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Fine Structure of the Band 3 Protein in Human Red Cell Membranes: Freeze-Fracture Studies

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The major red cell membrane protein, band 3, is a glycoprotein which extends across the membrane from the extracellular space into the cytoplasmic compartment. It is widely held that band 3 is a component of the intramembrane particles (IMP) which can be demonstrated by freeze-fracture electron microscopy. In this study, we find that the outer surface poles of the IMP can be seen by freeze-etching after they are unmasked by proteolysis under conditions which excise the surrounding sialopeptides from the membrane. The poles appear as distinctive projections, 30–50 Å in diameter, the "ES particles." The ES particles remain associated with the outer surface of the membrane following cleavage of the band 3 polypeptide by chymotrypsin or pronase. This is consistent with previous biochemical studies which have shown that the 38,000-dalton outer surface segment of band 3 is intercalated in the lipid bilayer. A granulofibrillar component at the inner surface of the membrane is provisionally identified as the 40,000-dalton inner-surface domain of band 3.

Key words: band 3 protein; freeze-fracture electron microscopy; glyceraldehyde-3 phosphate dehydrogenase; integral membrane protein; intramembrane particle; membrane proteins, unmasking; spectrin

The band 3 polypeptide demonstrated on polyacrylamide gels represents the major protein constituent of the red cell membrane. It comprises approximately one-quarter of the total membrane protein [1] and exists in the membrane as a noncovalent dimer [2–4]. The 93,000-dalton band 3 polypeptide can be proteolytically dissected into three large subfragments. These have been partially characterized and consist of outer-surface, transmembrane, and cytoplasmic surface domains with apparent molecular weights of approximately 38,000, 17,000, and 40,000 daltons respectively (Fig 1) [5, 6]. The fragment generated at the outer surface is a glycopeptide which intercalates into the bilayer, where it is anchored by hydrophobic associations. It remains firmly embedded within the lipid bilayer after proteolysis; however, the association of this segment with the remainder of the molecule is unknown. The transmembrane segment extends through the

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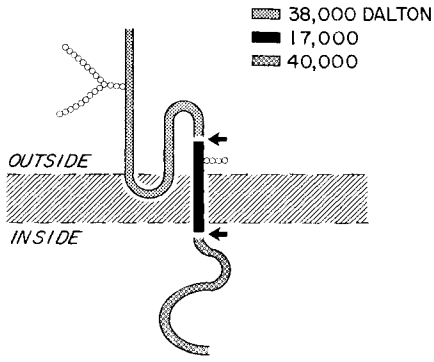


Fig 1. Highly schematic representation of the red cell protein band 3 showing the disposition of three large subfragments which are generated by proteolysis with chymotrypsin. Arrows indicate the sites of proteolysis. This is based on biochemical data reviewed by Steck [22] and ultrastructural data described in this paper.

hydrophobic core of membrane and can only be liberated from the bilayer with detergents. In contrast, the inner cytoplasmic domain is readily released from the membrane by proteolysis [5, 6].

Although the three-dimensional configuration of the band 3 protein within the membrane is unknown at the present time, it is established that this polypeptide spans the membrane asymmetrically and that it extends into the aqueous compartments at both membrane surfaces [1]. The studies described below represent an effort to visualize the morphologic counterparts of the band 3 polypeptide at the outer and inner membrane surfaces by freeze-fracture deep-etch electron microscopy.

MATERIALS AND METHODS

Red Blood Cells

Blood was obtained by venipuncture from a single human donor (TLS), washed three times with 150 mM NaCl–5 mM sodium phosphate buffer, and processed immediately for ultrastructural and biochemical analyses. The results of some experiments were confirmed on outdated blood from the blood bank. Unless otherwise stated, experimental procedures were performed at 0–5°C, and centrifugations were performed in a Sorvall SS-34 rotor.

