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FREEZE-ETCH LOCALIZATION OF CONCANAVALIN A RECEPTORS TO THE MEMBRANE INTERCALATED PARTICLES OF HUMAN ERYTHROCYTE GHOST MEMBRANES

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SUMMARY

Freeze-fracture, freeze-etch and molecular labeling techniques localize concanavalin A receptors to the membrane intercalated particles of human erythrocyte ghost membranes. The concanavalin A receptor is a Band III component [14]. We propose that the membrane intercalated particle represents an oligomeric structure containing, at least, the principal integral proteins [glycophorin and Band III component(s)] interacting with the protein spectrin at the membrane inner surface. Previous work implies Band III component(s), integral membrane proteins and the membrane intercalated particles in a variety of transmembrane phenomena. We hypothesize that the membrane intercalated particles represent an amphipatic structure ("permeaphore."*) which topologically and structurally interrupts hydrophobic bilayer membrane domains.

INTRODUCTION

The human erythrocyte membrane contains a variety of protein components [1–3], a few of which are glycoproteins exposed at the outer surface [4–6]. Two major, exposed glycoproteins are integral membrane components (i.e., stabilized by hydrophobic interactions in the membrane interior [7]), as it has been shown that portions of these components are intercalated into the lipid bilayer while other regions of their structures are expressed at either membrane surface [8–10]. The molecular weight and number [1–3] of these glycoproteins and the fact that they are the only major proteins expressed at the outer surface [4–6, 11] indicate that the erythrocyte outer surface is predominantly lipid with the two glycoproteins interrupting the lipid bilayer and displaying oligosaccharide containing regions. These glycoproteins have

* Permeaphore: from the Latin verb "permeare" ("to go through", cf. permeate) and the Greek and modern Latin suffix "phorus" ("bearing", "bearer", cf. semaphore: Shorter Oxford English Dictionary [1964] [Little, W., Fowler, H. W. and Coulson, J., eds], 3rd Edition, revised, Oxford Clarendon Press).

been separated and identified by sodium dodecylsulphate equilibrated polyacrylamide gel electrophoresis as Band III component [1, 2, 12] (also called 'band a' component [11]) and the sialoglycoprotein or glycophorin [13]. Glycophorin (molecular weight approx. 50 000* [ref. 1]) has been characterized as an amphipatic sialoglycopeptide structure (approx. 60 % carbohydrate, 40 % protein [9, 13] with multiple oligosaccharide chains attached near its N-terminal peptide end. The oligosaccharide chains are basically of two types and bear approx. 80 % of the total membrane sialic acid, AB(H) and MN blood group antigens, lectin binding sites and other surface receptors [8–10, 13, 14]. The primary sequence of glycophorin indicates that the oligosaccharides are part of a highly hydrophilic peptide region [10]. The C-terminal region of glycophorin's primary structure mainly consists of hydrophilic amino acids, while an internal region is predominantly composed of apolar amino acids and is intercalated into the membrane hydrophobic interior [8–10]. Much less is known about Band III component(s). It is a larger molecule than glycophorin (molecular weight approx. 90 000–100 000 by sodium dodecylsulphate equilibrated polyacrylamide gel electrophoresis [1, 2]; 150 000 by gel filtration [15]) and contains less carbohydrate (6–8 %, [see refs 3 and 15]). Recent studies implicate this glycoprotein in a variety of membrane functions [15–18].

