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# LOCALIZATION OF GLOBOSIDE AND FORSSMAN GLYCOLIPIDS ON ERYTHROCYTE MEMBRANES

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**Using the freeze-etch technique, the membrane localization of globoside, a principal glycolipid in human erythrocytes, and Forssman antigen, the chief glycolipid in sheep erythrocytes was evaluated using ferritin and colloidal gold as morphological markers for rabbit antibodies prepared against these glycolipids. Brief trypsinization of human red cell ghosts markedly aggregated intramembranous particles and permitted labeling of globoside, which appeared in a clustered arrangement. The aggregates of ferritin-anti-globoside differed from those of ferritin-wheat germ agglutinin, a label for glycophorin, which corresponded with the aggregates of intramembranous particles. Double-labeling of human trypsinized ghosts with anti-globoside/*Staphylococcal* protein A-colloidal gold and ferritin-wheat germ agglutinin indicated that the patterns of labeling were different and that the aggregates of globoside did not bear a direct relationship to the intramembranous particles, which represent transmembrane proteins. Resealed sheep erythrocyte ghosts labeled with ferritin-conjugated rabbit anti-Forssman showed small clusters of Forssman glycolipid on the erythrocyte surface, which could be markedly aggregated with a second goat anti-rabbit antibody, indicating relative mobility of the small glycolipid domains. The distribution of ferritin-anti-Forssman label in sheep ghosts treated at pH 5.5 to aggregate intramembranous particles also did not show definite correspondence between intramembranous particles and the clusters of ferritin-anti-Forssman.**

## Introduction

In contrast to the rather large amount of information available on the molecular organization of phospholipids and proteins in membranes, relatively little is known about how glycosphingolipids are organized in bilayers. In most systems studied, glycolipids have been localized predominantly in the outer leaflet of the plasma membrane [1–3], although exceptions have been noted [4].

Experiments in which neutral glycosphingolipids have been incorporated into multilamellar or unilamellar liposomes suggest that glycosphingolipids are organized in domains rather than being uniformly dispersed throughout the phospholipid bilayer. The glycosphingolipid asialo-GM1 showed a linear localization in dimyristoylphosphatidylcholine liposomes below the main transition temperature and appeared to be organized into small clusters above the main transition temperature [5]. Glucocerebroside incorporated into phosphatidylcholine bilayers, studied by differential scanning calorimetry and by kinetics of transfer between phospholipid bilayers, appeared to form gel-like domains distributed in a continuous liquid crystalline phosphatidylcholine phase [6,7].

It is of interest, therefore, to study the organization of glycosphingolipids in biological membranes to determine if they are present on the

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outer surface in domains and whether they might have any relationship to membrane glycoproteins or to structural features of the membrane, such as intramembranous particles. Using the freeze-etch technique, the membrane localization of the glycosphingolipids globoside * and Forssman ** antigen on human and sheep erythrocytes, respectively, was evaluated using ferritin and colloidal gold as morphological markers for rabbit antibodies prepared against these glycolipids. We have also compared the localization of globoside and Forssman glycolipids to that of ferritin-wheat germ agglutinin, a marker for the major erythrocyte sialoglycoprotein, and to the distribution of intramembranous particles on the freeze-fractured erythrocyte membrane.

## Materials and Methods

*Materials.* Wheat germ agglutinin and ferritin-conjugated goat anti-rabbit IgG were obtained from Miles Laboratories Inc. EM grade ferritin and 8% ultra-pure glutaraldehyde were obtained from Polysciences Inc., gold trichloride acid from ICN Pharmaceuticals, and *Staphylococcal* protein A from Pharmacia. The Forssman glycolipid and globoside were isolated from goat and human erythrocytes, respectively, and purified as previously described [10].

*Sera.* Anti-Forssman and anti-globoside sera were prepared by injecting New Zealand white rabbits with mixtures of the purified glycolipid and bovine serum albumin emulsified in Freund's adjuvant, as previously described [11]. After ammonium sulfate precipitation of the globulin fraction, antibodies against the albumin were removed by passage through a bovine serum albumin-Sepharose column. Each antiserum was specific for its respective glycolipid immunogen, as previously described [12].

*Conjugation of ferritin to wheat germ agglutinin.* The ferritin conjugation of wheat germ agglutinin was similar to the procedure previously described [13,14], except that the conjugation was carried out in phosphate-buffered saline containing 0.1 M *N*-acetylglucosamine. The reaction was stopped with 0.1 M glycine; then, the mixture was centrifuged for 20 min at $40\,000 \times g$. The supernatant was dialyzed overnight against phosphate-buffered saline, (0.15 M NaCl/0.005 M phosphate buffer, pH 7.3) and then concentrated with an Amicon filtration apparatus. The conjugate was applied to a Biogel A 5m column ($1 \times 167$ cm ) equilibrated with phosphate-buffered saline, and fractions were read for absorbance at 440 nm to determine ferritin concentrations. Wheat germ agglutinin was monitored by agglutination of human erythrocytes.