Ghosts and Vesicles

Sealed ghosts were prepared by lysing red cells in 40 volumes of 5 mM sodium phosphate buffer (pH 8). After centrifugation, the pellet was incubated in an equal volume of 0.3 M NaCl–10 mM Na phosphate buffer (pH 8) at 37°C for 1 h and washed twice in 150 mM NaCl–5 mM sodium phosphate buffer. Unsealed ghosts were prepared by lysing cells in 40 volumes of 5 mM sodium phosphate buffer (pH 8) and washing twice in the same buffer [7, 8]. To prepare inside-out (IO) vesicles, open ghost pellets were mixed with 39 volumes of 0.5 mM sodium phosphate buffer (pH 8.3). After 30 min on ice, the suspension was centrifuged at 15,000 rpm for 30 min. The supernatant was re-

moved and the pellets were incubated overnight at 5°. Pellets were homogenized by five passes through a 27-gauge hypodermic needle [7, 9]. Aliquots (1.2 ml) of homogenate were layered over a 2-ml barrier of Dextran 110 (Pharmacia) in 0.5 mM sodium phosphate buffer (pH 8) (density 1.01 g/ml). The samples were centrifuged at 15,000 rpm for 1 h at 5°. The band at the top of the dextran barrier was taken to be the IO vesicle fraction. The membranes were washed in 0.5 mM sodium phosphate buffer (pH 8.0) by centrifugation at 18,000 rpm for 30 min. The pellet was resuspended in a small volume of 0.5 mM sodium phosphate buffer (pH 8.0). Acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase (G3PD) accessibility in the input homogenate as well as in the gradient-purified IO vesicle fraction were quantified to estimate the percentage of vesicles of inside-out orientation [7, 9].

Proteolysis of Intact Cells, Ghosts, and Vesicles

Packed suspensions of intact red cells, ghosts, or vesicles were digested for 1 h with an equal volume of fresh chymotrypsin (Sigma) or trypsin (Worthington) at a final concentration of 0.1 or 1.0 mg/ml. Diisopropylfluorophosphate (DFP: Sigma) was added (to a final concentration of 1 mM for red cells and ghosts and 0.5 mM for IO vesicles) to terminate enzyme activity. Other experiments were performed with pronase (Sigma) at a final concentration of 1 mg/ml. Following proteolysis, membrane preparations were incubated at 0°, 23°, or 37°C for 1–4 hours to enhance IMP clustering. Aliquots of each sample were taken for SDS-polyacrylamide gel electrophoresis, performed according to Fairbanks, Steck, and Wallach [8], as modified by Steck and Yu [10].

Freeze-Fracture Electron Microscopy

Samples were fixed in PBS-buffered 1% glutaraldehyde (pH 8.0) at 0°C for 30 min, and then washed three times in distilled water. Specimens were placed either in 20% glycerol or water and rapidly quenched in Freon 22 to −150°C [11]. Freeze fracturing and freeze etching were done in a Balzer Model BAF 301 freeze-etch unit. The stage temperature was maintained at −100° for 60–90 sec for deep etching. Replication was with platinum-carbon for 1 sec at a 45° angle, followed by carbon coating for 10–12 sec. Some specimens were rotary-shadowed at a 25° angle, with a Balzers rotary shadowing unit [12]. Replicas were floated on saline, cleaned with Chlorox, rinsed in water, and collected on 100-mesh copper grids. Replicas were photographed in either a Philips EM-300 or 301 electron microscope. For measurements of intramembrane particle diameters, electron micrographs of unidirectionally shadowed membranes were printed at a final magnification of 150,000 ×. Measurements were made perpendicular to the direction of platinum shadowing for 200–300 consecutive particles.

RESULTS

Effects of Proteolysis on Electrophorograms

Gel electrophoresis of red cell ghosts and IO vesicles dissolved in SDS showed that band 3 is not diminished by the vesiculation procedure, although the amount of spectrin (bands 1 and 2) is markedly reduced. Proteolysis of unsealed ghosts and IO vesicles with chymotrypsin or trypsin (0.1 or 1.0 mg/ml) at 0° for 1 h produced extensive digestion of the major membrane proteins (Fig 2).