Freeze-fracture and freeze-etch studies have localized glycophorin molecules in human erythrocyte ghost membranes. During freeze-fracture the bilayer regions of erythrocyte membranes are split, revealing the existence of numerous structural discontinuities, the membrane intercalated particles [19, 20]. To reveal outer and inner surfaces, membrane suspensions are frozen in distilled water or very dilute buffers and, after freeze-fracture, are exposed to a period of sublimation at low temperature (freeze-etching) [19–21]. Combined use of freeze-fracture, freeze-etch and molecular labeling techniques demonstrated exclusive association to the membrane intercalated particles of antigens, receptors and acidic groups expressed by glycophorin at the outer membrane surface (A and B antigens [20, 22]), influenza virus and phytohemagglutinin receptors [13, 23], and anionic sites labeled at pH 1.8 [24] or pH 5.5 [25]. Extensive labeling of the membrane intercalated particles and agreement of estimates of the number of membrane particles, antigen sites and glycophorin molecules per ghost led to the proposal that each particle contained a glycophorin molecule [22, 23, 26]. However, a comparison of the molecular weight of the glycophorin with the size of the membrane intercalated particles (approx. 85 Å uncorrected for shadow thickness) renders it possible that the erythrocyte membrane particles are not exclusively composed of single glycophorin molecules. However, it is not possible to estimate molecular weight from measurements of particle diameters (and vice versa) because: (1) the exact conformation and shape of the component(s) which constitute the particle as well as the position of these component(s) relative to the membrane profile are not known; (2) at present the extent to which a particle represents a freezing induced perturbation of membrane lipids in the vicinity of a proteic intercalation cannot be defined; (3) the particles seen on the fracture faces represent structural asymmetries along the plane of the membrane, and consequently, it is possible that a

* Glycoproteins are known to exhibit anomalous migration in sodium dodecylsulphate equilibrated polyacrylamide gel electrophoresis gels. Consequently, molecular weight determinations are subject to error (Glossman and Neville [53] and Segrest and Jackson [54]).

hypothetical protein intercalation formed by two opposed subunits meeting at the level of the bilayer juncture would not result in a freeze-fracture membrane particle; and (4) precise correction for the thickness of platinum–carbon replica is difficult.