*Conjugation of ferritin to anti-Forssman and anti-globoside.* The conjugation of ferritin to antibody was similar to the procedure for conjugation of ferritin to wheat germ agglutinin, except that the phosphate-buffered saline did not contain *N*-acetylglucosamine. The fractions eluted from the Biogel column were read for absorbance at 280 and 440 nm. The elution of ferritin-antibody conjugates was determined by hemagglutination assays.

*Preparation of colloidal gold bound to Staphylococcal protein A.* Colloidal gold (150A–180A) was prepared as described by Ackerman [15]. The gold/*Staphyloccal* protein A conjugation was as described by Romano and Romano [16]. To 10 ml colloidal gold, pH 6.9, with gentle swirling, was added 1 mg *Staphyloccal* protein A dissolved in 0.2 ml high purity water. The gold/*Staphyloccal* protein A was mixed for 8 min and then 0.1 ml poly(ethylene glycol) (20 000 $M_w$) was added. The mixture was centrifuged at $100\,000 \times g$ for 1 h at 4°C. The supernatant was discarded and the resulting gold/*Staphyloccal* protein A formed a dark red pellet which was resuspended in 2 ml 0.005 M sodium phosphate buffer, pH 7.4, containing 0.2 mg/ml poly(ethylene glycol).

*Preparation of erythrocyte ghosts.* Fresh human and sheep red cells were washed three times with phosphate-buffered saline. Resealed ghosts were prepared by lysing the intact red cells with 0.005 M sodium phosphate buffer, pH 7.4, at 0°C for 2 min, centrifuging and then resuspending in prewarmed phosphate-buffered saline at 37°C and incubating for 1 h at 37°C [17]. After the incubation, the ghosts were centrifuged and resuspended

* Glycolipid structures: Globoside [8], GalNAcβ1 → 3Galα1 → 4Galβ 1 → 4Glcβ1 → 1ceramide.

** Forssman [9], GalNAc α 1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1ceramide.

twice in ice-cold, phosphate-buffered saline and stored at 4°C.

Trypsinized ghosts were prepared by lysing intact red cells at room temperature in 0.005 M sodium phosphate buffer according to Dodge et al. [18]; then 0.5 ml of freshly packed ghosts were treated with 1 mg trypsin in 0.5 ml 0.01 M Tris-HCl buffer, pH 7.5, for 15 min at 37°C [13]. Lima bean trypsin inhibitor (2 mg) was then added and ghosts were centrifuged and resuspended three times in 0.005 M sodium phosphate buffer, pH 7.4.

pH-treated ghosts were prepared by using freshly-packed lysed red cells, prepared according to Dodge et al. [18], and suspending 0.5 ml of a 1 : 30 dilution of ghosts in 5 ml 0.005 M sodium phosphate, pH 8.5, for 24 h at 37°C [19–21]. Ghosts were then centrifuged and resuspended in 15 ml 0.005 M sodium phosphate buffer, pH 5.5, with a 30-min incubation on ice. The pH 5.5 buffer washing and incubation was repeated three times.

*Treatment of ghosts with ferritin and colloidal gold conjugates.* 50 μl of packed ghosts were incubated with 500 μl of the conjugate for 1 h at room temperature with occasional mixing. The ghosts were centrifuged in an Eppendorf microfuge for 15 min, resuspended and washed twice with 0.005 M sodium phosphate buffer, pH 7.4. The final pellet was resuspended in 8–10 μl buffer before freezing.

*Freeze-etching procedure.* 1-μl suspensions of treated ghosts were placed on gold alloy specimen carriers and quickly frozen in liquid Freon 22 cooled by liquid nitrogen. Specimens were freeze-cleaved at −103°C and deep-etched for 7–8 min at −103°C in a Balzers BAF 300 freeze-etching apparatus. The specimens were shadowed with platinum-carbon and stabilized with a layer of pure carbon. The replicas were floated off on distilled water and cleaned with concentrated chromic-sulfuric acid solution for 2 min, then rinsed several times with distilled water and picked

