inhibitors on the transport of NBD-PS in vesicles. Vesicles were generated from normal RBC as described in Materials and methods. Aliquots were then treated with inhibitors as described, washed, and their ability to transport NBD-PS in the absence (A) or presence (B) of DTT was determined. (+), control vesicles; (●), vesicles treated with PDA; (▼), vanadate; (■), diamide; (▲), NEM.

indicated that the band 7 proteins were relatively enriched in the vesicles and comprised about 14% of the vesicle membrane's protein compared to about 4% of the ghost membrane's protein (Fig. 5A). Analysis of these gels by autoradiography revealed, however, that in addition to iodine-labeled 32 kDa protein, several other proteins were released from the red cells into the vesicle membrane. For example, labeling with ^{125}I-PDA revealed that a substantial fraction of a diffusely labeled band in the range of 62–76 kDa was released from the red cells (Fig. 5C, lane 1) into the vesicles (Fig. 5C, lane 3). Similarly, a 92 kDa protein (Fig. 5C, lane 4), probably glycophorin A dimer, was released from ^{125}I-labeled RBC (Fig. 5C, lane 2).

Analysis of the distribution of ^{125}I-N_3-PS in irradiated vesicles showed crosslinking of the probe to 32 kDa band 7 polypeptides in vesicles derived from RBC containing increased (preincubated in buffer A) and normal ATP levels (Fig. 6, lanes 1 and 3, respectively). The fraction of probe crosslinked to 32 kDa polypeptides was decreased in vesicles derived from RBC pretreated with the transport

inhibitors, PDA (lane 4) and vanadate (lane 5) to levels obtained with nontransportable ^{125}I-N_3-PC (lane 2).

Because Rh polypeptides are a component of the band 7 proteins and have been implicated in lipid transport, the presence of these polypeptides in the vesicles was determined by immunoprecipitation with monoclonal antibodies. Fig. 5D and Fig. 6B show that iodine-labeled Rh was immunoprecipitable from ^{125}I-PDA-, ^{125}I-, and ^{125}I-N_3-PS-labeled vesicles. Immunoprecipitation was specific because monoclonal anti-D failed to precipitate protein from vesicles generated from these cells (not shown).

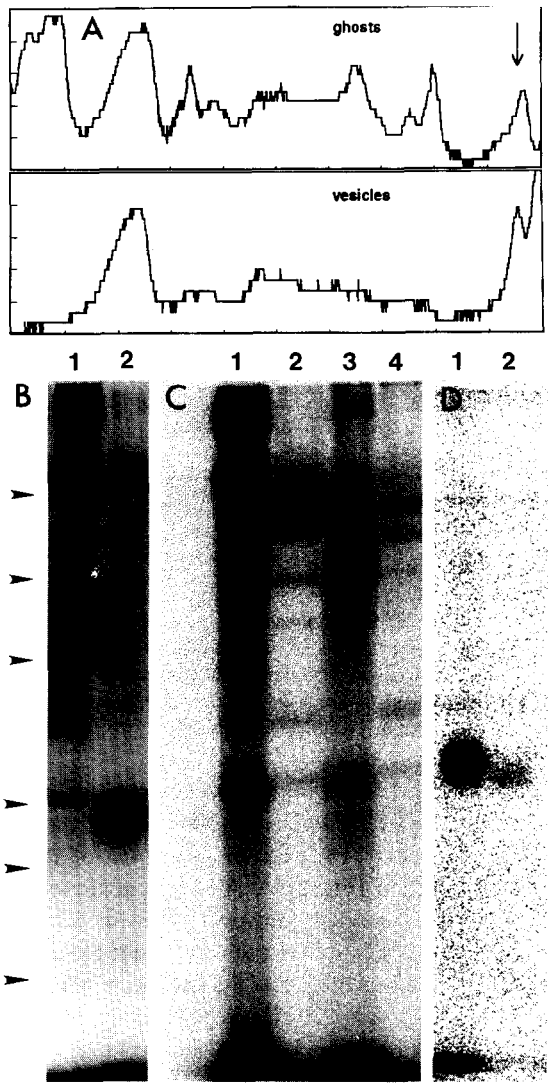

Fig. 5. SDS-PAGE analysis of DLPC-induced vesicles and immunoprecipitation of labeled Rh polypeptide with Rh antibody. RBC (rr cells) prelabeled with ^{125}I-PDA or with ^{125}I were vesiculated with DLPC and electrophoresed directly (B and C) or after immunoprecipitation with Rh (anti-c) antibodies (D). (A) shows densitometric scans of Coomassie blue-stained RBC ghosts and DLPC-induced vesicles from panel B (lane 1, ghosts; lane 2, vesicles). Panel C is an autoradiograph of ghosts (lanes 1 and 2) and vesicles (lanes 3 and 4) labeled with ^{125}I-PDA (1,3) or Na^{125}I (2,4). In Panel D, lanes 1 and 2 contain an immunoprecipitate of the same vesicles shown in panel C, lanes 3 and 4, respectively. Arrows mark the positions of molecular weight standards (97.4, 69, 46, 30, 21.5 and 14.3 kDa).

