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Oriented reconstitution of red cell membrane proteins and assessment of their transmembrane disposition by immunoquenching of fluorescence

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The two major membrane glycoproteins of human red cells, glycophorin and band 3, the anion exchange protein, were isolated from cells exofacially labeled with fluorescein and reconstituted into vesicles with defined transmembrane disposition. Uniform orientation of polypeptides was accomplished by two procedures: (i) Vesicles with single protein units were obtained by a one-step dilution of a protein/detergent suspension with a vast excess of phospholipid. Vesicles with uniform orientation of protein were selected by affinity chromatography on derivatized Sepharoses (organomercurial, wheat germ agglutinin, aminoethyl or diethylaminoethyl). (ii) Vesicles with multiple protein units with uniform orientation were generated by vectorial immobilization of detergent solubilized proteins on the above affinity matrices and in situ formation of proteoliposomes by detergent substitution for phospholipid. The proteoliposomes were released from the column by addition of excess free ligand. The orientation of band 3 and glycophorin in the reconstituted vesicles was first assessed by immunofluorescence quenching, using anti-fluorescein antibodies, to quantitatively quench fluorescein residues exposed on the outer surface of vesicles. Further assessment was achieved by chromatographing the vesicles through various affinity and ionic matrices. Vesicle populations of higher than 90% homogeneity in protein orientation (right-side-out or inside-out) were obtained with both procedures. The above methods provide a convenient experimental tool for the oriented reconstitution of proteins and the evaluation of their transmembrane disposition.

Introduction

Reconstitution of integral membrane proteins into artificial vesicles has provided one of the most useful means for studying their structure-function relationship and to a definitive identification of transport proteins [1–4]. An integral part of the reconstitution procedure is the solubilization of the membrane with a mild detergent, occasionally a selective step which is followed by partial purification of proteins with standard procedures and finally by incorporation of the proteins back into a membranous structure (e.g., lipid vesicle) by substitution of detergent for lipids. By its very nature, the procedure leads asymmetric proteins to orient

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Abbreviations: GPH, glycophorin; AEP, anion exchange protein; OSDS, one-step dilution in suspension; GDM, gradual dilution on matrix; F, fluorescein, CF, 5(6)-carboxyfluorescein; IFQ, immunofluorescence quenching; LR, lyssamine-rhodamine; FTSC, fluorescein-5-thiosemicarbazide; FA, fluorescein-5-amine; [^{3}H]H$_2$DIDS, 4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonic acid; WGA, wheat germ agglutinin; pHMB, *p*-hydroxymercuribenzoate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FITC, fluorescein-5-isothiocyanate; AE, amino ethyl; PBS, phosphate-buffered saline (147 mM NaCl, 20 mM sodium phosphate); 5P8, 5 mM sodium phosphate (pH 8.0); 36P7.4, 36 mM sodium phoshate (pH 7.4); high ionic strength buffer, 150 mM sodium phosphate, 150 mM NaCl, (pH 7.4); CMC, critical micellar concentration; PC, phosphatidylcholine; TLCK, tosyl-lysine-chloromethyl-ketone.

randomly across the vesicle membrane, that is, either as in the native system or in an inverted disposition. Although in several instances, reconstitution led preferentially to one particular orientation of protein in vesicles [5–10], lack of information about the factors governing oriented incorporation, precluded general application of the procedure to other systems.

In the present work we introduce methodologies for isolating vesicles with uniform orientation of transmembrane polypeptides and means for quantitative assessment of the orientation. We have chosen the glycophorins and the anion exchange protein, commonly referred to as band 3, as representative transmembrane polypeptides, since a wealth of information is available on their structure and function, and particularly on their asymmetric transmembrane disposition [11–14]. Vesicles containing uniformly oriented polypeptides were obtained either (i) by diluting the detergent solubilized protein which was supplemented with excess phospholipid, followed by affinity chromatography of vesicles, or (ii) by in situ formation of vesicles on polypeptides bound to affinity matrices. We refer to the first procedure as one-step dilution in suspension (OSDS) and to the second as gradual dilution on matrices (GDM). We have used fluorescein labels to tag the external carbohydrate-rich domain of the above glycoproteins. Based on their high fluorescein fluorescence quenching capacity, we used antifluorescein (anti-F) antibodies [15] as quantitative, selective and non-penetrating detectors of fluorescein present in the external surface of vesicles [16]. With this technique which is referred to as immunofluorescence quenching (IFQ), we provide firm evidence for the oriented reconstitution of two representative asymmetric transmembrane proteins of human erythrocytes.

Experimental procedures

The external components of human red cell membranes were tagged with fluorescein (F) or lyssamine-rhodamine (LR) label by the following labeling procedures: Oxidation of external carbohydrate moieties of intact cells or ghosts with galactose oxidase or with Na-periodate, followed by either fluorescein-5-thiosemicarbazide (FTSC) or fluorescein-5-amine + $NaCNBH_3$ as previously described [16]; oxidation of isolated glycophorin with sodium periodate followed by FTSC in conditions analogous to those used for labeling of cells, except that dialysis was used to remove excess reagents [16]; sulfonation of amino groups of ghosts (50% cell suspension) with LR-sulfonylchloride (1 mg/ml) for 4 h at 5°C, followed by extensive washings. Labeling of ghosts with LR was particularly useful for obtaining fluorescently labeled glycophorins used in various reconstitution studies. Specific labeling of the anion exchange protein (band 3) with [^{3}H]H_2DIDS was done as previously described [17]. Usually all preparations were first reacted with [^{3}H]H_2DIDS and subsequently with fluorescein-containing reagents. For isolation of band 3 alone, cells (20% hematocrit), prior to labeling were subjected to extensive trypsinization at 0.1 mg/ml trypsin for 1 h at 37°C in phosphate-buffered saline (pH 7.4), followed by washes with phosphate-buffered saline + bovine serum albumin 0.5%, soya bean antitrypsin (0.5 mg/ml), and finally with buffer.