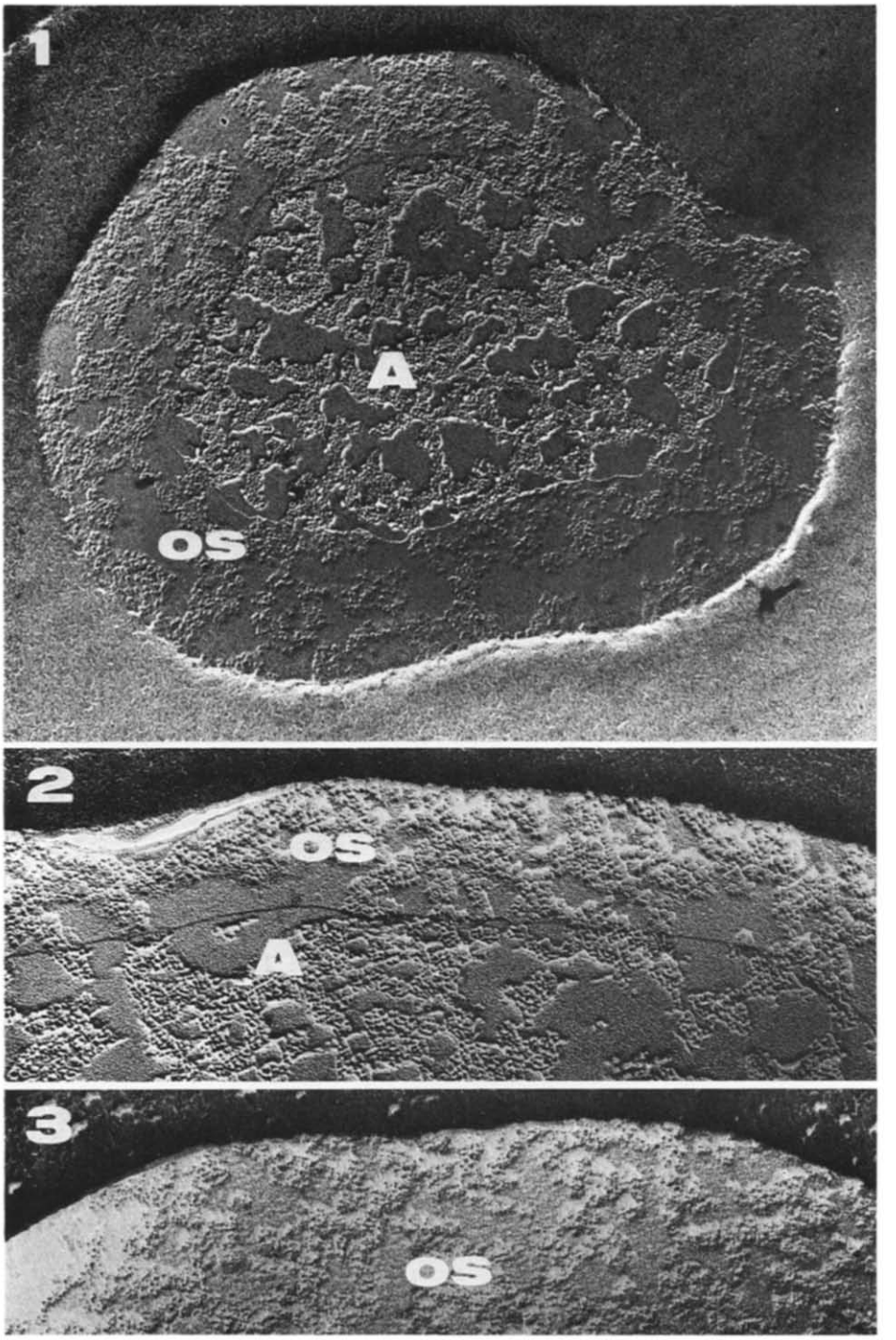
Because the number of Band III component copies per erythrocyte approximates the number of particles, component III has been alternatively hypothesized as the membrane intercalated particle [4]. However, it is also possible that the membrane intercalated particles are composed of glycophorin and Band III component molecules [3], possibly within each particle [15, 25]. Recent studies show that in human erythrocyte membranes, a Band III component is the major concanavalin A receptor while glycophorin interaction with concanavalin A is negligible or absent [15, 27]. We report here the distribution of concanavalin A-binding sites on the human erythrocyte outer surface by freeze-etch electron microscopy. Our results demonstrate coincidence of the distribution of concanavalin A binding sites with that of the particles and support the concept of the membrane intercalated particles of human erythrocyte ghost membranes as oligomeric structures containing at least glycophorin and component III molecules.

MATERIALS AND METHODS

Ferritin–concanavalin A conjugate was prepared [28, 29] using final concentrations of the following reagents: ferritin (11× crystallized; see ref. 30), 4 % concanavalin A (2× crystallized, Calbiochem), 1.5–2 %; glutaraldehyde (vacuum redistilled, Polysciences), 0.02–0.03 %; α-methyl-D-mannopyranoside (Calbiochem), 0.1 M in 0.2 M NaCl–0.05 M sodium phosphate buffer, pH 6.5. The conjugate was affinity purified on a 2.5 cm × 100 cm column of Sephadex G-200 (Pharmacia) using 0.1 M sucrose to elute the ferritin–lectin conjugate [29]. After extensive dialysis of each 1 ml fraction, the peak containing the conjugate was pooled and titrated against washed rabbit erythrocytes.