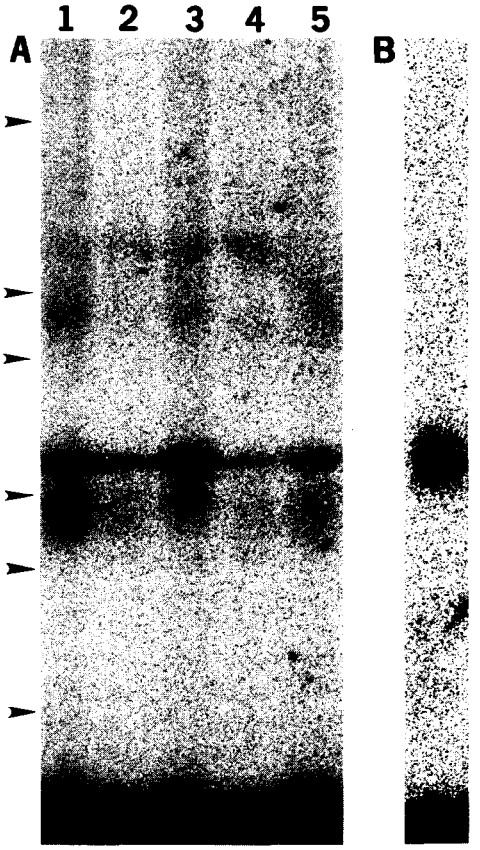

Fig. 6. Analysis of ^{125}I-N_3-lipid labeled vesicles by SDS-PAGE. Control and inhibitor-treated RBC (rr) were vesiculated with DLPC. The purified vesicles were then incubated with ^{125}I-N_3-PS or ^{125}I-N_3-PC in the dark at 37° C for 1 h, irradiated and analyzed by SDS-PAGE (A). Autoradiography of vesicles labeled with ^{125}I-N_3-PS were generated from RBC containing high ATP levels (lane 1), normal ATP levels (lane 3), PDA- (lane 4) and vanadate-treated RBC (lane 5). Lane 2 shows control vesicles incubated with ^{125}I-N_3-PC. (B) shows monoclonal anti-c immunoprecipitated Rh from vesicles generated from ^{125}I-N_3-PS labeled RBC. Arrows mark the positions of molecular weight standards (see Fig. 5).

3.4. Association of Rh polypeptides with the aminophospholipid transporter

The data presented to this point indicate that both Rh polypeptides and the aminophospholipid transporter distribute from the cell into the emerging vesicle upon DLPC-induced vesiculation. It also appears that labeling/inhibition of the aminophospholipid transporter with transport inhibitors did not affect the movement of the transporter into the budding vesicle. This is concluded from data showing that vesicles generated from diamide- or PDA-inhibited RBC can transport PS upon the addition of DTT.

To verify that these inhibitors did not affect the localization of Rh polypeptides in the vesicle membrane, a series of semi-quantitative hemagglutination inhibition experiments were carried out (Table 1). Rh(c) and Rh(D) antibodies were adsorbed with vesicles generated from Rh(c) (rr cells)- and Rh(D)-expressing (R_1R_1 cells) RBC

Table 1
Presence of Rh polypeptides in vesicles derived from inhibitor-treated RBC [a]

Phenotype	RBC treatment	Hemagglutination titer	
		anti-Rh(c)	anti-Rh(D)
	unadsorbed antibody	2^{11}	2^{12}
rr vesicles (cde/cde)	control	2^{7}	2^{12}
	PDA	2^{8}	–
	NEM	2^{7}	–
	diamide	2^{7}	–
R_1R_1 vesicles (DCe/DCe)	control	2^{11}	2^{6}
	PDA	–	2^{6}
	NEM	–	2^{6}
	diamide	–	2^{6}

[a] RBC were treated with the indicated inhibitors as described in Materials and methods. Vesicles were generated from 2 ml of packed inhibitor-treated RBC. The vesicles were purified by sequential centrifugation and pelleted at 30 000 × *g*. Antibody adsorption was done by incubating polyclonal anti-c or anti-D with the vesicles for 1 h at 20° C followed by 30 min at 0° C. Antibody titers were then determined on papain-treated R_1r (DCe/cde) cells which express both the c and D alleles. Hemagglutination is expressed as $\log_2$ titers.

after treatment with the various inhibitors. Preincubation of antibody with DLPC-induced vesicles from control (untreated) RBC resulted in specific antibody adsorption. This can be seen by the approx. 30-fold reduction in titer after adsorption of the antibody with vesicles derived from RBC of the appropriate phenotype (Table 1). Thus, only anti-c was adsorbed by c-expressing rr vesicles (cde/cde) because these vesicles do not express the D allele. Likewise, only anti-D was adsorbed by D-expressing R_1R_1 vesicles (DCe/DCe) because these vesicles do not express the c epitope. Similar reductions in antibody titers were observed after adsorption on DLPC-induced vesicles derived from PDA-, NEM-, or diamide-treated RBC indicating that these inhibitors had no effect on the distribution of Rh polypeptides into the vesicle membrane.