Affinity matrices were prepared from Sepharose 4B (Pharmacia) or α-cellulose (Sigma) activated with CNBr, as described [18], or activated with nitrophenyl chloroformate, as described [19]. Wheat germ agglutinin (WGA) was coupled directly on the activated matrix [17], while *p*-hydroxymercuribenzoate (pHMB) was coupled with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) after saturating the activated group with diaminopropane [17]. Aminoethyl-Sepharose 4B (AE-Sepharose) was prepared by the CNBr-triethylamine method [18] by reacting 15 g CNBr with 100 ml wet Sepharose 4B for 5 min at 5°C in 60% acetone, slowly adding 210 mmol triethylamine washing out the acetone and subsequently washing in cold distilled water and $NaHCO_3$ (pH 9), and coupling the activated gel with excess diaminoethane. The final number of 280 nmol NH_2/ml wet gel was obtained by reacting FITC (1 mM) with 1 ml AE-Sepharose in sodium borate (0.2 M), pH 9.0, for 1 h at room temperature, and 12 h at 4°C, followed by extensive washings with distilled water, 0.1 M sodium phosphate (pH 6.9), and reading the absorbance in 60% glycerol in 0.1 M sodium phosphate (pH 6.9), against an equivalent concentration of AE-Sepharose suspended in

the same solvent (ϵ_M^{1cm} of fluorescein at 491 nm, 61 000).

Isolation of band 3 and glycophorins

Membranes were prepared by hypotonic lysis of red blood cells in 30 volumes of 5 mM sodium phosphate, pH 8.0 (5P8) and centrifugation (10 000 × *g*, 10 min) and repetitive washes until the pellet was free of hemoglobin. Glycophorin was usually isolated by the chloroform-methanol method [20] in conjunction with affinity chromatography on WGA-Sepharose [21]. Band 3 and glycophorin were co-isolated in detergent (Triton X-100, 1%, or octylpolyoxyethylene, 3%) after solubilization of alkali-EDTA treated membranes and ultracentrifugation at 100 000 × *g* for 1 h [17]. The supernate was concentrated on an Amicon filtration unit, chromatographed on DEAE-cellulose and eluted with high ionic strength buffer in the presence of detergent [17]. Band 3 was separated from glycophorin by affinity chromatography, either on a WGA-Sepharose column (to sequester glycophorin or a pHMB-Sepharose column (to sequester band 3) [17]. The detergent used for loading the sample was either Triton X-100 (1%) or octylpolyoxyethylene (3%); the protein loaded column was washed extensively with detergent solution and the protein eluted after addition of the appropriate ligand (100 mM cysteine or *N*-acetylglucosamine) in either 2% octylpolyoxyethylene or 1% octylglucopyranoside [17].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli [22], and the gels were fixed with methanol (50%), acetic acid (10%) or stained with Coomassie blue and subsequently sliced, digested with H_2O_2 and counted for radioactivity, as detailed elsewhere [17]. Protein was determined by fluorescence [23], phospholipids by a chemical reaction [24] and band 3 by [^{3}H]H_2DIDS label [17]. All measurements of fluorescence were carried out with a Spex Fluorolog II spectrometer, and measurements of radioactivity with a Prias II scintillation counter. All solutions were checked for osmolarity in a Wescor II osmometer.

Chemicals were obtained from the following sources: FTSC, fluoresceinamine (FA), carboxyfluorescein (CF) and lyssamine-rhodamine (LR) from Molecular PRobes, Inc.; EDC, $NaCNBH_3$ and CNBr, from Aldrich; Sepharose 4B from Pharmacia; DEAE-cellulose (DE52) from Whatman; egg L-α-phosphatidylcholine from egg yolk, type V-EA, from Sigma, all others were from Sigma. A sample of octylpolyoxyethylene (C_8E_{3-12}) was prepared [25] and generously provided by Dr. J.P. Rosenbusch (Basel and Heidelberg).

Results and Discussion

Labeling

The labeling of cells with the various reagents gave in gel radioelectrophoretograms or fluorograms the following results: With [^{3}H]H_2DIDS, more than 95% of the label was found associated with band 3, as previously reported [26]; with FTSC or FA-$NaCNBH_3$ reacted with cells previously trypsinized (to remove exofacial domains of glycophorin) and then galactose oxidase treated (to oxidize galactosyl groups) about 90% of fluorescein was in band 3 and the remaining was associated with organic solvent extractable material [16], presumably lipid. Omission of the trypsinization step resulted in that about 1/3 of the labeled material (i.e., ghosts) was associated with glycophorins, while the remaining was on band 3. The labeling efficiency in ghosts derived from cells treated with [^{3}H]H_2DIDS + trypsin + galactose oxidase + either FTSC or FA-$NaCNBH_3$, was usually 1 fluorescein label per 2 band 3 monomers. Fluorescein was routinely determined by fluorescence, using fluorescein-albumin as standard (excitation 493 nm, emission 518 nm), while band 3 was assessed by radioactivity based on the specific and stoichiometric labeling of [^{3}H]H_2DIDS. Ghosts derived from periodate-treated cells which were subsequently reacted with FTSC gave a higher than 10 fluorescein labels per glycophorin monomer labeling yield.