Human erythrocyte ghosts were prepared from outdated human blood [31]. For each experiment 100 μl of packed ghosts were incubated in 15 ml of 8 mM potassium phosphate buffer–1 mM $CaCl_2$, pH 5.5 at 35 °C for 30 min to aggregate the membrane intercalated particles [32]. The ghosts were centrifuged and incubated in ice for 10 min in: (1) 0.7 ml conjugate (≈ 1 mg protein/ml; 500 HA units*; dialysed in Ca^{2+}-containing potassium phosphate buffer (pH 5.5) or (2) 5 ml concanavalin A (200 μg/ml) solution in Ca^{2+}-containing potassium phosphate buffer (pH 5.5). The ghosts were then washed in 4 mM Ca^{2+}-containing potassium phosphate buffer (pH 5.5) and frozen in the liquid phase of partially solidified Freon 22 cooled by liquid nitrogen. Control ghosts were labeled with ferritin–concanavalin A conjugate as described and then incubated in 0.1 M α-methyl-D-glucopyranoside at room temperature for 15 min. Freeze-fracture and etching were carried out in Balzer's device (stage temperature −100 °C). The specimens were shadowed and the replicas recovered, cleaned and observed with a Hitachi HU-12 electron microscope. The micrographs are mounted with shadow from bottom to top. Shadows are white.

* HA, hemagglutination units determined with rabbit erythrocytes as described by Nicholson and Blaustein [58].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RESULTS

Observation of the outer surface of erythrocyte ghost membranes preincubated at pH 5.5 and labeled with ferritin–concanavalin A conjugate demonstrates massive labeling over the surface regions which correspond to aggregates of membrane intercalated particles: (1) where extensive areas of membrane surface are exposed (Fig. 1), the pattern of aggregation and distribution of ferritin–concanavalin A molecules closely resembles the random, continuous network formed by aggregation of the membrane intercalated particles induced by incubation in Ca^{2+}-containing potassium phosphate buffer (pH 5.5) at 35 °C; (2) where areas of fracture face A are observed, contiguous to the outer surface, the patterns of particle aggregation are contiguous and continuous with similar pattern of distribution of the ferritin–concanavalin A conjugate (Figs 1 and 2). The label is not observed on particle-free areas of the membrane; (3) treatment of ferritin–concanavalin A labeled membranes with α-methyl-D-glucopyranoside results in removal of almost all of the label.

Observation of the outer surface of erythrocyte ghost membranes treated with non-conjugated concanavalin A shows raised particulate areas with a distribution similar to that of the aggregates of membrane intercalated particles (Fig. 3). Such areas can be distinguished from the slight protrusions of the particles in non-labeled membranes (see Figs 3, 4 of ref. 25 or Fig. 3 of ref. 49). However, due to the small size of concanavalin A molecules, resolution of individual concanavalin A molecules is generally difficult except on areas with a particularly favorable angle of shadow (Fig. 3).

DISCUSSION

Our study localizes the concanavalin A receptors sites to the membrane intercalated particles of human erythrocyte ghost membranes. Because ferritin–concanavalin A molecules are larger than the membrane particles, aggregation of the latter was essential in order to relate the label to smooth regions (bilayered domains)

* When the experiments demonstrating pH-induced translational aggregation of the membrane intercalated particles were performed, it was also observed that no such aggregation could be induced in intact erythrocytes or pink ghosts by incubation at 37 °C in 8 mM potassium phosphate buffer at pH 5.5, 1 mM $CaCl_2$ (Pinto da Silva, P., unpublished data), conditions which normally produce intense membrane intercalated particle aggregation in hemoglobin-free ghosts. It was not clear at that time whether this observation was due to a different internal pH of erythrocytes of pink ghosts or whether the removal of some peripheral membrane component (such as spectrin) was necessary to allow for translational movement of the membrane intercalated particles.

Fig. 1. Human erythrocyte ghost membranes labeled with ferritin–concanavalin A. The pattern of aggregation of the label on the outer surface (OS) closely resembles the random network of aggregated membrane intercalated particles on the fracture face (A). The pattern of distribution of ferritin–concanavalin A is contiguous and continuous with that of the membrane intercalated particles. × 40 000.

Fig. 2. Legend is the same as in Fig. 1. × 70 000.

