Physical Determinants of Intermembrane Protein Transfer†

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ABSTRACT: Intermembrane protein transfer between erythrocytes and phospholipid vesicles was examined under a variety of conditions to investigate physical factors governing this process. Human erythrocytes were incubated with sonicated dimyristoylphosphatidylcholine vesicles containing trace [^{14}C]dipalmitoylphosphatidylcholine. Protein−vesicle complexes were separated from cells and from membrane fragments by density gradient centrifugation. The yield of isolated protein vesicles was determined from the ^{14}C-vesicle marker; protein compositions were analyzed by SDS−polyacrylamide gel electrophoresis. Enzymatic removal of portions of the cytoplasmic or exoplasmic domains of cell membrane proteins had little effect on the extent of protein transfer. Membrane additives such as cholate produced a 2-fold increase in protein−vesicle yield. The selectivity of protein transfer from erythrocytes was influenced by the lipid composition of recipient vesicles: inclusion of cholesterol increased band 3 content while the presence of anionic phospholipids reduced transfer. Proteins transferred from ^{32}P-labeled cells differed in specific radioactivity from bulk cell proteins: glycophorin, highly phosphorylated in the cell membrane, showed no detectable labeling in the corresponding protein−vesicle band. These observations suggest that cell-to-vesicle protein transfer is insensitive to bulk steric and electrostatic properties of cell membranes, but enhanced by membrane defects. Recipient membrane composition influences the selectivity of transferred proteins and may reveal subtle differences in the membrane association of protein subpopulations.

Integral membrane proteins of erythrocytes transfer to phospholipid vesicles during cell−vesicle incubations (Bouma et al., 1977; Cook et al., 1980). The protein−vesicle complexes formed consist of unilamellar vesicles containing transmembrane proteins, including the anion transporter band 3 (Newton et al., 1983; Huestis & Newton, 1986; Sato et al., 1990). Spontaneous movement of integral proteins between membrane bilayers is an unexpected finding, since such a process is inconsistent with maintenance of stable cell membrane composition. However, diverse membrane proteins undergo spontaneous intermembrane transfer. Cell-to-cell transfer of the epidermal growth factor receptor has been observed in the absence of fusogenic agents (Bishayee et al., 1982), and extraction of proteins from cells into phospholipid vesicles has been reported for spleen cells (Dunnick et al., 1976) and for adipocytes (Hallet & Campbell, 1980). Cytochrome b_5 transfers from vesicle to vesicle (Enoch et al., 1977; Roseman et al., 1977) and from cell to cell (George et al., 1991). Plasma membrane proteins transfer to vesicles from murine lymphoma cells (BL/VL3) (Newton & Huestis, 1988a) and from human platelets (Okumura et al., 1994). Trypanosome variant surface glycoproteins, which are anchored to the membrane by phosphatidylinositol containing glycolipid anchors, transfer spontaneously to erythrocytes (Rifkin & Landsberger, 1990). Tumor surface antigenic proteins transfer directly from leukemia cells to vesicles, providing an effective liposomal vaccine (Shibata et al., 1991). Additionally, decay-accelerating factor is reported to be extracted from erythrocytes by DMPC vesicles (Walter et al., 1992).

Transfer of band 3 from erythrocytes to dimyristoylphosphatidylcholine (DMPC)[1] vesicles provides a useful system for investigation of the phenomenon of intermembrane protein transfer, since details of band 3 structure and several characteristics of band 3−vesicle complexes are known. Band 3, the anion transporter responsible for chloride−bicarbonate exchange, is the major transmembrane protein in the erythrocyte. Analysis of its cDNA sequence predicts that the polypeptide chain traverses the membrane bilayer 14 times (Kopito & Lodish, 1985). The N-terminus of band 3 includes an elongated cytoplasmic domain (Low, 1986). Band 3 is functional in protein−vesicle complexes, exhibiting DIDS-inhibitable chloride transport (Newton et al., 1983). Proteolytic studies showed that band 3 is inserted into vesicles in native orientation, with its cytoplasmic domain in contact with the contents of the vesicle lumen (Huestis & Newton, 1986). These studies also suggest that band 3 transfer occurs without cell−vesicle fusion and without exposure of the cytoplasmic tail to the bulk aqueous phase. Further, proteoliposomes formed by intermembrane protein transfer are capable of delivering proteins into cells. Functional erythrocyte anion transporters were transferred from protein vesicles into erythrocytes whose anion transporters had been inhibited, as well as into lymphocytes, which have little native anion transport capability (Newton et al., 1983;

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[1] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DPG, 2,3-diphosphoglycerate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; bbPS, bovine brain phosphatidylserine.

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Newton & Huestis, 1988b). A working model was proposed (Huestis & Newton, 1986) in which the hydrophobic core of the protein moves from the cell into the vesicle during close apposition of their membranes.