Reconstitution

The reconstitution of systems containing fluorescein-labeled polypeptides with a defined transmembrane orientation was attempted by two different procedures, referred to in this work as OSDS, vesicle formation by one-step dilution in suspension, and GDM, vesicle formation by gradual dilution on solid matrices.

Vesicle formation by the OSDS method

This method is essentially an extension of a previously described technique of reconstitution [27]. The proteins (band 3 and/or glycophorin) isolated in detergent of a relatively high CMC (e.g., octylglucopyranoside with a CMC of 22 mM or octylpolyoxyethylene with a CMC of 6.7 mM) were supplemented with a large excess of egg PC (100–1000 mg PC/mg protein) to ensure formation of vesicles with a single protein unit per vesicle. If turbidity appeared, it was eliminated by the addition of trace amounts of detergent. The clear suspensions were subsequently diluted with buffer or water, so as to bring the detergent below the CMC level (about one-third the CMC concentration). Osmolarities were normally readjusted to 100 mosM with concentrated NaCl. After 20 min at 5°C, the suspensions were centrifuged (170000 × g, 40 min) to give a pellet which contained 50–60% of the protein and a 1 : 300 (w/w) protein/lipid ratio which, for the case of band 3 [34], corresponded to less than one band 3 dimer per vesicle (using 400 Å as the vesicle diameter found by electron microscopy of negatively stained samples and a PC surface area of 50 $Å^2$). After freezing and thawing, followed by a 30 s sonication in a bath sonicator [28], the vesicles appeared homogeneous in size, monolamellar and sealed to phosphotungstic acid.

Various fluorescein-labeled protein preparations were used with this reconstitution technique: (a) band 3 + glycophorin (20 μg/ml protein) fraction eluted from the DEAE-cellulose or AE-Sepharose column with detergent (polyoxyethylene or octylglucopyranoside) in high ionic strength buffer; (b) glycophorin (300 μg/ml) in polyoxyethylene or octylglucopyranoside; and (c) band 3 (10 μg/ml) eluted from pHMB-Sepharose with octylglucopyranoside and cysteine.

The orientation of fluorescein-polypeptides in the reconstituted vesicles was assessed by the immunofluorescence quenching (IFQ) technique. As previously described [16], addition of anti-F antibodies to fluorescein-labeled red cells or membranes isolated thereof, resulted in a total quenching of fluorescence, indicating the external location of the label, its accessibility to quencher and the efficiency of quenching of fluorescence by the antibody. When added to detergent solutions of

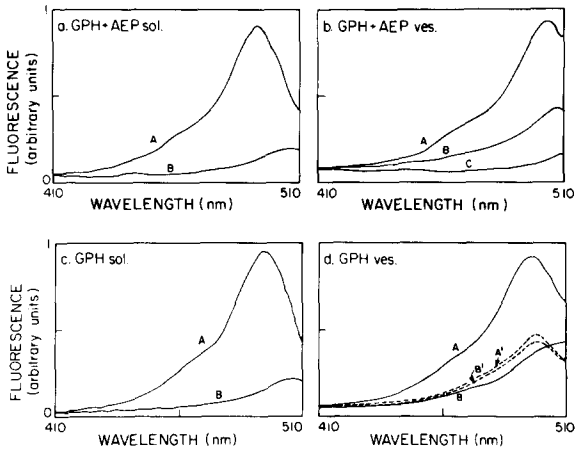

Fig. 1. Immunoquenching of fluorescein-labeled glycophorins (GPH) and anion exchange protein (band 3, AEP) reconstituted proteoliposomes. Excitation spectra of fluorescein-labeled GPH and AEP before (A) and after (B) addition of anti-F (1 μl of original immunoglobulin fraction per 1 nM fluorescein concentration). All spectra were recorded at 20°C and normalized to the fluorescein peak intensity at 494 nm before addition of anti-F. (a) The fraction eluted from the DEAE column with high ionic strength buffer in 2% octylpolyoxyethylene (i.e., fluorescein-labeled GPH + AEP, 20 μg/ml) was diluted 1 : 40 in 36P7.4 buffer supplemented with detergent to prevent protein aggregation. (b) GPH + AEP were coreconstituted by the OSDS method, centrifuged and resuspended in 36P7.4. Curve C, represents the spectrum of the reconstituted systems after addition of the detergent Nonidet P-40 (0.01%). (c) Isolated GPH (300 μg/ml) was dissolved in 36P7.4 containing 3% polyoxyethylene and (d) reconstituted by the OSDS method, as mentioned above and described in the text. Curves A′ and B′ represent analogous samples derived from GPH reconstituted vesicles which were subjected to proteolysis, removing protein fragments exposed at the outer surface of the vesicles (0.1 mg/ml trypsin, 1 h 37°C, trypsin inactivation by 0.1 mg/ml soya bean antitrypsin inhibitor + 1 mM TLCK, centrifugation and resuspension to the same original volume).