Fig. 3. Human erythrocyte ghost labeled with unconjugated concanavalin A. The pattern of distribution of the label is similar to that of the membrane intercalated particles or ferritin–concanavalin A (see Figs 1 and 2, above). In regions with low angle of shadow, sharp protrusions probably represent individual concanavalin A molecules. × 70 000.

or to the membrane particles [22, 25]. Incubation of the ghosts at pH 5.5 in presence of Ca^{2+} causes aggregation of the particles into random, almost continuous networks [32*]. Our results demonstrate extensive and exclusive association of ferritin–concanavalin A or unconjugated concanavalin A molecules with the regions at the outer membrane surface which correspond to membrane particle aggregates. Addition of α-methyl-D-glycopyranoside results in almost quantitative removal of the label from the membrane surface [28, 29]; therefore, the binding was specific.

The dense labeling of ferritin–concanavalin A or unconjugated concanavalin A molecules over regions of membrane particle aggregates indicates that most or even all the membrane intercalated particles contain receptors for concanavalin A. This is consistent with quantitative binding experiments with ^{125}I-concanavalin A which have determined that the number of concanavalin A binding sites per erythrocyte ($0.8–1 \cdot 10^6$, see ref. 33) approximates the number of membrane particles per ghost. Because the major concanavalin A receptor at the surface of erythrocyte membranes is contained in a Band III component [15] and concanavalin A receptors are exclusively associated with the membrane particles, it follows that a Band III component is a part of the membrane intercalated particles. Freeze-etch localization of antigen, lectin and influenza virus receptors and negative charges of glycophorin to the membrane intercalated particles [22, 23, 25] demonstrates that glycophorin is also a component of the particles. Consequently, we envisage the membrane intercalated particles as oligomeric structures [34, 35], containing, at least, a Band III component(s) and a glycophorin molecule [15, 25, 36]. Such a unit would have a minimum molecular weight of approx. 150 000–200 000 which could more easily account for the 85 Å average diameter of the membrane particles*.

Chemical and enzymatic labeling of the two major protein components exposed at the surface of the erythrocyte and exclusive association of a variety of surface sites contained in these two major glycoproteins with the membrane intercalated particles makes it likely that most, if not all, of the protein components displayed at the outer surface are confined to this structure and implies extensive exposure of lipid regions [7, 20, 36, 37]. Lipid exposure at the outer surface might also be suggested by observation of the etched outer surface of erythrocyte membranes over the surface regions which correspond to bilayer domains (particle-free areas) because these surface regions do not reveal the presence of slight protrusions which might suggest the presence of other globular components. Indeed, these areas look identical to the etched surfaces of phospholipid vesicles or of vesicles prepared from purified erythrocyte membrane lipids (Pinto da Silva, unpublished results). However, the relatively low resolution of shadowing techniques (size of grain about 2 nm) and the possibility of a thin coat of nonsublimable water molecules at the cell surface makes it likely that protein molecules spread at the surface will escape detection. For instance, individual anti-A antibody molecules (IgG, ≈ 160 000 molecular weight;

* The notion that each membrane particle contains a Band III component and glycophorin is further supported by the uniform behavior of the particles (i.e., all aggregate at pH 5.5; or upon proteolytic digestion [55] and the absence of at least two distinctly sized subpopulations. Differences in size and/or properties would be expected if the two proteins did not co-exist in each membrane particle. Furthermore, because the low molecular weight of glycophorin and the fact that a large part of the molecule is expressed at the outer surface, glycophorin might originate a much smaller particle (possibly undetectable; see introduction).