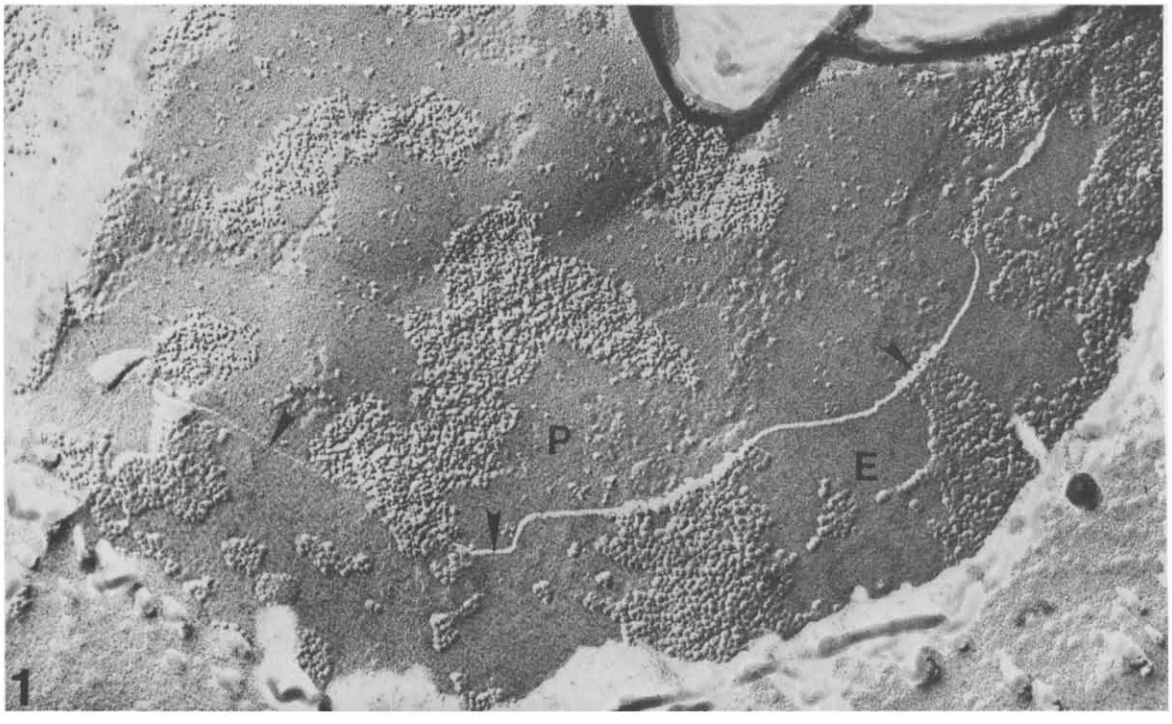
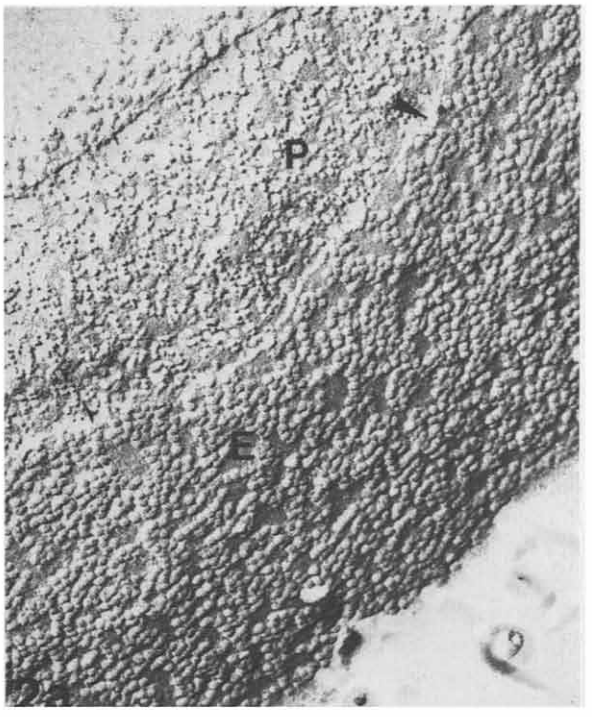



Fig. 1. Freeze-etch electron micrograph of trypsinized human erythrocyte ghost labeled with ferritin-anti-globoside. The intramembranous particles are aggregated on the P-face (P) and the ferritin-anti-globoside is also clustered on the external surface (E) of the membrane. The concordance of aggregates of intramembranous particles and ferritin-anti-globoside across the fracture line (arrows) measured an average of 37% in this and other micrographs of similar preparations. Magnification is approx. 70000×.



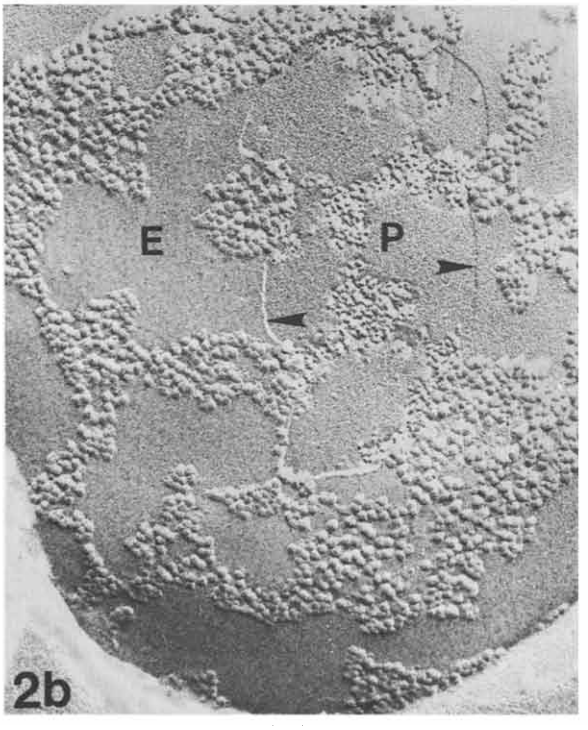




Fig. 2. Freeze-etch electron micrographs of normal (a) and trypsinized (b) human erythrocyte ghosts labeled with wheat germ agglutinin-ferritin. Both preparations show close correspondence of wheat germ agglutinin-ferritin binding sites on the external ghost surfaces (E) and the intramembranous particles on the P-faces (P). The concordance of aggregates of intramembranous particles and ferritin-wheat germ agglutinin in the trypsinized ghosts (Fig. 2b) was 88% in this and other micrographs. Fracture lines are indicated by arrows. Magnification is approx. 75000×.

up on 200 or 300 mesh copper grids. They were examined in an Hitachi HU12A electron microscope.