glycophorin + band 3 (Fig. 1a), or glycophorin (Fig. 1c) or band 3 (not shown), the anti-F antibody led also to complete quenching of fluorescence. However, when added to the same preparations after isolation of vesicles by the OSDS technique (Fig. 1b and d), only about half of the fluorescence signal could be reduced, even with surplus addition of antibodies. Moreover, vesicle disruption by addition of detergent exposed virtually all of the fluorescein label to macromolecular quencher. We interpret these results to indicate the formation of vesicles, half of which contained the

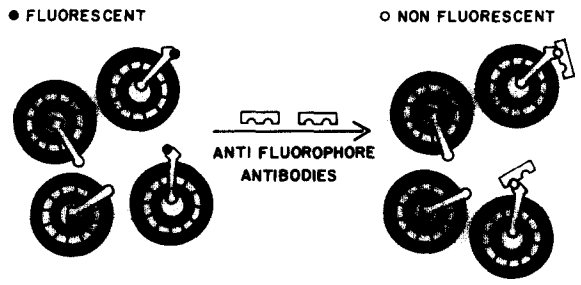

Fig. 2. Schematic representation of the immunofluorescence quenching technique (IFQ) applied to a population of proteoliposomes isolated by the OSDS technique. The asymmetric transmembrane polypeptide is fluorescein-labeled at the carbohydrate domain (black dot). Fluorescence is quenched upon addition of anti-F antibodies.

proteins in the native membrane orientation (right-side-out), while the remaining half contained proteins in inside-out orientation. This is schematically portrayed in Fig. 2. However, so as to strengthen this interpretation, we subjected the fluorescein-labeled vesicles to: (a) affinity chromatography on either pHMB-Sepharose (for band 3 vesicles) or on WGA-Sepharose (for glycophorin vesicles) and (b) ion-exchange chromatography on either DEAE-Sepharose or AE-Sepharose (for both types of protein vesicles) (Fig. 3). The rationale of this approach is based on the idea that any vesicle containing polypeptides in the two transmembrane orientations ought to be sequestered by a matrix with a high affinity for either an exo- or endofacial domain of the polyppetide. We verified this to be indeed the case with the OSDS procedure applied to either band 3 (AEP) or glycophorin (GPH) at 1 : 10 (w/w) protein/lipid ratio and the reconstituted vesicles chromatographed on pHMB-Sepharose or DEAE-Sepharose, respectively. Moreover, vesicles containing polypeptides with uniform orientation should be sequestered only, if the transmembrane orientation is such that the ligand is exposed at the outer surface of the vesicle. However, it should be stressed that this technique does not allow differentation between vesicles containing one, two, or more uniformly aligned polypeptides. In Fig. 4 we demonstrate its application to F-AEP proteoliposomes, using the IFQ to assess the orientation of the polypeptide in the vesicles. Band 3 was bound to the affinity column via the SH-groups present in the cytoplasmic domain,

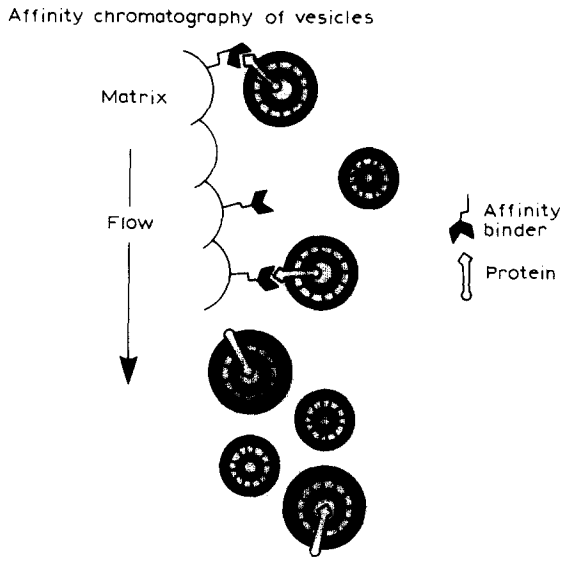

Fig. 3. Schematic representation of affinity chromatography of vesicles. A mixed population of vesicles, each containing a single protein (prepared by the OSDS method), is sieved through an affinity matrix containing an affinity binder specific for a particular domain of the protein. Protein-free vesicles and vesicles containing protein with the ligands facing the vesicle interior are filtered through, while the remaining are sequestered in the matrix and can be eluted by addition of appropriate free ligand.

while the fluorescein group was located on the exofacial domain. Sufficient antibody was added to ensure full quenching of all accessible fluorescein-fluorophore, as demonstrated for carboxyfluorescein and for proteoliposomes dissolved in detergent. As shown before, prior to vesicle separation, the maximal quenching efficiency was 50%, indicating a random distribution of polypeptides. However, the vesicle fraction which did not bind to the affinity gel, presumably because it consisted of vesicles with band 3 in the right-side-out orientation (i.e., with unexposed SH-groups), showed more than 90% of the fluorescein signal susceptible to quencher (after correction for background and scattering effects). On the other hand, the vesicles with band 3 in an inverted orientation, which bound to the affinity gel and which were eluted with cysteine, showed more than 85% of the fluorescein label inaccessible to quencher. An essentially complementary picture was obtained by chromatography of vesicles containing fluorescein-labeled glycophorin or fluorescein-