In this investigation, we modify several properties of the erythrocyte and the recipient vesicle membranes and examine any changes in transfer of band 3. The effects of (1) removal of electrostatic and steric barriers on the exoplasmic cell surface, (2) alterations to band 3 to ease its passage through hydrophobic membrane domains, (3) perturbation of the erythrocyte cytoskeleton to which band 3 is partially attached, (4) creation of lipid bilayer packing defects using cholate, and (5) alteration of recipient vesicle membrane composition were examined. Results suggest that transfer of band 3 is insensitive to bulk steric and electrostatic properties of the erythrocyte plasma membrane. Enhanced transfer is achieved upon disruption of cell membrane organization, and specificity is dependent on recipient membrane composition.

EXPERIMENTAL PROCEDURES

Materials. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylglycerol (DPPG), bovine brain phosphatidylserine (bbPS), cholesterol, Pronase E (protease type XXV from *Streptomyces griseus*), trypsin, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]DPPC was purchased from Amersham Radiochemicals (Arlington Heights, IL). [^{32}P]H_3PO_4 (carrier free) was purchased from New England Nuclear (Wilmington, DE), and Na^{125}I was from ICN (Irvine, CA). IODO-GEN was obtained from Pierce Chemical Co. (Rockford, IL). The ionophore A23187 was purchased from Calbiochem (La Jolla, CA). All other chemicals were reagent grade.

Cells. Human blood was obtained by venipuncture from healthy adult volunteers and collected in 15 mM citrate anticoagulant. Erythrocytes were pelleted by centrifugation (3000*g*, 5 min) and washed 3 times with 5 volumes of 150 mM NaCl and once with 5 volumes of phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4). Washed cells were stored at 4 °C in PBS and used within 24 h.

Radiolabeling of Cells. For some experiments, cells were prelabeled by phosphorylation or radioiodination to facilitate quantification of transferred protein. In experiments where no direct protein modification was employed, or where the exofacial domains of proteins were to be cleaved, proteins were labeled in cytoplasmic domains by phosphorylation. Phosphorylated cells were prepared by incubation for 6 h at 37 °C with 0.25−1.0 mCi of ^{32}P/mL of cells, in phosphate-free buffer (TBS: 20 mM Tris-HCl, 150 mM NaCl, and 5 mM glucose, pH 7.4) supplemented with 10 mM inosine, 10 mM glucose, and 1 mM adenosine. Cells were isolated by centrifugation and washed 4 times in 50 volumes of TBS. For experiments where the cytofacial domains of proteins were to be cleaved, cells were radiolabeled by surface iodination, as described previously (Huestis & Newton, 1986). Briefly, Na^{125}I (100 μCi in 10 μL) was added to an IODO-GEN-coated vial (150 μg) containing 2 mL of cells at 50% hematocrit. The vial was kept at room temperature for 15 min, with occasional swirling. Cells were washed 4 times in 50 volumes of 1% bovine serum albumin in PBS, followed by three washes in 50 volumes of PBS to remove the bovine serum albumin.

Vesicles. Small unilamellar vesicles were prepared by suspending dry lipids in PBS and sonicating to clarity using a bath sonicator. Lipids in chloroform/methanol solution were dried using a nitrogen stream before suspension in buffer. Sonication was done at temperatures above the phase transition temperature of the lipid being used. As determined by transmission electron microscopic examination, this procedure yielded largely monodisperse small unilamellar vesicles averaging 20 ± 3 nm in diameter (Waters, 1993). Sonicated vesicle compositions were 24 mM DMPC, 24 mM DMPC plus 0.8 mM DPPG, 21.6 mM DMPC with 2.4 mM bovine brain phosphatidylserine, or 21.6 mM DMPC with 2.4 mM cholesterol. Vesicles of these lipid compositions have been shown to be closely similar in size (Zucker et al., 1994). Anionic lipid molecules have been demonstrated to be present in the outer monolayer of cosonicated phosphatidylcholine and phosphatidylglycerol vesicles (Michaelson et al., 1973). Consistent with this report, line widths of lipid peaks increased in the presence of Mn^{2+} in ^{31}P NMR spectra of cosonicated DMPC and DPPG vesicles (data not shown). [^{14}C]DPPC was added as vesicle marker at concentrations of <1 μCi mL^{-1}.

Pronase Treatment of Erythrocytes. Intact cells were pretreated in the presence and absence of 25 μg/mL Pronase in PBS at 25% hematocrit for 30 min at 37 °C. Proteolysis was stopped by the addition of 0.25 mg/mL phenylmethanesulfonyl fluoride from ethanol stock (final ethanol concentration was less than 1%). Cells were then washed 3 times in PBS containing 15 mM EDTA.