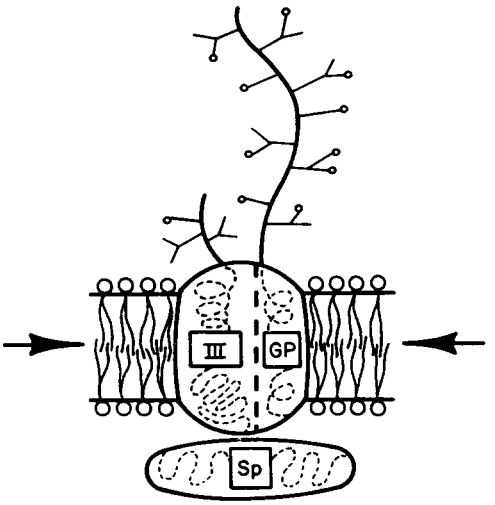

Fig. 4. Diagrammatic representation of "permeaphore", the oligomeric structure observed as the freeze-fracture membrane intercalated particles in human erythrocyte ghost membranes. GP, glycophorin; III, Band III component(s); Sp, spectrin. These components do not represent the total composition of the permeaphore, but they probably account for the major integral units. Additional spectrin molecules are present in intact cell membranes, possibly forming a network with other spectrin molecules at the inner membrane surface. Arrows indicate the cleavage plane of the bilayer regions of the membrane during freeze-fracture. Topological distribution of hydrophilic and hydrophobic spaces is depicted elsewhere [32, 50].

or IgM, $\approx$ 900 000 molecular weight) cannot be identified when bound to the membrane particles at the outer surface of human erythrocyte ghosts, although their collective detection is possible [38].

Visible rugosities have been observed on the inner etched surface which co-aggregate with the membrane intercalated particles under conditions which cause their aggregation (see ref. 25, Fig. 7). These rugosities are probably due to peripheral membrane proteins such as spectrin, an inner surface peripheral protein [30] of approx. 250 000 molecular weight [39–41]. Spectrin has been shown to be structurally linked to glycophorin from anti-spectrin-induced aggregation experiments [42, 45] and lectin-induced transmembrane effects on the organization of spectrin (measured by enhancement of chemical crosslinking of spectrin at the inner surface [44]). In consequence, the membrane intercalated particles probably represent an oligomeric structure containing both integral and peripheral membrane components (Fig. 4).

Functional implications

A variety of transmembrane functions seems to be associated with the membrane intercalated particles of human erythrocyte membranes: (a) Band III component(s) have been identified as the phosphorylated protein during ATP hydrolysis by a membrane-associated, Mg^{2+}-dependent Na^+, K^+-stimulated ATPase [16] and as a membrane component involved in anion permeability [17]; (b) integral erythrocyte membrane proteins are also probably involved in the transmembrane passage of glucose as this process is not affected by proteolytic digestion [18, 45, 46] or by non-enzymatic removal of 50–75 % protein [47], but it is inhibited during the

initial phase of binding (at or near the outer surface) by slowly permeating sulphydryl reagents (*p*-chloromercuribenzoate sulphate and chlormerodrin) [48, 49]*; and (c) the membrane intercalated particles probably represent preferred sites for transmembrane passage of water molecules subliming at low temperature (−100 °C) [50].

Functions dependent on transmembrane events may require a structure which provides topological and structural continuity of hydrophylic spaces between the outer and inner membrane surfaces, possibly through specific pathways. Although it is clear that the presence, per se, of such a structure cannot account for the functional complexity of the erythrocyte membrane, we propose that a transmembrane structure (a "permeaphore") may represent, topologically and structurally, a necessary differentiation interrupting hydrophobic erythrocyte membrane bilayer domains. The membrane intercalated particles of human erythrocyte ghosts probably fulfill the requirements of a "permeaphore" because they represent amphipatic structures which are intercalated across the hydrophobic regions of the membrane and are in polar contact with the hydrophylic spaces at the outer and inner surface [25, 32, 50]. We are aware that our hypothesis rests, in part, on results obtained for erythrocytes and a variety of ghost preparations which may not be strictly structurally or functionally comparable [51, 52]. Furthermore, extrapolation of this hypothesis to other systems is, at present, impossible because of the incomplete understanding of the topological, structural and functional nature of particles in other plasma membranes.

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* It is interesting that phloretin inhibits both anion permeability and sugar transport across the erythrocyte membrane (Weith et al. [56] and LeFevre [57]).

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