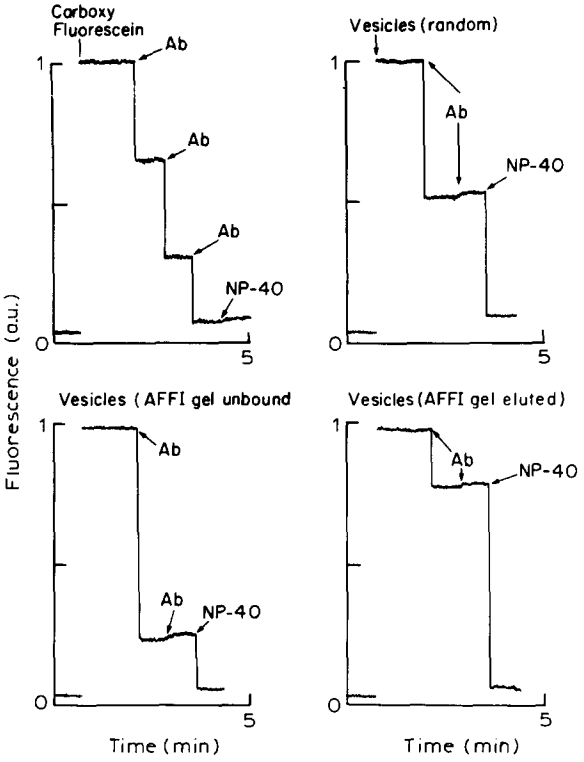

Fig. 4. Determination of band 3 (EAP) orientation in vesicles reconstituted by the OSDS method. Vesicles containing F-labeled band 3 were prepared by supplementing purified band 3 suspended in 2% polyoxyethylene with egg PC (1:1000, w/w protein to lipid ratio) and subsequently diluting the suspension 1:30 with 36P7.4. The newly formed vesicles were centrifuged (100000 × *g*, 50 min) and resuspended in 36 P7.4, then frozen in liquid N_2, thawed at room temperature and briefly bath sonicated (random vesicle population). For IFQ, the antibody (Ab) was calibrated with solutions containing equivalent fluorescence intensity of carboxyfluorescein (top left). Addition of the detergent Nonidet NP-40, 0.01%) did not interfere with the fluorescein quenching. The vesicles with random disposition of F-AEP showed only about 50% of the fluorescence accessible to Ab (top right). After sieving through pHMB-Sepharose (AFFI gel) column (5 ml gel, 10 ml/h) bottom left), the quenching efficiency on F-AEP proteoliposomes increased to more than 90% (after subtraction of background fluorescence due to light scattering by vesicles). The vesicle fraction eluted from the column with cysteine (100 mM) in 36P7.4 (bottom right) showed only 15% quenching, even with excess Ab. All fluorescence intensities (excitation 494 nm, emission 518 nm) were normalized to 1 after addition of vesicles to the cuvettes.

labeled band 3 on AE-Sepharose, using a high ionic strength buffer for their elution from the column (not shown). AE-Sepharose or DEAE-Sepharose sequestered the vesicles of exclusive right-side-out orientation of polypeptides. For chromatography of glycophorin vesicles, we have found the ionic sieves to give more reproducible results than WGA-Sepharose matrix. This is apparently due to the fact that the fluorescein moiety on the glycopeptide interfered with binding of glycophorin vesicles or isolated glycophorin to the immobilized lectin.

An additional tool used for assessing the orientation of fluorescein-labeled band 3 reconstituted by the OSDS consisted of immunoelectromicroscopic staining of vesicles. Indirect staining with gold-protein A on vesicles treated with anti-F antisera (Fig. 5) revealed gold particles associated with membranes only on the vesicle fraction which did not bind to the pHMB-Sepharose column. Although these results provide a visual demonstration of band 3 orientation, they also underscore some technique limitations of the immunoelectromicroscopic approach for the quantitative assessment of transmembrane disposition of glycoproteins.

Vesicle formation by gradual dilution on gel matrices (GDM)

This method consists essentially of the asymmetric binding of proteins in mixed lipid detergent suspension on solid affinity matrices (Fig. 6A). The detergent is gradually substituted with phospholipid, while the protein is still immobilized on the matrix (Fig. 6B), serving as a nucleation point for in situ formation of proteoliposomes with defined transmembrane protein orientation (Fig. 6C). These vesicles can be eluted from the column by addition of excess ligand or another suitable agent (Fig. 6D). The results with band 3 or glycophorin indicate that about 10–20% of the originally applied labeled polypeptide is usually recovered from the column after dilution, exchange of detergent for phospholipid and elution from the column. The eluted vesicles were frozen, thawed and sonicated, and as in the OSDS method, they appeared monolamellar and demonstrably sealed in negatively stained preparations. The protein-lipid content (w/w) was considerably higher than with vesicles prepared by the OSDS method. It varied with the preparations from 1/100 to 1/200 in line with the idea that no protein-free vesicles are likely to be released from the column by the free ligand.