Elevation of Intracellular 2,3-Diphosphoglycerate Levels. Erythrocytes were incubated in buffer containing 10 mM inosine, 10 mM sodium pyruvate, 50 mM Na_2HPO_4, and 75 mM NaCl, pH 7.4, for 60 min at 37 °C. This resulted in a (5.0 ± 0.8)-fold ($n = 3$) increase in DPG concentration as measured using a DPG assay kit from Sigma Chemical Co. This is consistent with previous reports (Deuticke et al., 1971). Cells were washed in PBS before incubation with vesicles.

Cell to Vesicle Protein Transfer. Vesicles (concentrations as noted) were incubated with cells at 50% hematocrit, for 60 min, at 37 °C. In the indicated experiments, 100 μM sodium cholate was added to the incubation. Cell−vesicle suspensions were centrifuged at 3000*g* for 5 min at 25 °C to pellet erythrocytes. If necessary, a second spin was done to ensure complete removal of cells. Linear density gradients were prepared by freezing 4 mL aliquots of 18% (w/v) solutions of sucrose in PBS, and thawing them at 5 or 25 °C (Baxter-Gabbard, 1972). Supernatants were layered onto the gradients, which were centrifuged at 200000*g* for 2 h at 5 °C. Under these conditions, cell membrane fragments migrate to the bottom of the tube, protein−vesicles form a band at an intermediate density, and unaltered vesicles and cytosolic proteins remain near the top of the gradient. Fractions (200 μL) were collected from the top of the gradient using a manual pipettor, or from the bottom after tube puncture, and assayed for the vesicle marker, [^{14}C]-DPPC, by liquid scintillation counting. The fractions containing protein−vesicles were collected, diluted with PBS, and centrifuged for 15−30 min at 200000*g*. The washed protein−vesicle pellets were resuspended in a small volume of PBS.

Cell Membrane Fragments. Cell membrane fragments were generated by three different methods.

(1) Trypsin-Induced Cell Membrane Fragments. Packed radioiodinated cells were lysed in an equal volume of 5 mM sodium phosphate buffer, pH 8.0, in the presence of 60 μg/mL trypsin. Proteolysis was allowed to proceed at 37 °C for 15 min, before addition of 3 mg/mL soybean trypsin inhibitor. The lysed cell suspension was diluted in 10 volumes of PBS and then centrifuged at 5000*g* for 10 min to pellet the cell membrane fragments. The pellet was washed 3 times in 10 volumes of PBS before use in protein transfer experiments. Cleavage of band 3 was confirmed by PAGE analysis of membrane fragments, which were found to contain the 55 kDa transmembrane domain of band 3 (Lepke & Passow, 1976).

(2) Calcium-Induced Cell Membrane Fragments. Cell membrane fragments containing intact band 3 were prepared by incubation of radioiodinated cells with 0.15 mM Ca^{2+} and 0.01 mM A23187 in phosphate-free buffer (20 mM HEPES, 145 mM NaCl, pH 7.4) at 37 °C for 1 h (Ferrell & Huestis, 1982). Cells were pelleted by centrifugation (3000*g*, 5 min), and membrane fragments were isolated by centrifugation of cell-free supernatants at 30000*g* for 30 min. In some experiments, cell membrane fragments were made from unlabeled cells using an identical protocol; fragment concentrations in these preparations were determined by cholesterol assay (Zlatkis et al., 1953).

(3) DMPC-Induced Cell Membrane Fragments (Buds). Packed ^{32}P-labeled cells were incubated with 24 mM DMPC at hematocrit 50% for 60 min at 37 °C. Cell−vesicle suspensions were centrifuged at 3000*g* for 5 min at 25 °C to remove erythrocytes, and the resulting supernatant was fractionated on a linear sucrose density gradient as described above. Membrane buds were isolated from the bottom of the gradient, diluted in PBS, and centrifuged for 15−30 min at 49000*g*. The washed membrane buds were resuspended in a small volume of PBS and subsequently subjected to SDS−PAGE, autoradiography, and densitometric analysis as described below.

Protein Transfer from Cell Membrane Fragments to DMPC Vesicles. Radioiodinated cell membrane fragment preparations containing either intact or trypsin-cleaved band 3 were incubated with DMPC vesicles for 90 min at 37 °C. The entire suspension was layered onto 14% (w/v) sucrose gradients. Gradient centrifugation, fractionation, and protein−vesicle isolation were carried out as described for cell to vesicle protein transfer.

Preparation of Cell Membranes. Erythrocyte membranes (stroma) were prepared by the method of Sheetz and Singer (1977). Cells were lysed in 20 volumes of ice-cold 5 mM sodium phosphate buffer, pH 8.0, for 10 min. The membranes were then pelleted at 20000*g* for 10 min.