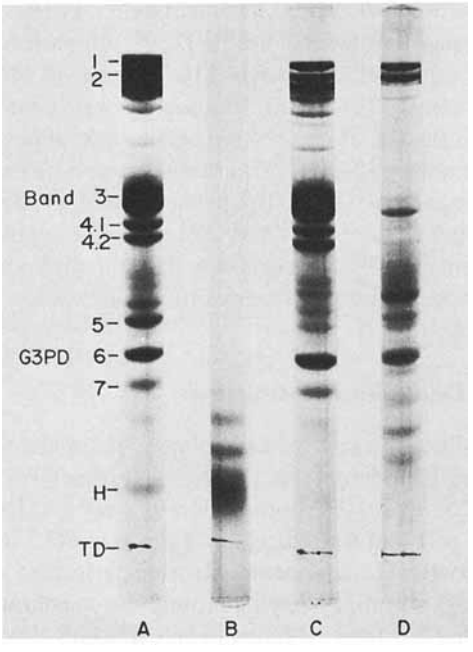


Fig 2. SDS-polyacrylamide gels of membrane preparations. H) residual hemoglobin; TD) tracking dye. Gel A, fresh ghosts. Gel B, unsealed ghosts digested with chymotrypsin (1 mg/ml) for 1 h at 0°C; there is extensive digestion of all major membrane proteins. Gel C, sealed inside-out vesicle fraction. Acetylcholinesterase [7] and G3PD accessibility assays [9] demonstrated that 88% of the vesicles had an inside-out orientation. The remainder were right-side-out vesicles. Most of the spectrin (bands 1 and 2) and actin (band 5) have been eluted. Gel D, sealed inside-out vesicle fraction digested with trypsin (5 μg/ml) for 1 h at 0°. Band 3 is markedly reduced. The residual band 3 protein may be in right-side-out vesicles which contaminate the fraction.

Freeze-Fractured Red Cell Membranes

Four membrane surfaces are revealed by freeze-fracture electron microscopy. According to recently standardized nomenclature, the natural surfaces of the membrane are designated ES for the extracellular or outer surface, and PS for the protoplasmic or inner surface [13]. The fracturing process generates two novel surfaces: PF, the fracture face of the protoplasmic leaflet of the membrane; and EF, the fracture face of the extracellular leaflet of the membrane. The two fracture faces bear populations of intramembrane particles (IMP), designated IMP_P on PF and IMP_E on EF surfaces [14, 15]. An asymmetrical distribution of the IMP persists when membranes are fragmented into right-side-out and inside-out vesicles and thus serves as a useful marker of membrane orientation [16].

Ultrastructure of Intramembrane Particles

In high-quality replicas of intact red cell membranes, the IMP_P and IMP_E appeared somewhat pleomorphic. In unidirectionally shadowed replicas, there was variability in the apparent diameters of the IMP_P (Fig 3), although this could occur if the IMP are asymmetrical. However, this variability of diameters was also observed in rotary-shadowed preparations in which the majority of IMP_P appeared symmetrical in the plane of the membrane (Fig 4). Therefore, the variability cannot be ascribed to IMP asymmetry alone.

Measurements of IMP diameters confirmed the impression that the IMP are somewhat variable in size as is reflected in the standard deviations of the measurements. In 1% glutaraldehyde-fixed, glycerinated intact red cells, the mean IMP_P diameter was 66 ± 12 Å (±SD). The mean diameter of IMP_E was 74 ± 13 Å. Although the large majority of IMP on a given fracture face fall within a relatively narrow size range, there may be subpopulations which have a different morphology. This was supported by the demonstration of previously undescribed short rods at fracture faces by rotary shadowing (Fig 4).

The ultrastructure of the IMP in isolated red cell membranes was examined before and after protease digestion. Proteolysis with chymotrypsin, trypsin, and pronase cleaved the membrane polypeptides into small segments and released 50–80% of the protein from the membrane. However, the IMP remained morphologically intact (Figs 5–8).

IMP clustering was not observed in intact red cells, although there was occasionally mild clustering in undigested isolated ghost membranes. Proteolysis of isolated membranes at 0° produced significant clustering, which was enhanced by incubation of isolated membranes at 23° or 37° either during or subsequent to proteolysis. Proteolysis of intact human cells did not induce a redistribution of IMP_P.

Unmasking of the Outer Surface Pole of the IMP

The extracellular surface (E surface) of deep-etched intact red cells, isolated membranes, and IO vesicles appeared smooth or finely granular (Fig 5). There was a suggestion