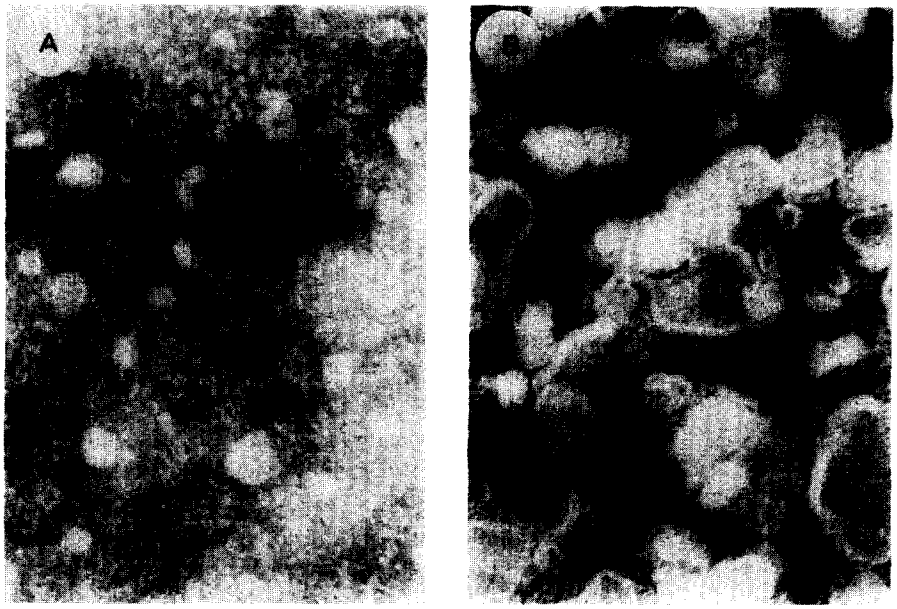

Fig. 5. Immunoelectronmicroscopy of F-AEP vesicles after affinity chromatography on pHMB-Sepharose. Vesicles isolated by the OSDS method and chromatographed as mentioned in Fig. 4, were centrifuged (100000 × *g*, 50 min), mildly sonicated (1 min in a bath sonicator), adhered on Cu grids (300 mesh), coated with Formvar and carbon for 60 s and washed with 1% bovine serum albumin in phosphate-buffered saline. The grids were transferred to bovine serum albumin (BSA) in phosphate-buffered saline containing 1 : 20 diluted anti-F, and after 15 min, washed thrice with phosphate-buffered saline (PBS) and incubated for 15 min with gold-protein A (20 nm particles, prepared as described elsewhere, Refs. 32, 33). The grids were carefully washed thrice with BSA/PBS, thrice with phosphate-buffered saline, and thrice with water, and stained with 2% phosphotungstic acid at pH 6, subsequently washed with phosphate-buffered saline and water. (A) Represents vesicles bound and eluted from the AFFI-gel column with cysteine while (B) represents the vesicle fraction which did not bind to the column.

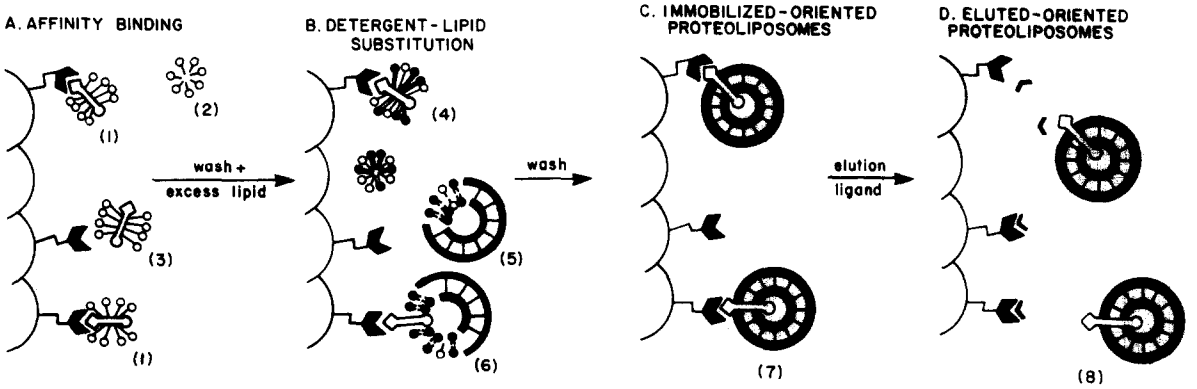

Fig. 6. Schematic representation of in situ formation of vesicles with uniform orientation of proteins by gradual dilution on matrices (GDM). Specific and oriented binding (1) of asymmetric protein molecules (arrows) solubilized in detergent (open symbols) (2) to gel matrices is followed by extensive washings with detergent to remove native lipids and unspecifically-bound proteins (3). Gradual substitution of detergent for lipids induces formation of mixed micelles of protein/detergent/lipid (4) which are bound to the column and of detergent/lipid micelles which are free (5). Upon washing with buffer and lipid, the remaining detergent/lipid micelles are washed off the column, while (4) remain bound asymmetrically and gradually form vesicles around the immobilized proteins (6 and 7). Finally, elution with free ligand (V symbol) releases a population of proteoliposomes with uniform orientation of proteins, the affinity ligands facing the external surface of the vesicles.

This is supported by the fact that all the protein content was consistently found in the membrane fraction, i.e., the 100 000 × *g* pellet.