SDS−Polyacrylamide Gel Electrophoresis. The protein composition of protein−vesicles, membrane fragments, and stroma was examined by PAGE. Samples were prepared for electrophoresis by adding 1 part dye buffer (3.03% Tris, 12.6% SDS, 31.5% glycerol, 0.007% bromophenol blue, and 5% β-mercaptoethanol, pH 6.8) to 2 parts sample and heating for 2 min at 100 °C. Protein components were resolved on 10% acrylamide or 12.5% acrylamide gels (Ames, 1974), and proteins were visualized by silver staining (Merril et al., 1980). ^{32}P- or ^{125}I-labeled proteins were detected by autoradiography at −70 °C, using Kodak X-Omat film with DuPont Cronex intensifying screens. Protein staining and incorporation of radiolabel were quantified by densitometric analysis of PAGE gels or autoradiograms.

Analysis of Lipid Yield in Protein Transfer Experiments. The nontransferable lipid label [^{14}C]DPPC was used to monitor vesicle location on sucrose gradients (Figure 3). The fraction of label in the protein−vesicle band (intermediate density region of the gradient) was used to determine protein−vesicle yield. Results are reported as mean ± standard deviation with n = number of experiments.

Analysis of Pronase-Digested Protein Bands. Densitometric analysis of band 3 transfer was complicated by its cleavage, since staining of the native heterogeneously glycosylated protein was different from that of its 60 kDa fragment (consisting of some of the transmembrane helices and the cytoplasmic tail). The concentration and staining of spectrin should be unaffected by Pronase treatment of intact cells; thus, spectrin was used to standardize densitometric scans of control and Pronase stroma lanes with respect to the amount of material loaded. Using spectrin-standardized stroma lanes, the relative staining of band 3 and its Pronase fragment was determined. On silver-stained gels, the band 3-to-Pronase fragment staining ratio was 1.54 ± 0.57 (n = 8); the variability may be due to donor glycosylation differences. To determine relative transfer of band 3 and its Pronase fragment, protein−vesicle gel lanes were integrated, standardized such that vesicles from equal cell−vesicle incubation volumes were compared, and corrected for background and for relative staining intensities.

RESULTS

Cells and vesicles were subjected to a panel of enzymatic and chemical treatments designed to alter their physical properties. The effects of these treatments on subsequent cell-to-vesicle protein transfer were evaluated by two criteria: the yield of lipid recovered in the proteoliposome complexes (protein vesicle yield) and the band 3 or band 3 fragment content of those complexes. The results are summarized in Table 1.

Electrostatic and Steric Alterations of the Erythrocyte Membrane. Pronase treatment was used to remove the bulk of exposed protein from the exofacial surface of erythrocytes. This procedure cleaved band 3 into a monodisperse 60 kDa band (consisting of some of the transmembrane helices and the cytoplasmic tail) and a 35 kDa glycosylated fragment; the smaller fragment was lost to further proteolysis under the conditions employed (Cabantchik & Rothstein, 1974). This treatment effects a significant change in both the charge and steric blockage of the cell membrane and increases the hydrophobicity of the cell surface (Darmani & Coakley, 1991). Pronase treatment did not alter the yield of protein vesicles significantly upon subsequent incubation of cells with sonicated DMPC vesicles (Table 1). Proteins recovered in protein vesicles were analyzed by PAGE (Figure 1). Transfer of the 60 kDa Pronase fragment of band 3 was equal to or slightly less than that of intact band 3. Densitometric analysis of silver-stained gels revealed that 83 ± 39 (n = 7) band 3 fragments transferred for every 100 intact band 3 molecules (Table 1). These results indicate that removal of protein and glycoprotein components from the exofacial surface of the cell membrane does not alter band 3 transfer.

Electrostatic Alterations of the Recipient Membrane. Close apposition between the negatively charged erythrocyte

Table 1: Protein−Vesicle Yields from Modified Membrane Incubations

modification	protein−vesicle lipid yield[a] (%)		ratio (treated/ control)	protein−vesicle band 3 yield, ratio (treated/ control)
	treated cells	control cells		
Pronase treatment: reduction in electrostatic and steric barriers on cell surface	18.0 ± 7.3 (n = 25)[b]	19.4 ± 10.2 (n = 15)	0.93	0.83 ± 0.39 (n = 7)
anionic recipient vesicles: addition of DPPG to vesicle membrane	21.2 ± 7.2 (n = 11)	18.3 ± 3.6 (n = 11)	1.16	0.85 ± 0.29 (n = 4)
trypsinolysis: removal of cytoplasmic domain of band 3	16.4 ± 9.3 (n = 8)[c]	14.9 ± 8.7 (n = 8)[d]	1.10	1.05 ± 0.08 (n = 3)
elevation of 2,3-DPG: disruption of membrane cytoskeleton	18.5 ± 4,9 (n = 3)	18.8 ± 4.7 (n = 3)	0.98	1.07 ± 0.14 (n = 2)
cholate addition: formation of membrane defects	44.6 ± 11.2 (n = 2)	21.9 ± 4.1 (n = 2)	2.04	2.33 ± 0.14 (n = 2)

[a] As percent of total vesicle lipid. [b] Mean ± sd; n = number of experiments. [c] Membrane fragments with cleaved band 3. [d] Calcium ionophore-induced membrane fragments with intact band 3.