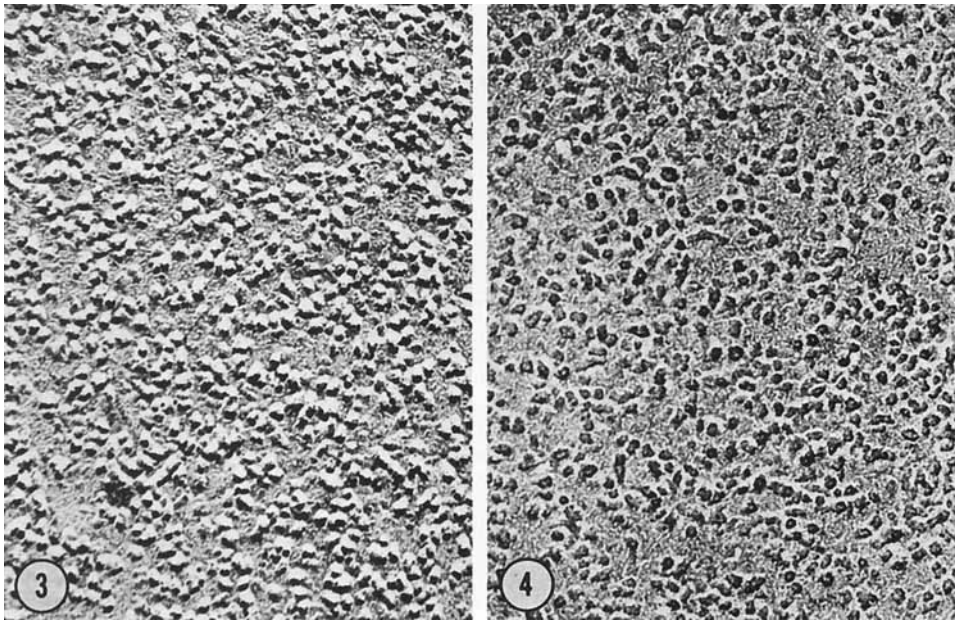
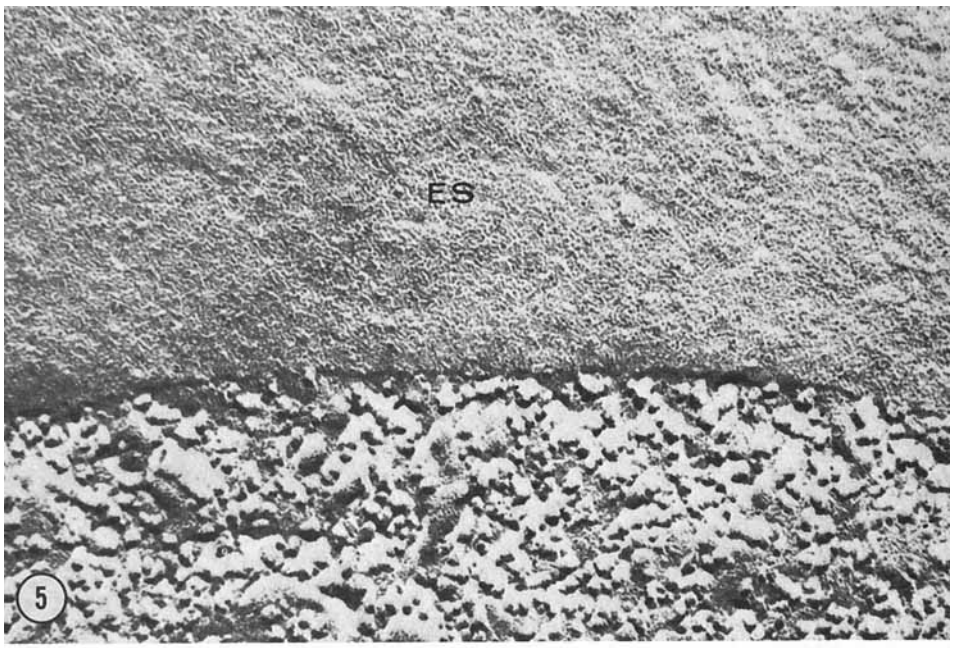


Fig 3. Electron micrograph of the PF surface of a human red cell fixed in 1% glutaraldehyde and glycerinated prior to freeze-fracturing. The surface was unidirectionally shadowed with platinum-carbon at a 45° angle. The IMP_P vary in diameter and cast shadows of different lenghts (× 150,000).

Fig 4. PF surface of a human red cell prepared as in Figure 3 but rotary-shadowed at a 25° angle. The variability in IMP_P sizes is confirmed. In addition to the round particles, many short rods are demonstrated at the PF surface by rotary shadowing (× 150,000).