The high protein/lipid ratio clearly indicates that the vesicles contained more than one polypeptide per vesicle. So as to study the orientation of these polypeptides, we conducted IFQ studies in conjunction with affinity chromatography. Using GPH on WGA-Sepharose, we obtained results virtually analogous to those observed with band 3, except that vesicles eluted with *N*-acetyl-D-glucosamine were of right-side-out orientation with respect to glycophorin disposition in the native membrane. Using the IFQ technique on glycophorin vesicles eluted from WGA-Sepharose, we observed (Table I) that about 90% of the fluorophore was accessible to immunoquencher, indicating that about that fraction of glycophorin was

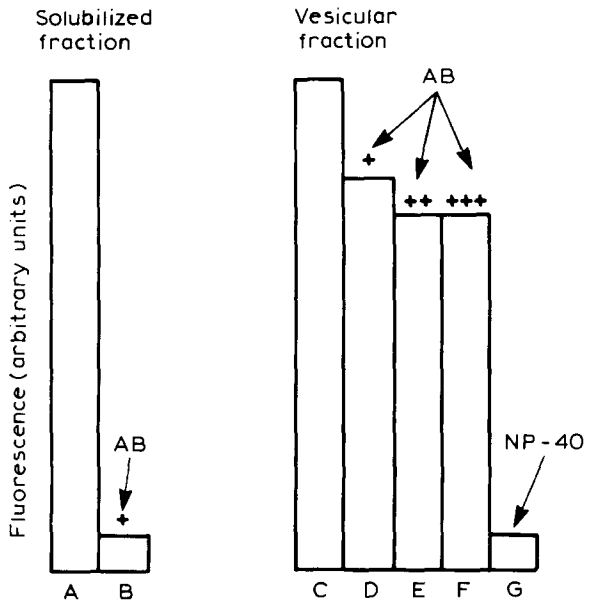

Fig. 7. Determination of the orientation of proteoliposomes generated by the GDM technique. Band 3 which was F-labeled on the carbohydrate domain, was solubilized in 36P7.4 containing 2% octylglucopyranoside. The preparation contained fluorophores (A) which were fully accessible to anti-F (AB), as demonstrated by the high quenching attained (B). After formation of vesicles by the GDB technique and elution from pHMB-Sepharose with cysteine (100 mM) in 36P7.4, most of the fluorescein fluorophore (C) is shown to be inaccessible to anti-F (D, E, F). Upon addition of the detergent Nonidet P-40 (NP-40, 0.01%), the fluorescence intensity decayed to the same level as that obtained in B (G).

TABLE I

ANTI-F QUENCHING EFFICIENCY (%) OF FLUORESCEIN-LABELED GLYCOPHORIN VESICLES GENERATED BY THE OSDS AND GDM PROCEDURES

Fluorescein-labeled glycophorin vesicles generated by the two indicated procedures were chromatographed and assessed for fluorescence quenching with anti-F antibodies, as described in the figures. Vesicles (original) were suspended in 36P7.4 buffer. OSDS method: 0.7 ml of vesicles containing 300 μg glycophorin were chromatographed on 2 ml WGA-Sepharose, washed with 36P7.4 and eluted with 35 ml *N*-acetyl-D-glucosamine (GlcNAc) (100 mM) in 36P7.4. Vesicles were centrifuged, washed, resuspended in 36P7.4, chromatographed on 5 ml DE-52 cellulose and eluted with 10 ml high ionic strength (HIS) buffer. GDM method: 300 μg glycophorin; all steps were essentially the same as those described above. OSDS, one-step dilution in suspension; GDM, gradual dilution on matrix.

OSDS		GDM
55	original	> 90
> 90	WGA-Sepharose bound and GlcNAc eluted	> 95
> 95	DE-cellulose bound and HIS eluted	> 95
no F	DE-cellulose filtered	no F

presumably in the right-side-out orientation. This was further substantiated by the observations that the vesicles eluted from the lectin column were fully sequestered by DEAE-cellulose when in low ionic strength media, but released with high ionic strength media in a form displaying full accessibility to anti-fluorescein. This conforms with the idea that the negatively charged groups of glycophorin which play a role in electrostatic binding to DEAE-Sepharose, are exposed on the outer surface of cells as well as of the reconstituted vesicles with right-side-out orientation, containing the intrinsic proteins glycophorin and band 3. Virtually the same results were obtained with glycophorin-containing vesicles isolated by the OSDS method (Table I).

Discussion

The successful isolation of reconstituted systems with defined and uniform orientation of transmembrane polypeptides depends on the methods of isolation of proteoliposomes as well as