4. Discussion

It is well established that membrane phospholipids of eukaryotic cells are not randomly distributed across the plasma membrane, but certain species and lipids of specific molecular composition are distributed asymmetrically across the membrane bilayer [29]. This is especially evident for the aminophospholipids, which preferentially reside in the plasma membrane's inner leaflet. Although it is still uncertain whether an active process is required to maintain lipid asymmetry, it is clear that an energy-dependent aminophospholipid translocase is responsible for the movement of aminophospholipids across the membrane and generates unequal transmembrane lipid distributions [3,4].

Although considerable progress has been made in understanding the molecular requirements of aminophospholipid movement in RBC, the proteins responsible for transport have not been unequivocally identified. The available data are, however, compatible with at least two proteins. Based on PS and sulfhydryl labeling studies, we have identified a 32 kDa protein that may be part of the Rh complex [2,25]. Using vanadate sensitivity and PS-stimulated ATPase activity, Devaux and colleagues have identified a 120 kDa Mg-ATPase with characteristics compatible to those known for the aminophospholipid translocase [20,21]. Although these proteins are distinct, they are not mutually exclusive; both may be members of a more complex structure that requires the participation of several proteins. Indeed, purification of a 120 kDa candidate aminophospholipid-transporting Mg-ATPase copurified with substantial amounts of 32 kDa band 7 proteins [30].

Because RBC vesicles contain a limited number of red cell proteins yet still transport exogenously supplied PS analogs [23], we used this system as a model to investigate the relationship between PS transport and Rh polypeptides. Although red cells can be induced to vesiculate by a variety of techniques, the protein composition of the vesicle membrane seems to be independent of the mechanism responsible for its formation. SDS-PAGE analysis of membrane-derived vesicles invariably shows an absence of cytoskeletal proteins and varying amounts of other membrane components, including what appear to be disproportionate amounts of proteins in the band 3 and band 7 regions [6,7,31]. The results shown here demonstrate that Rh polypeptides and the aminophospholipid transporter segregate into budding RBC vesicles generated upon incubation of the cells with DLPC. This was concluded from experiments showing that, (1) Rh antibody specifically immunoprecipitated Rh polypeptides from vesicles, (2) vesicles can specifically adsorb Rh antibodies, and (3) vesicles can transport PS. Because the restricted diffusion of cytoskeleton-linked integral membrane proteins prevents their segregation into emerging vesicles [13], these experiments suggest that at least a fraction of cell's Rh polypeptides and aminophospholipid transporter are not associated with the membrane cytoskeleton.

PS transport was ATP- and temperature-dependent, and dependent on the oxidative state of membrane sulfhydryls

in the initial red cell population as well as in the membrane vesicles. This was concluded from the experiments shown in Fig. 3, in which remnant RBC and vesicles generated from inhibitor-treated or ATP-depleted red cells did not transport PS. Similarly, transport was blocked when vesicles derived from control RBC were treated with the same inhibitors (Fig. 4). In both cases, inhibition by the disulfide exchange reagents was reversible upon reduction with DTT (Figs. 3 and 4).

The data presented in Fig. 3 and Table 1 show that there is a direct relationship between the localization of Rh polypeptides in the vesicle membrane and the ability of the vesicles to transport PS. For example, while vesicles derived from PDA- and diamide-treated RBC did not transport PS, the inhibited transporter did localize in the vesicles because transport inhibition was reversed with DTT (Fig. 3). To determine whether the inhibitor treatments affected the movement of Rh polypeptides into the vesicles, Rh protein was semi-quantified by antibody adsorption assay (Table 1). Data from these experiments suggest that similar amounts of Rh polypeptides were present in all the tested vesicles. It should be noted that the reduction in the ability of vesicles derived from PDA-treated RBC to adsorb Rh(c) but not Rh(D) antibodies (see Table 1) was a consistent finding. Although this could indicate that these vesicles contain less Rh polypeptide, related studies have shown that PDA treatment specifically reduces Rh(c) antigenicity (unpublished observations) similar to the known effects of certain sulfhydryl reagents on Rh(D) antigenicity [32,33]. In conclusion, the combined SDS-PAGE analysis and immunochemical and functional data indicate that Rh polypeptides and the aminophospholipid transporter colocalize in RBC vesicles.

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