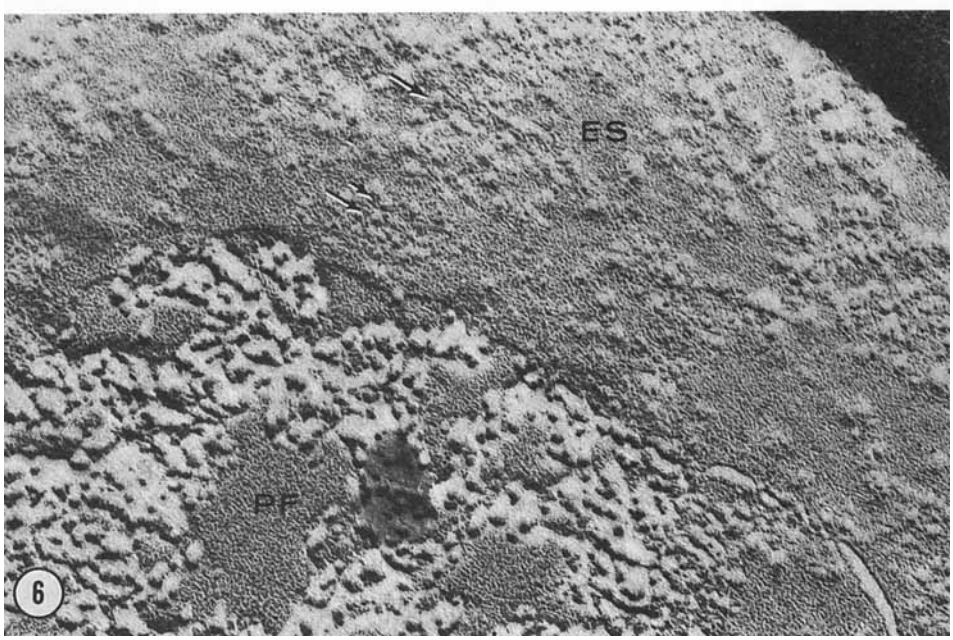
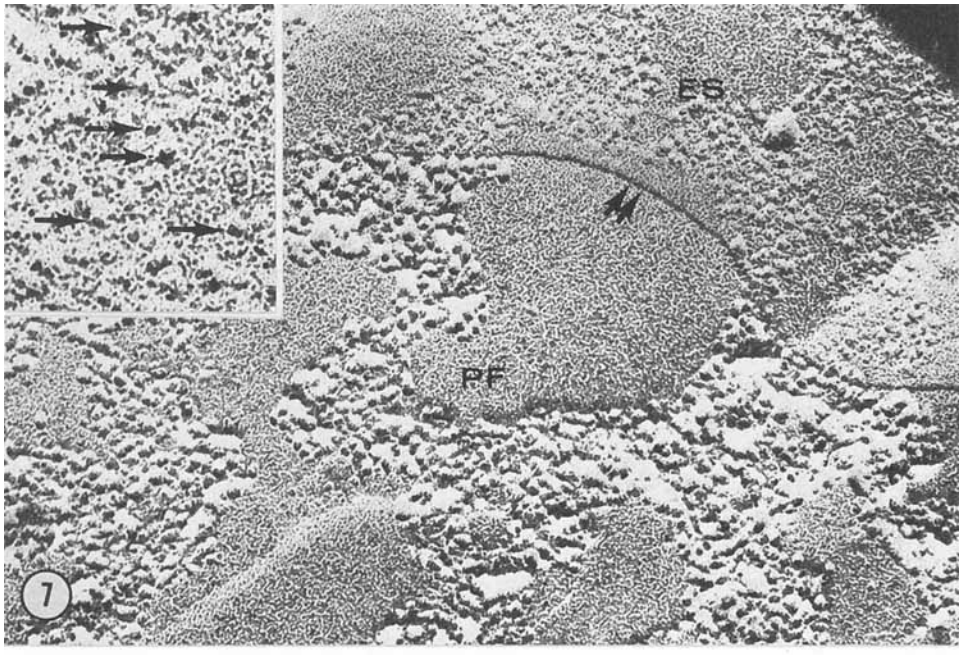


Fig 5. Freeze-fractured, deep-etched open ghost without proteolysis. The outer or extracellular surface (ES) of the membrane is nearly devoid of substructure. IMP_P (bottom) are non-clustered (× 156,000).

Fig 6. *Open ghost* following digestion with chymotrypsin (1 mg/ml) for 1 h at 0° C. Many 30- to 50-Å particles (arrows) are present at the E surface (ES). The ES particles are mildly clustered, as are the IMP_P at the P face (PF), following proteolysis at 0° (× 156,000).