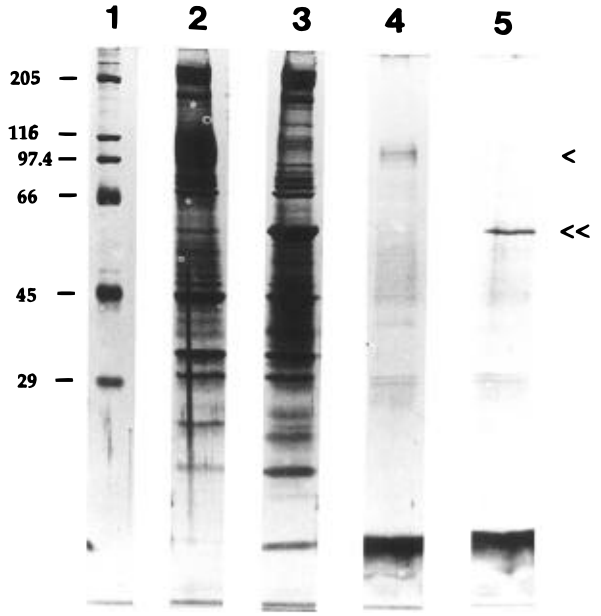

FIGURE 1: SDS−polyacrylamide gel (7.5%, silver-stained) of protein vesicles from Pronase-treated and control erythrocytes. Lane 1, molecular mass standards (in kDa); lanes 2 and 3, cell membranes from control and Pronase-pretreated erythrocytes, respectively; lane 4, protein vesicles from control erythrocytes; lane 5, protein vesicles from Pronase-treated erythrocytes. Positions of band 3 (<) and band 3 fragment following Pronase treatment (<<) are marked.

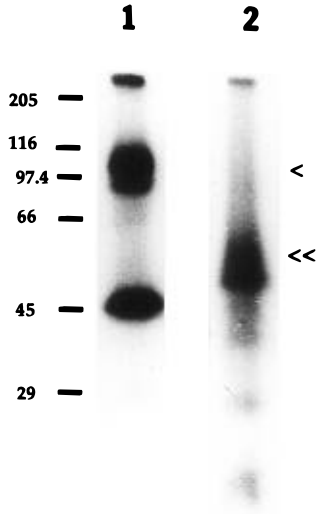

FIGURE 2: Autoradiogram of protein vesicles from radioiodinated cell membrane fragments. Lane 1, proteins transferred from cell membrane fragments with intact band 3; lane 2, proteins transferred from cell membrane fragments after cleavage of the cytoplasmic domain of band 3. Positions of intact band 3 (<) and band 3 following cleavage of the cytoplasmic domain (<<) are indicated.

cell surface and vesicle bilayers would be expected to be inhibited if the vesicles were negatively charged. Recipient membranes were prepared containing a small amount of a negatively charged lipid (3 mol % DPPG/97 mol % DMPC). This alteration had little effect on protein−vesicle yield (Table 1), and the ratio of band 3 content in protein vesicles from DPPG/DMPC samples relative to control was 0.85 ± 0.29 (n = 4) (Table 1).

Removal of the Cytoplasmic Domain of Band 3. Trypsin treatment of lysed erythrocytes cleaves off the cytoplasmic N-terminal domain of band 3 and hydrolyzes membrane skeletal proteins, resulting in vesiculation of the membrane (Shields et al., 1987). The cytoplasmic N-terminus of band 3 is a hydrophilic domain whose passage through a phospholipid bilayer would present a thermodynamic barrier to protein transfer. The effect of removal of this domain on band 3 transfer to vesicles was examined, using the trypsin-induced membrane fragments as donor membranes. (Membrane fragments used in these experiments were derived from radioiodinated cells to facilitate protein quantification.) Incubation of these plasma membrane fragments with DMPC vesicles yielded protein−vesicle complexes containing the 55 kDa band 3 transmembrane domain. Transfer from trypsin membrane fragments was compared with transfer from membrane fragments generated by calcium loading; these species contain intact band 3. Protein−vesicle yield was similar from the cell membrane fragments containing intact band 3 and from membrane fragments with the cytoplasmic domain of band 3 removed (Table 1). The protein content of the protein−vesicle complexes was analyzed by autoradiography (Figure 2). Surface-iodinated proteins of erythrocytes include band 3 (broad band at ∼100 kDa), PAS 1 (80 kDa), and PAS 2 (38 kDa) (Thompson et al., 1980). Densitometric analysis revealed that transfer of band 3 with its cytoplasmic domain removed (lane 2) was similar to transfer of intact band 3 (lane 1). The ratio of the integrated intensities of the intact (∼100 kDa) versus cleaved (55 kDa) band 3 was 1.05 ± 0.08 (Table 1), suggesting that the cytoplasmic domain of band 3 does not present a significant barrier to band 3 transfer into recipient DMPC vesicles.