*Calculations.* The degree of concordance of patches of ferritin or colloidal gold label on the external surface of freeze-etched ghosts with the aggregates of intramembranous particles on P − faces of the replicas was determined using a map distance measuring device (Alvin No. 1112). Concordance was quantitated according to the following equation:

$$\text{Percent concordance} = \frac{A}{A+B} \times 100$$

where $A$ = the length along the fracture line where intramembranous particles and label were in apposition across the fracture line and $B$ = the length where either intramembranous particles or label were not apposed.

## Results

*Localization of globoside in human erythrocyte membranes*

Ferritin conjugates of rabbit antibodies prepared against globoside did not label intact human erythrocytes or resealed ghosts prepared from human adult erythrocytes. This result is consistent with previous observations of Hakomori [22] and Gahmberg and Hakomori [1], which indicated that globoside is not exposed in adult human erythrocytes (see Discussion). This result also indicated that there was no nonspecific labeling of the erythrocyte membrane by either ferritin or colloidal gold conjugates and that there was no cross-reaction of the anti-globoside antibodies with exposed membrane glycoproteins.

In contrast to resealed human ghost preparations, ghosts treated by brief trypsinization or by depletion of spectrin followed by incubation at pH 5.5 showed the characteristic aggregation of intramembranous particles on the freeze-fracture faces of the membrane and showed marked label-


Fig. 3. Freeze-etch electron micrograph of a trypsinized human erythrocyte ghost treated with rabbit anti-globoside followed by ferritin-anti-rabbit IgG. The large clusters of anti-globoside label on the external surface (E) show very little concordance (approx. 22%) across the fracture line (arrows) with the aggregated intramembranous particles on the P-face (P). The ferritin label is less distinct in this micrograph because it is layered over patches of rabbit anti-globoside antibodies. Magnification is approx. 80000×.

ing by ferritin conjugates of the anti-globoside antibodies (Fig. 1). The ferritin-anti-globoside conjugate was present on the outer membrane surface exposed by deep-etching in clusters or patches, which bore minimal relationship to the patches of intramembranous particles present on the P-fracture faces of the trypsinized erythrocyte membranes (Fig. 1). Although the distribution of ferritin-anti-globoside and the intramembranous particles were both in clusters of approximately similar size, careful measurement of the amount of concordance in many micrographs did not show close correspondence of ferritin labeled anti-globoside to the patches of intramembranous particles across fracture lines (Fig. 1, legend), such as that seen with ferritin conjugates of wheat germ agglutinin and other lectins that label specific glycoproteins on human erythrocytes [13,23,24]. The close correspondence of ferritin-wheat germ agglutinin, which labels the major sialoglycoprotein of the human erythrocyte [13,25] to the distribution of intramembranous particles on normal and trypsinized ghost membranes is shown for comparison in Fig. 2. Even larger aggregates of globoside may be induced on the outer surface of trypsinized human erythrocyte ghosts by a double-labeling procedure in which the ghosts were first treated with rabbit anti-globoside and then by a ferritin conjugate of goat anti-rabbit IgG (Fig. 3). The ferritin was present on the ghost surfaces in larger clusters that bear little apparent direct relationship to the aggregates of intramembranous particles. Although there were some areas along the membrane fracture line in which patches of anti-glycolipid ferritin label were partially in apposition to aggregates of intramembranous particles, quantitation of the areas of concordance (Fig. 3, legend) always indicated much less concordance than that seen with labels for erythrocyte membrane glycoproteins.