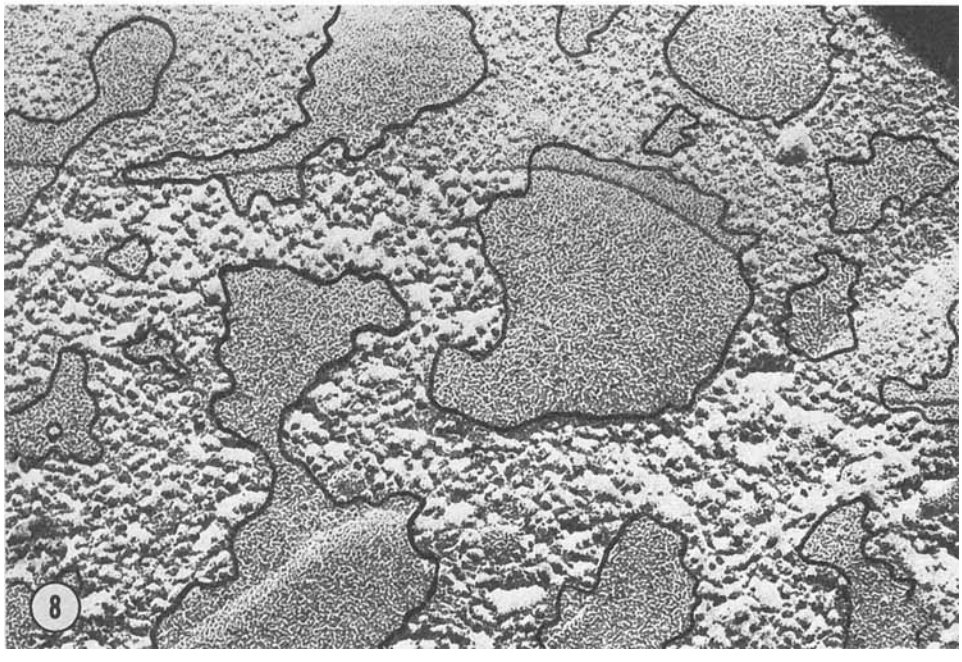


Fig 7. Top: Open ghost following digestion with pronase (1 mg/ml) at 0° for 4 h. Double arrows show where the fracture passes from the interior of the membrane (PF) to the extracellular surface (ES). The IMPp and the ES particles are clustered (× 156,000). Insert: High magnification of ES particles (arrows). The particles are small and cast long shadows (× 310,000).

Fig 8. Duplicate print of the electron micrograph in Figure 7 (× 156,000). Boundaries have been drawn with a pen between ES-particle-containing areas and smooth areas on the extracellular surface (ES), and between IMPp-rich areas and smooth areas on the protoplasmic face (PF). Particle-containing areas have the same topographical distribution at the PF and ES surfaces.

of discrete particles at the E surface, but particles casting long shadows were not observed. Amorphous mounds were present overlying the IMP_P aggregates at the E surface of isolated membranes in which mild IMP_P clustering was induced by incubation at 37°C in the absence of protease [15].

A discrete population of 30- to 50-Å particles, designated ES particles, was observed at the outer surface of the membranes following proteolysis (Figs 6, 7). These ES particles cast long shadows, indicating that the structures they represent project well above the adjacent membrane. The ES particles were somewhat difficult to quantitate in freeze-fracture replicas because of their small size, but they appear to be represented in numbers similar to the IMP_P (Weinstein, Khodadad, and Steck, manuscript in preparation). Their topography also closely resembled that of the IMP_P (Figs 7, 8). Based upon these observations, we can hypothesize that the ES particles are the outer surface poles of the IMP_P, revealed by proteolysis.

Since the IMP_P have been ascribed to band 3, it follows that the ES particles may contain the outer surface domain of the molecule. It is conceivable that following proteolysis, the constituent polypeptides of the IMP_P and ES particles might be dissociated by lateral diffusion in the membrane if they are merely linked covalently (Fig 9). Therefore, we subjected isolated red cell membranes to proteolytic digestion with pronase or chymotrypsin at 0° under conditions which cleave every copy of band 3. Following proteolysis, we encouraged the differential redistribution of the IMP_P and ES particles by incubating the digested membranes at 23° or 37° for up to 4 h. The two populations of particles consistently cosegregated, implying that there are stable noncovalent associations between the IMP_P and the ES particles. Furthermore, that segment of the band 3 polypeptide represented in the ES particles may contribute to the image of the IMP_P. The emergence of ES particles following proteolysis is consistent with the observation that IMP_P morphology is unaffected by such treatment.