on the availability of techniques for the assessmentof protein orientation in membranes. The IFQ procedure, in conjunction with selective labeling of superficial domains introduced in the previous work for cells [16] and applied in the present one to reconstituted proteoliposomes, served as a highly sensitive and quantitative means for determining the property in question. The couple anti-F antibodies and fluorescein label tagged on the carbohydrate containing moiety of glycoproteins, provided the tool for identification of the exofacial domain of band 3 and glycophorins. Based on the observation that anti-F antibodies led to almost complete (more than 90%) quenching of fluorescence in fluorescein-labeled cells or ghosts, we set out to demonstrate that the degree of quenching attained in sealed vesicles can be used to define quantitatively the relative number of polypeptides oriented as in the intact cell. Evidently, with this information alone, we could not define unequivocally the disposition of polypeptides bearing inverted transmembrane orientation, as lack of quenching could not be discerned from conformational changes interfering with accessibility of label to antibody. To some extent, this obstacle was overcome by demonstrating that after solubilization of vesicle with non-ionic detergents (Figs. 1, 4 and 7) or better after sonication of vesicles in the presence of antibodies (not shown), full quenching could be obtained. However, only when the above studies were complemented with separations of vesicle by affinity chromatography (Figs. 3 and 4), the IFQ technique provided definitive information about the sidedness of polypeptide domains in reconstituted vesicles. For transmembrane glycoproteins lacking enzymatic activities, which are usually confined to either the exo- or the endofacial membrane domain, identification of the latter could be achieved only with exogenously added probes. Classical immunochemical detection of endogenous and/or exogenous markers are usually semiquantitative in nature, as demonstrated also in the present work (Fig. 5). However, the IFQ approach is definitely quantitative, displaying also high sensitivity in terms of requisite fluorescent labeled material, and simplicity by circumventing the need for separating vesicles from medium in order to quantitatively evaluate the degree of fluorophore exposure. Regarding the possibility of labeling differentially the cytoplasmic domain of band 3, this was achieved by alkylation of the endofacial SH-groups [29] with either fluorescein-5-maleimide, fluorescein-mercuric acetate or iodoacetamidofluorescein (not shown). In principle, selective labeling of the cytoplasmic domain of various red cell membrane proteins is easily achieved by reacting impermeant reagents with inside-out vesicles derived from erythrocyte ghosts [30]. An additional fluorescent label which can be used for analogous IFQ studies is nitrobenzyldiazole (NBD) for which specific antibodies were also raised [31] and a variety of covalent reacting reagents are commercially available. However, caution has to be exercised with the application of all these procedures, since not all superficial labels are easily accessible to antibodies, thus limiting the scope of the IFQ technique to the most exposed domains of membrane surfaces.

Although in principle protein disposition in proteoliposomes formed upon detergent removal is likely to be of a random nature, several examples of fortuitous oriented reconstitution were obtained [35]. The orientation of proton pumping activity in bacteriorhodopsin reconstituted into cardiolipin containing vesicles, was clearly demonstrated to be determined by the medium pH [5]. The state of aggregation of the spike protein of Semliki forest virus dissolved in octylglucopyranoside was assumed to determine the transmembrane disposition of the protein in reconstituted proteoliposomes [6]. While no electrostatic factors were shown to affect the random orientation of M13 virus coat protein, the critical temperature of lipid phase transitions was suggested to play a key role in inducing orientation and full incorporation of the reconstituted polypeptide [7]. The reconstitution procedure was also shown to affect orientation, such as in the case of mitochondrial ATPase which showed right-side-out orientation when reconstituted by cholate dialysis [8], as opposed to random orientation when done by a single step cholate dilution [9]. Similar results were recently reported for band 3 (Schubert, D., private communication). Oriented reconstitution was also obtained by protein transfer and insertion into preformed acidic liposomes such as in the case of cytochrome oxidase and H^+-ATPase from mitochondria [8,10]. However, taken in toto, the above reconstitution

schemes provided no explanation for the published experimental results nor were they shown to be of general applicability.

The main feature of the GDM reconstitution procedure reported here is that protein orientation in proteoliposomes can be predetermined by appropriate selection of an affinity ligand, tagged on or localized at either the exo or endofacial domain of the polypeptide (Fig. 6). In this fashion, vesicles with inside-out orientation of band 3 were isolated by using pHMB-Sepharose, whereas vesicles with right-side-out orientation of band 3 and/or glycophorin were obtained with the aid of cationic matrices or of lectin-Sepharose. The method is of general applicability, particularly if specific antibodies are used both to select the protein and to fix its orientation with respect to the affinity matrix. Uniform orientation of polypeptides was accomplished by isolating vesicles, either with single protein molecules generated by 'infinite' dilution of protein with lipid (FIg. 3) or with several protein molecules generated in situ on the affinity matrix (Fig. 6).

Since with both approaches the affinity matrices played an essential role in the purification of polypeptides and/or generation of proteoliposomes, their design demanded special attention in our preliminary studies. First, the arm by which the affinity ligand was coupled to the matrix was shown to affect the elution of the proteoliposomes. Hydrophobic arms (e.g., hexyl chain), while efficient in increasing the binding affinity, render the protein virtually unelutable from the matrix, even in the presence of mild, non-ionic detergents. Introduction of a positively charged group into the hydrophobic chain brought improvement, although not sufficient to justify a change from the 2C commonly used or 3C alkyl spacer. Second, the relatively spacious α-cellulose was not any better than Sepharose 4B or 6B as the basic matrix, indicating that vesicle formation on the column is not seriously hindered by the carbohydrate matrix of the Sepharoses. However, what remains to be evaluated is the density of affinity ligands on the matrix as a factor which determines both the density and the oligomeric state of the polypeptide in the vesicle, generated by the GDM method. Nevertheless, in the form the methods are presented here, they offer new opportunities for exploring structure-function relationships of a variety of membrane proteins which have otherwise been inaccessible to study with the presently available methodologies.

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