Elevation of Intracellular 2,3-Diphosphoglycerate Levels. Linkages between the erythrocyte membrane and its skeleton are affected by intracellular DPG (Moriyama et al., 1993). At higher than normal levels, DPG increases the lateral diffusion of integral membrane proteins, dissociates spectrin−actin−band 4.1 complexes, and decreases the number of attachments between the skeleton and the membrane (Schindler et al., 1980; Sheetz & Casaly, 1980). The impact of such disruption of the erythrocyte−cytoskeletal protein interactions on intermembrane protein transfer was studied in erythrocytes with elevated DPG levels. No difference in protein−vesicle yields was found from cells with DPG levels elevated 5-fold compared to control cells. Densitometric analysis of silver-stained gels showed no changes in the protein content of protein−vesicle complexes (Table 1).

Effect of Membrane Defects. Cell-to-vesicle protein transfer was examined in the presence of an additive that creates membrane defects. Cholate and related bile salts, when used in amounts below their critical micelle concentrations (e.g., <1 mM), bind to vesicles and destabilize the packing of membrane lipids (Nichols, 1986). Addition of 100 μM sodium cholate to cell−vesicle incubations produced a significant increase in both lipid (Figure 3A) and protein (Figure 3B) yield in protein vesicles: protein−vesicle yields were 2-fold higher from cholate-treated cells (Table 1). The ratio of integrated intensities of transferred band 3 from cholate-treated versus control cells was 2.33 ± 0.14 (n = 2).

Vesicle Composition and Selectivity of Protein Transfer. Varying the lipid composition of recipient vesicles had a significant effect on the transfer of specific proteins (Table 2). The three principal phosphorylated proteins transferred were band 3, band 4.5, and the band at 43 kDa. Protein vesicles formed using DMPC vesicles containing bovine brain phosphatidylserine (10 mol % bbPS, 90 mol % DMPC) were found to be enriched in phosphorylated band 4.5 and depleted in phosphorylated band 3, relative to those obtained with pure DMPC vesicles. In contrast, recipient vesicles containing cholesterol (10 mol % in DMPC) were enriched in phosphorylated band 3 and depleted in phosphorylated band 4.5, relative to pure DMPC vesicles.

Subpopulations of Proteins in Protein−Vesicles. The phosphorylated protein populations of protein vesicles derived from phosphorylated cells were significantly different from the populations found in either stroma or DMPC-induced membrane buds (Figure 4). Glycophorin (80 kDa) and band 4.5 (broad band ~55 kDa) were phosphorylated to different extents in protein vesicles and in membrane bud fractions; glycophorin present in protein vesicles was not phosphorylated detectably. Relative to protein staining, phosphorylated material in the band 4.5 region of protein vesicles was more heavily radiolabeled than that in either erythrocyte stroma or buds.

DISCUSSION

Plasma membrane proteins transfer from erythrocytes into phospholipid vesicle bilayers during cell−vesicle incubations. Other events accompany intermembrane protein transfer: DMPC monomers transfer from vesicles to cells, intercalate in the outer monolayer of the plasma membrane, and convert discoid cells to echinocytes (Ferrell et al., 1985; Margolis, 1984). Consequently, cells being incubated with DMPC vesicle eventually transform to spherocytes with loss of sealed membrane fragments. Density gradient fractionation of cell-free supernatants allows separation of transferred protein-containing vesicles from membrane fragments and