Band 3 at the Protoplasmic Surface

In an attempt to visualize band 3 protein at the protoplasmic surface (P surface) of the membrane, we examined deep-etched inside-out vesicles which were depleted of nearly all of their spectrin and actin. The P surface of the vesicles contained two prominent components: a granulofibrillar component; and a population of discrete particles (PS particles) having an average diameter of approximately 90 Å (Fig 10). Digestion with trypsin, chymotrypsin, or pronase under conditions which result in the release of the 40,000-dalton segment of band 3 from the cytoplasmic surface, without producing a further reduction in residual spectrin and actin, also caused the removal of the granulofibrillar component leaving intact the 90-Å PS particles (Fig 11).

DISCUSSION

These freeze-fracture studies support the view that the red cell membrane protein band 3 is a component of the IMP_P as postulated previously by many other investigators [1, 17–19]. In the past, it has been difficult to reconcile the relatively flat, structureless appearance of the outer surface of the red cell membrane with the concept of glycopeptides extending into the extracellular compartment [14]. A number of explanations which might account for the lack of detailed structure at the outer surface of the mem-

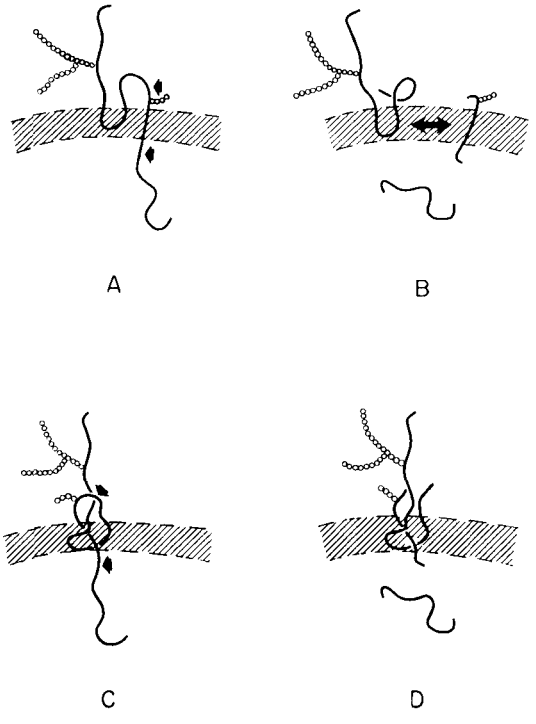


Fig 9. Scheme depicting two possible fates of a proteolytically cleaved glycoprotein, corresponding to the IMP and the ES particles which are visualized by freeze-fracture and deep-etch electron microscopy. A) Glycoprotein *without* noncovalent associations between the extracellular and transmembrane domains of the polypeptide. Arrows show the locations of extracellular and intracellular protease-sensitive sites. B) Molecule corresponding to "A" following proteolysis. The extracellular domain has dissociated from the membrane-spanning segment, but remains intercalated in the lipid bilayer. The inner surface segment, which does not intercalate into the bilayer, is shed. C) Glycoprotein *with* noncovalent associations between the extracellular and transmembrane domains of the polypeptide. Arrows show the locations of protease-sensitive sites. D) Molecule corresponding to "C" following proteolysis. The extracellular domain remains noncovalently associated with the transmembrane domain. The inner surface segment is shed.

brane have been considered on theoretical grounds, but none have been fully substantiated experimentally [20].

We have now demonstrated that a distinctive particulate component, the ES particle, can be "unmasked" at the outer surface of the red cell membrane by proteolysis. There are several observations which indicate that these outer surface particles and the IMP_P are structurally related. First, the ES particles appear to overlie the IMP_P and coaggregate with them when IMP_P clustering is produced experimentally. Secondly, these two populations of particles seem to be present in approximately equivalent numbers, although precise quantitation of the ES particles is difficult because of their small size. These observations suggest that the ES particles may be the outer surface poles of the IMP_P. If the band 3 polypeptide is represented in the IMP_P (an idea which is consistent with available evidence), then the band 3 molecule is likely to be a component of the ES particles as well.