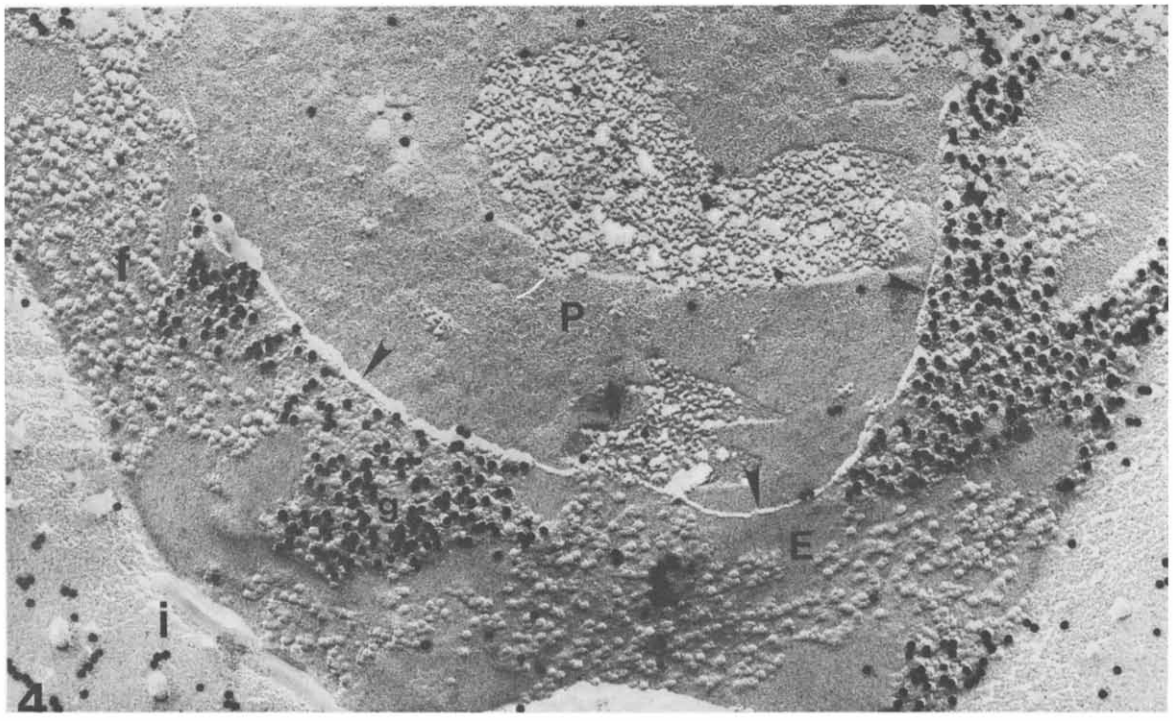



Fig. 4. Freeze-etch electron micrograph of a trypsinized human erythrocyte ghost double-labeled with ferritin-wheat germ agglutinin (f) to indicate sialoglycoprotein distribution and rabbit anti-globoside/*Staphyloccal* protein A-colloidal gold (g) to localize glycolipid. The ferritin-wheat germ agglutinin and colloidal gold/anti-globoside labels appear to be in separate clusters on the external surface (E). Concordance of wheat germ agglutinin-ferritin is nearly 100% with aggregates of intramembranous particles and colloidal gold/anti-globoside shows almost no concordance with intramembranous particles across the fracture line (arrows). Scattered individual colloidal gold particles adhere to the back of the platinum-carbon replica and appear under the P-face (P) and surrounding ice (I) and can be recognized because they cast no shadow during replication. Magnification is approx. 80000×.

In order to demonstrate directly that globoside and glycoproteins are distributed differently in trypsinized erythrocyte membranes, two morphologically different labels were applied sequentially to the same human trypsinized ghosts. A ferritin conjugate of wheat germ agglutinin, a label for the sialoglycoprotein component of the membrane, showed a different distribution on the outer membrane surface than the label for globoside, in this instance rabbit anti-globoside followed by protein A conjugated to colloidal gold (Fig. 4). It thus appears that the aggregation of the intramembranous particles induced by treatment of ghosts with trypsin or by spectrin depletion followed by pH alteration causes the glycolipids to become aggregated in the areas between the glycoproteins, and no direct interrelationship between the carbohydrate receptors on the glycolipids and those on the membrane glycoproteins can be demonstrated.

*Localization of Forssman glycolipid in sheep erythrocyte membranes*

In contrast to the masking of globoside molecules on normal human erythrocyte membranes, Forssman glycolipid is exposed on the surface of sheep erythrocytes and antibody to Forssman glycolipid will agglutinate these red cells. Resealed sheep erythrocyte ghosts treated with ferritin-conjugated antibodies to Forssman glycolipid showed small clusters of ferritin label distributed over the entire outer surface of the ghosts (Fig. 5). The small clusters of ferritin-anti-Forssman glycolipid are separated by larger areas of membrane which are unlabeled. The distribution of the label is different than the more uniform and dense distri-