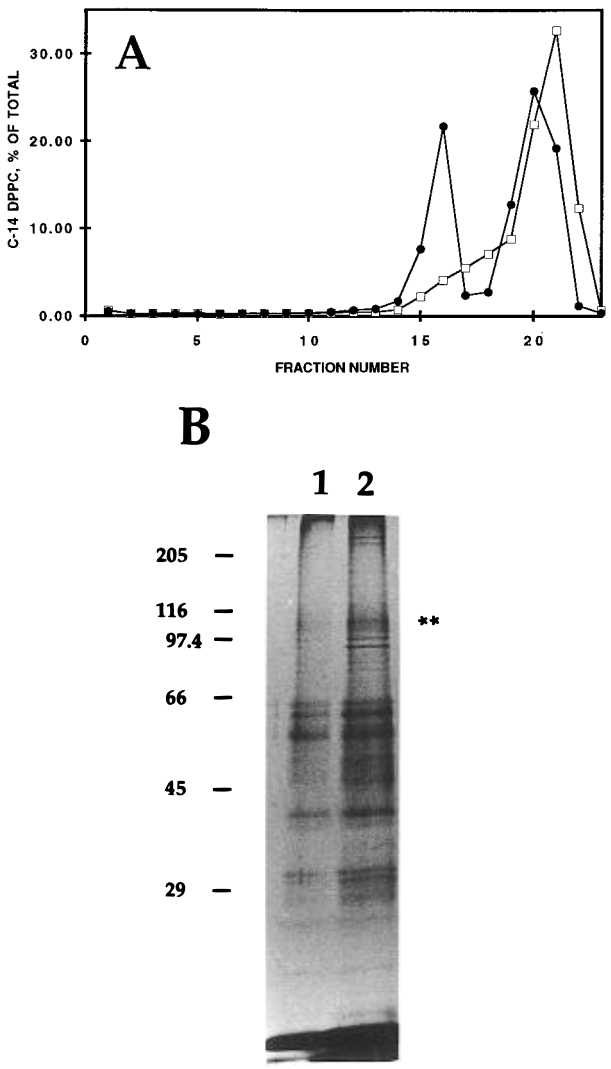

FIGURE 3: Intermembrane protein transfer in the presence and absence of cholate. (A) Sucrose density gradient profiles for the cell-free supernatants from erythrocytes incubated with DMPC vesicles in the presence (circles) or absence (squares) of 100 μM sodium cholate. (B) SDS−polyacrylamide gel (silver-stained) of protein vesicles. Lane 1, protein vesicles from a cholate-free incubation; lane 2, protein vesicles formed in the presence of cholate. Position of transferred band 3 is marked (**). Molecular masses (in kDa) are indicated on the left.

Table 2: Effect of Recipient Vesicle Composition on Intermembrane Transfer of Phosphorylated Proteins

	vesicle lipid[a] (%)		
protein	DMPC	DMPC/bbPS	DMPC/cholesterol
band 3	22.7 ± 1.8	17.6 ± 1.2	33.1 ± 1.3
band 4.5	49.3 ± 0.6	56.5 ± 1.7	35.0 ± 3.2
band at 43 kDa	28.0 ± 1.3	26.0 ± 2.7	32.0 ± 3.3

[a] Values presented represent percentage of phosphorylated proteins present in samples of protein vesicles derived from incubation of phosphorylated erythrocytes with liposomes of varying lipid composition as described under Experimental Procedures. Replicate samples were subjected to SDS−PAGE and subsequent autoradiography on separate gels. The autoradiograms were scanned using a high-resolution flat-bed scanner and Adobe Photoshop (version 2.5.1) software. Scans were analyzed using NIH Image (version 1.52) gel scanning software. Errors represent the standard deviation of the data.

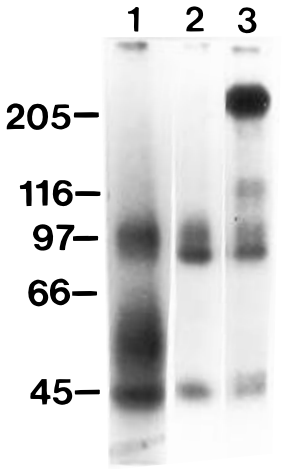

FIGURE 4: Autoradiogram of phosphorylated proteins in protein vesicles (lane 1), membrane buds (lane 2), and stroma (lane 3). Positions of molecular mass markers (in kDa) are indicated on the left.

other constituents of the incubation mixture. The present study examines various properties of erythrocyte and vesicle membranes that affect the nature and extent of intermembrane protein transfer.

Modification of Electrostatic or Steric Properties. Neither Pronase cleavage of erythrocyte surface constituents nor inclusion of anionic lipid in the recipient vesicle membrane alters protein−vesicle yield (Table 1), suggesting that steric and electrostatic factors do not limit intermembrane transfer. In a similar finding, removal of sialic acid groups from the plasma membrane of erythrocytes did not influence transfer of trypanosome surface glycoprotein to erythrocytes (Rifkin & Landsberger, 1990).