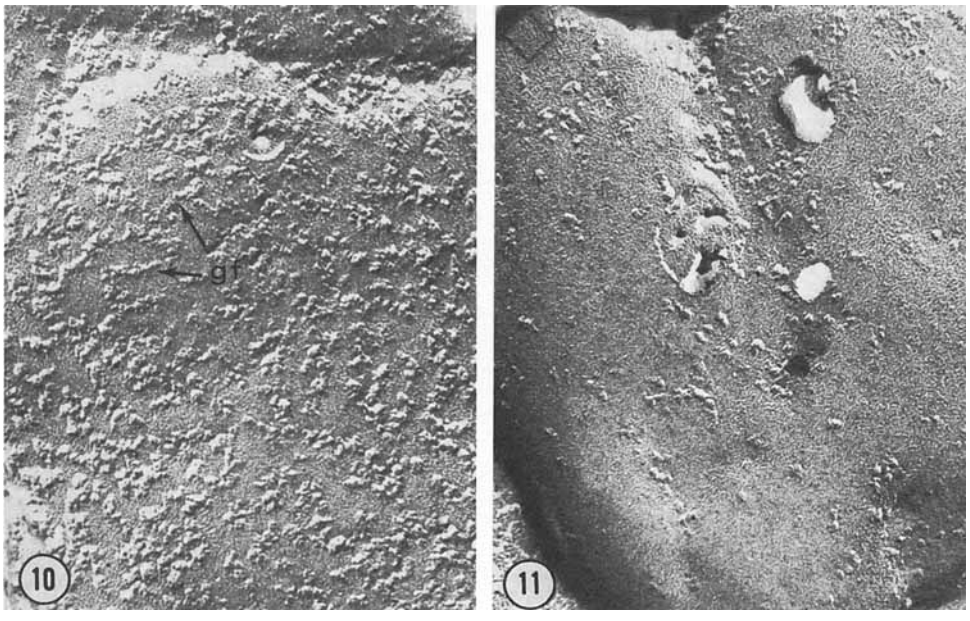


Fig 10. P surface of an inside-out vesicle. A granulofibrillar component (gf) is prominent on this deep-etched surface. PS particles are present, but are relatively inconspicuous prior to proteolysis (× 110,000).

Fig 11. Inside-out vesicle after digestion with trypsin (5 μg/ml). The granulofibrillar component of the P surface (inner surface) of the membrane has been cleaved away. Protease-resistant 90-Å particles are prominent after trypsinization has removed the granulofibrillar component (× 110,000).

The mechanism by which partial proteolysis reveals the ES particles is uncertain. However, there are several possibilities that merit discussion. The first possibility is that the ES particles represent residual proteolytic enzymes; this possibility was eliminated by polyacrylamide gel electrophoresis of SDS-solubilized membranes. Bands corresponding to the molecular weights of trypsin, chymotrypsin, and pronase were not represented on these gels. A second possibility is that the emergence of ES particles is due to an intramolecular rearrangement of partially digested band 3 molecules. This is unlikely because the ES particles are made visible by proteolysis of resealed ghosts by trypsin under conditions which do not permit cleavage of the band 3 polypeptide [5, 6, 21]. A third possibility is that proteolytically sensitive sialoglycoproteins are released from the membrane, unmasking the stable outer surface domains of band 3. This explanation is the most plausible in view of the differential sensitivity of band 3 and sialoglycoproteins to proteolysis at the extracellular surface of the cell [21].

Several lines of evidence support the hypothesis that the granulofibrillar component at the inner surface of the membrane contains the 40,000-dalton intracytoplasmic extension of the band 3 polypeptide.

1. The granulofibrillar component persists after all peripheral membrane proteins, including spectrin and actin, are removed from vesicle membranes with either dimethylmaleicanhydride or para-chloromercuribenzene sulfonate [15].

2. The granulofibrillar component can be selectively decorated with glyceraldehyde-3-phosphate dehydrogenase (G3PD) [15], which binds exclusively to the cytoplasmic

domain of band 3 [3]; with proteolysis, binding as well as decoration with G3PD is abolished concomitantly with the release of the 40,000-dalton fragment.

3. The band 3 polypeptide and the granulofibrillar component are both exquisitely sensitive to proteolysis.

4. The topographical distribution of the granulofibrillar component of the PS array at the inner surface of the membrane and the IMP_P within the membrane are similar.

In summary, proteolytic digestion has different effects on the ultrastructure of the outer surface, the inner surface, and the interior of the red cell membrane. The images of individual IMP_P are unaffected by proteolysis at 0°. Structures which we have tentatively identified as the outer surface projections of the IMP_P, the ES particles, become apparent after proteolysis, whereas the corresponding PS projections, the granulofibrillar component, disappears. This behavior can be accounted for by current knowledge of the disposition of the band 3 polypeptide in the membrane (see Steck [22]).

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