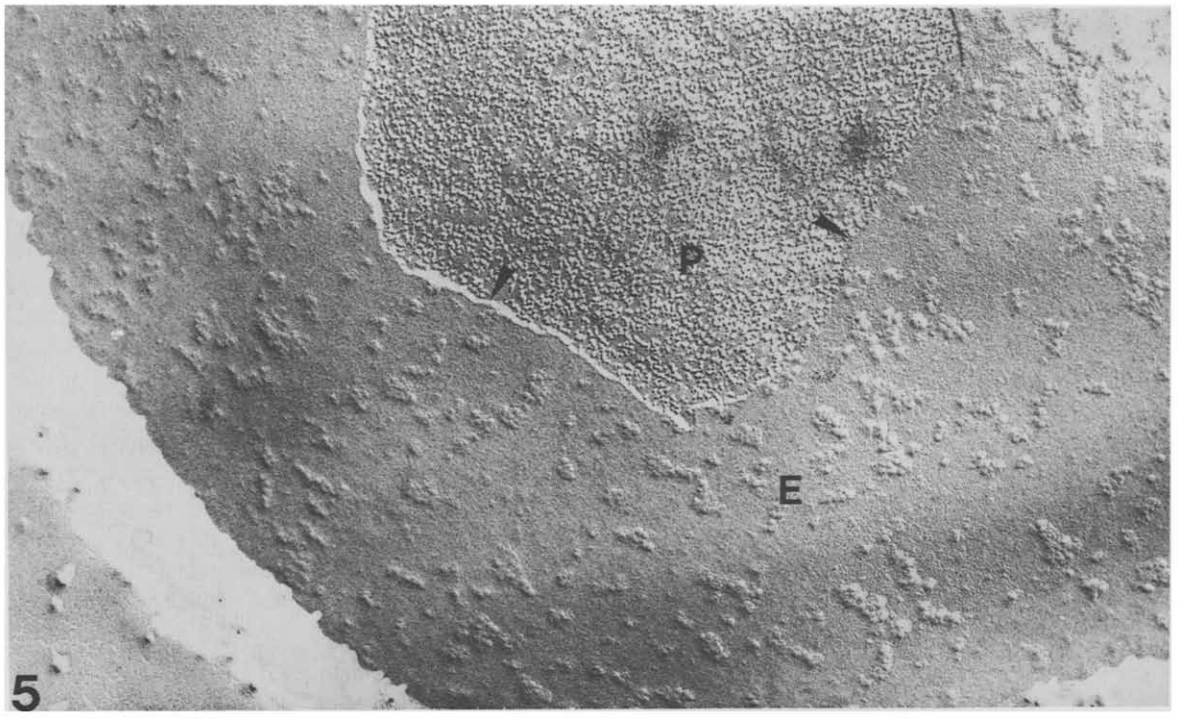



Fig. 5. Freeze-etch electron micrograph of a resealed sheep erythrocyte ghost labeled with ferritin-anti-Forssman antiserum. The anti-Forssman label is in small clusters over the external surface (E) of the ghost, while the intramembranous particles are randomly oriented on the P-face (P). Fracture line is indicated by arrows. Magnification is approx. 68000×.

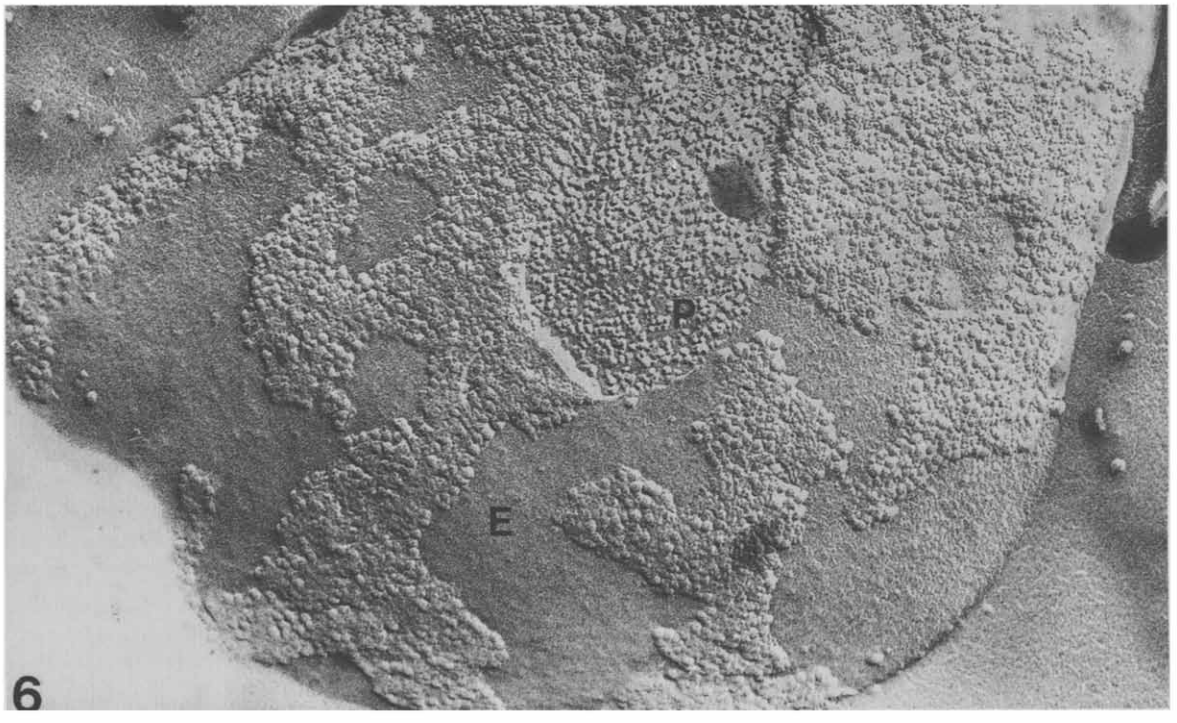



Fig. 6. Freeze-etch electron micrograph of a resealed sheep erythrocyte ghost treated with rabbit antiserum to Forssman glycolipid followed by ferritin-anti-rabbit IgG. The anti-Forssman label is in large clusters on the external surface (E), in no apparent relationship to the randomly oriented intramembranous particles on the P-face (P). Magnification is approx. 100000×.