Removal of the Hydrophilic Cytoplasmic Domain of Band 3. A working model (Huestis & Newton, 1986) of intermembrane transfer requires passage of the cytoplasmic tail of band 3 through hydrophobic membrane domains, first as it leaves the erythrocyte and again as it inserts into the vesicle membrane in native orientation. Removal of the hydrophilic cytoplasmic domain of band 3 would be expected to facilitate this process, yet proteolytic cleavage of the tail does not alter the yield of transferred band 3 (Table 1). It is conceivable that increased penetration by water molecules into highly curved lipid bilayers reduces the hydrophobicity of membrane domains. This characteristic has been implicated in the 200-fold preference of cytochrome b_5 for small unilamellar vesicles over larger vesicles (Greenhut et al., 1986). These authors conclude that looser surface packing of lipids in a highly curved domain leads to deeper water penetration in small vesicles. In the current study, both participant membranes (small unilamellar vesicles and spiculate erythrocytes) possess regions of high surface curvature. Enhanced water penetration into their hydrocarbon domains may ease passage of hydrophilic segments of integral membrane proteins.

Involvement of Membrane Protein Subpopulations in Protein Transfer. Native band 3 interacts with the erythrocyte cytoskeleton; however, cytoskeletal dissociation induced by elevated levels of DPG does not affect transfer of this protein. This result is consistent with transfer of a population of band 3 not attached to the membrane cytoskeleton. Echinocytic spicules are also free of cytoskeletal constraints, since the cytoskeleton does not follow the membrane out to the tips of spicules during membrane vesiculation (Liu et al., 1989). Approximately 40% of total cell band 3 is found in microvesicles released during amphipath treatment of erythrocytes (Hagelberg & Allan, 1990). It is possible that this subpopulation of band 3 in echinocytic spikes transfers more readily.

The existence of a band 3 subpopulation susceptible to transfer is consistent with the differences in radiolabeling of transferred proteins, as compared with labeling of bulk cell protein (Figure 4). Heterogeneity in band 3 and glycophorin A populations has been reported by several workers. Experiments with stroma have shown that the subset of band 3 that binds to Con A−Sepharose is not phosphorylated (Roses, 1976). In addition, only a small percentage of band 3 is readily phosphorylated (Boivin, 1988). Band 3 heterogeneity was also suggested by phase partitioning studies in Triton X-114 (Swanson et al., 1988). The functional significance of band 3 heterogeneity is unclear, due to the difficulty of discriminating between different subsets of the protein in intact cells.

Varying Lipid Composition of Recipient Vesicles. The lipid composition of recipient vesicles influences the transferred protein population: inclusion of anionic phospholipid reduces the percentage of radiolabeled band 3 in protein vesicles, while inclusion of cholesterol increases radiolabeled band 3 transfer relative to pure DMPC vesicles (Table 2). These results may reflect thermodynamic preferences of transferred proteins. The activity and thermal stability of human erythrocyte band 3 have been shown to vary with its lipid environment (Maneri & Low, 1988; Kohne et al., 1983), showing most stability in lipid bilayers formed from 24 acyl carbon lipids. Also, lipids that copurify with band 3 are found to be enriched in long-chain fatty acids, suggesting an in vivo preference for long-chain lipids (Manieri & Low, 1989). Lipid headgroups also affect the stability of reconstituted band 3; the protein is much less stable in the anionic lipids phosphatidylserine (PS) or phosphatidylglycerol (PG) than is protein reconstituted into neutral lipids. Cholesterol, which thickens the bilayer (Nezil & Bloom, 1992), has been shown to increase band 3 stability. The protein transfer results suggest that band 3 is less stable in DMPC vesicles than it is in the cell membrane. An artificial lipid with increased hydrogen bonding capability, dimyristoylamidodeoxyphosphatidylcholine, has been reported to increase protein transfer (Sunamoto et al., 1990). These results suggest that the selectivity of protein transfer may be modulated by appropriate manipulation of recipient vesicle composition.

Disruption of Lipid Packing Using a Membrane Additive. The presence of cholate in cell−vesicle incubations causes a significant enhancement in intermembrane protein transfer (Table 1). This is consistent with reports that spontaneous insertion of purified integral membrane proteins into vesicle membranes is mediated by agents that create membrane packing defects (Jain & Zakim, 1987). Intercalation of DMPC monomers provides an additional source of packing defects in the erythrocyte membrane. Bilayer destabilization by soluble, easily transferred lipids may explain the observation of intermembrane transfer with DMPC and not longer chain lipids (Cook et al., 1980).

CONCLUSION

These studies and previously published reports suggest that cell-to-DMPC vesicle protein transfer is induced by perturba-

tion of the lipid environment of band 3 as DMPC monomers insert into the plasma membrane. The resulting morphological alterations produce regions of high membrane curvature with altered lipid packing and increased penetration of the lipid bilayer by water molecules. Membrane perturbants such as cholate influence the distribution of band 3 between different lipid environments by altering lipid packing. Varying the recipient membrane lipid composition has some effect on selectivity of protein transfer. While DMPC promotes protein transfer by perturbing the donor membrane, other phospholipids may be better choices for improving band 3 yield. The process of intermembrane protein transfer is relatively insensitive to specific characteristics of donor cells as well as transferred proteins, resulting in general utility of this process for a variety of cells.

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