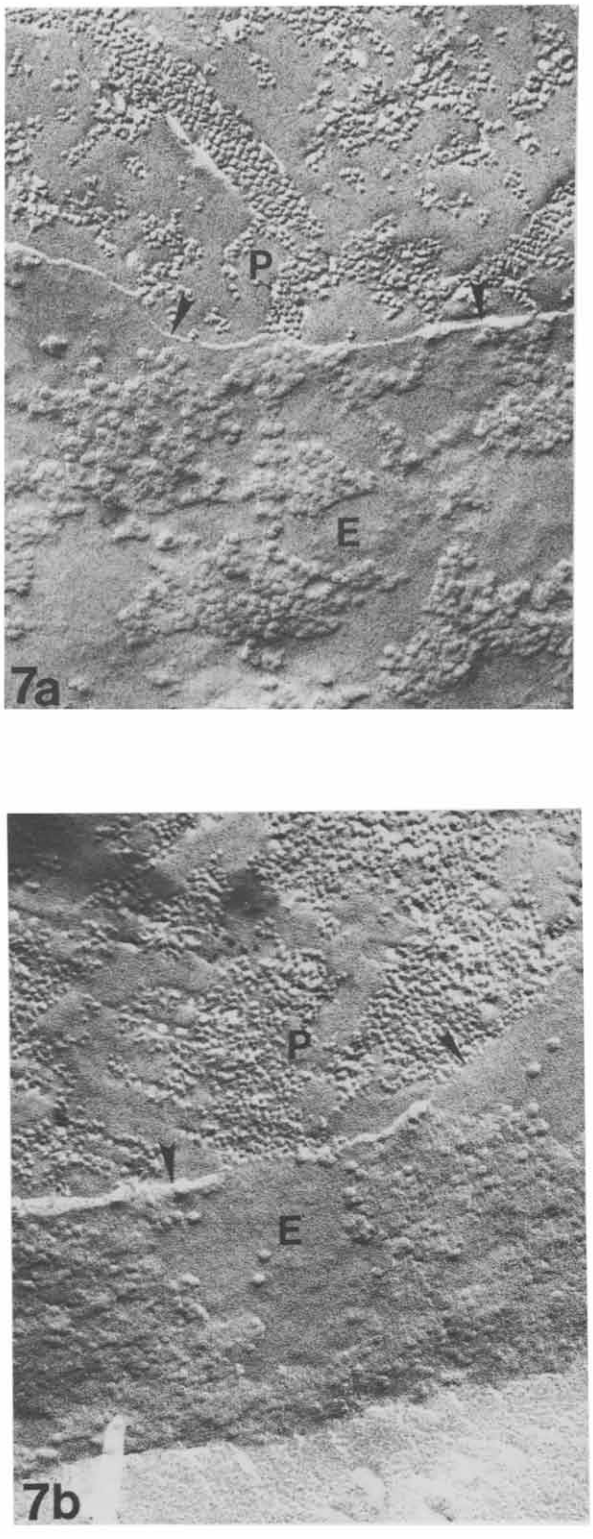



Fig. 7. Freeze-etch electron micrographs of pH 5.5-treated sheep erythrocyte ghosts labeled directly with ferritin-anti-Forssman (a) and with anti-Forssman antiserum followed by ferritin-anti-rabbit IgG (b). The anti-glycolipid label on the external surfaces (E) of the ghosts shows only 37% concordance with the aggregates of intramembranous particles on the P-face (P). The ferritin label is less distinct in Fig. 7b, since it is layered over the antibodies to Forssman glycolipid. Fracture lines are indicated by arrows. Magnification is approx. 100000 ×.

bution of the intramembranous particles on the P-fracture face of the normal sheep ghosts. Thus, the distribution of this glycolipid in normal sheep erythrocytes appears to be in small patches or clusters, rather than distributed evenly as individual molecules.

The organization of Forssman antigen on intact resealed sheep erythrocyte ghosts can be dramatically altered by applying a second binding reagent to aggregate the glycolipid into large patches. Resealed sheep ghosts treated with antibodies to Forssman glycolipid followed by a ferritin conjugate of goat anti-rabbit IgG showed large aggregates separated by large areas free of label (Fig. 6). The intramembranous particles on the P-fracture faces of these ghosts showed no alteration in their arrangement induced by this aggregation of glycolipids. This indicates that the glycolipids are freely moveable in the plane of the outer half of the membrane bilayer and that membrane proteins, represented by the intramembranous particles, do not restrict this movement.

Sheep erythrocyte ghosts, incubated overnight at pH 5.5, show aggregation of intramembranous particles into patches. Brief trypsinization has a similar effect on intramembranous particle aggregation. Treatment of these ghost preparations with ferritin-anti-Forssman conjugate shows the label to be in relatively large clusters, which bear minimal visible relationship to the aggregates of intramembranous particles on the P-fracture face (Fig. 7a). Even larger aggregates of anti-Forssman label can be induced by incubation of pH 5.5-treated ghosts with anti-Forssman antibodies followed by ferritin-anti-rabbit IgG conjugate (Fig. 7b). Again, measurements of concordance of patches of anti-Forssman glycolipid label and aggregates of intramembranous particles show no convincing evidence of overlap.

## Discussion

Previous studies using cholera toxin as a label for the ganglioside GM1 have indicated that this glycolipid can be capped in vitro on lymphocyte membranes; these studies used fluorescinated

cholera toxin or antibodies to cholera toxin to localize GM1 binding sites at the light microscope level [26,27]. The multivalency of cholera toxin or the presence of a cross-linking antibody to cholera toxin could have facilitated the clustering and capping that were seen in those experiments. In experiments using various tissue culture cells with exogenously added Forssman glycolipid treated with monoclonal anti-Forssman antibody, Stern and Bretscher showed that the Forssman glycolipid could be capped if cross-linked by antibody [28]. Thin-section ultrastructural examination of these Forssman antigen positive cells treated with monoclonal anti-Forssman antibody and a ferritin-labeled second antibody showed the glycolipid to be present in small patches rather evenly distributed on the surface of cells at 4°C in the presence of azide [28]. The explanation of how glycosphingolipids, which do not extend through the lipid bilayer to interact with cytoskeletal proteins, can cap has been speculated to be a result of a flow mechanism of membrane constituents [28], or by association of the glycosphingolipid with integral membrane proteins, which are themselves linked to underlying cytoskeletal proteins [26,27]. A clustered or patched arrangement of exogenously added biotinylated gangliosides was also observed on the surface membrane of aldehyde-fixed rat thymocytes when labeled with ferritin-conjugated avidin [29].

Our studies have utilized the freeze-etch technique combined with morphologically identifiable markers to examine the distribution of the neutral glycosphingolipids globoside and Forssman in human and sheep erythrocyte membranes, respectively, where they represent major native glycosphingolipid components of the membrane. Previous experiments have demonstrated the usefulness of this approach in localization of specific membrane glycoproteins, such as the band 3 and sialoglycoprotein molecules in human erythrocyte membranes [13,23,24]. These studies have indicated that these membrane glycoproteins are associated with the intramembranous particles seen on fractured erythrocyte membranes. We also used ferritin-labeled lectins and freeze-etching to study the organization of glycosphingolipids in liposomal systems; those experiments indicated that the neutral glycosphingolipid asialo-GM1 was organized in domains or clusters when incorporated into phospholipid bilayers [5]. The finding in the present study that Forssman glycolipid is present in small clusters scattered over the surface of sheep erythrocyte membranes is consistant with previous ultrastructural studies of glycosphingolipid localization in mammalian cells [28,29] and with the organization of asialo-GM1 in liposomal systems above their main phase transition temperature [5]. There is no apparent relationship between the Forssman glycolipid clusters and the underlying intramembranous particles, which are randomly and individually disposed on the membrane fracture faces. The addition of a second bridging antibody induces large aggregates of these smaller clusters, still without affecting the organization of the intramembranous particles, and by inference, the major membrane glycoproteins, whose transbilayer components probably comprise the major portion of the intramembranous particles. Labeling of globoside in human erythrocytes requires that the membranes be treated briefly with a proteolytic enzyme to expose its antigenic sites, in keeping with the previous observation that antisera to globoside could not agglutinate adult human erythrocytes unless erythrocytes were first treated with trypsin or neuraminidase [1,22]. Trypsin treatment of erythrocytes removes approx. 40% of their sialic acid [30], and trypsin treatment of ghosts causes marked aggregation of intramembranous particles [20,23]. We found in these experiments that trypsinization or treatment of ghosts to remove spectrin followed by incubation at pH 5.5 allowed the ferritin- or colloidal gold-conjugated reagents to adequately label the glycosphingolipids. Neuraminidase treatment of ghosts permitted only sparse labeling. We could not observe any distinct relationship between either globoside or Forssman glycolipid and intramembranous particles when the aggregated distributions of ferritin label on the outer surface of the membrane were compared with the aggregates of intramembranous particles on the fracture faces of the erythrocyte membranes. Even though both the ferritin label and intramembranous particles were similarly aggregated, careful inspection and measurement of many electron micrographs did not show consistent correspondence of the aggregates across the fracture lines. This conclusion was sub-

stantiated by the double-labeling experiments, in which aggregates of ferritin-wheat germ agglutinin, which bind to the membrane sialoglycoproteins and did correspond closely to intramembranous particle distribution, showed separate and distinct labeling from colloidal gold-protein A, which bound to the anti-globoside antibody. It thus appears that the markedly clustered arrangement of glycosphingolipid molecules directly labeled by ferritin conjugates of specific antibodies seen in membranes in which intramembranous particles have been aggregated could be induced by the movement of membrane glycoproteins into large patches, forcing the relatively mobile glycosphingolipids into intervening areas.

As suggested by Dr. Thomas E. Thompson, organization of glycosphingolipids, which have only one binding site per molecule, into domains could enhance their function as recognition sites on biological membranes by creating patches of complex carbohydrate composition similar to membrane glycoproteins. These domains could consist of one species of glycosphingolipid or conceivably could contain more than one glycosphingolipid, giving a mixture of carbohydrates that could function as a receptor site distinct from carbohydrate-specified receptors on membrane glycoproteins. In addition, glycosphingolipid transfer experiments in phosphatidylcholine bilayers suggest that the organization of glycosphingolipids in biological membranes might be in gel-like domains that would resist the tendency of these molecules to transfer readily to other membranes, thus preserving their receptor function [7].

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