

DEMONSTRATION OF THE OUTER SURFACE OF FREEZE-ETCHED RED BLOOD CELL MEMBRANES

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Freeze-etching promises to be a valuable technique for the ultrastructural study of cells and subcellular components. The tissue specimen is cleaved while frozen by liquid nitrogen, and the exposed faces are replicated by platinum-carbon shadowing while under high vacuum. Thus, this technique eliminates the artifacts of thin-section microscopy produced by fixation, dehydration, embedding, and heavy metal staining.

Through the use of this technique, a new feature of cell membrane ultrastructure has been revealed. Cleavage of cell membranes results in the appearance of regularly spaced globular units approximately 85 Å in diameter on the exposed membrane face (1, 2). The chemical composition and possible functional properties of the globular units are as yet unknown, and there is also some controversy as to the anatomical location of these structures within the membrane.

This controversy is based on a fundamental disagreement as to which face of the membrane is exposed by the cleavage process. Moor and Mühlenthaler (1) and others (3, 4) have suggested that membranes are cleaved along planes which expose either their true outer or cytoplasmic

surfaces. According to this view, the 85-Å particles are located on the outside surface of the cell membrane; some are also present on the cytoplasmic side of the membrane. It has even been suggested that the particles extend completely through the thickness of the membrane (5).

However, studies by Branton (2, 6) question the above interpretations. An analysis of the appearance of membranes in freeze-etched preparations and freeze-cleaving experiments with lipid bilayers has led Branton to conclude that the freeze-fracture process actually splits membranes along a plane *within* the membrane itself rather than cleaving along its outer or cytoplasmic surface. If this is the case, then the globular particles seen on freeze-cleaved membrane faces would be located somewhere within the interior of the membrane rather than directly on the surface.

The usefulness of the freeze-etching technique in the study of membranes clearly requires that the controversy over the location of the cleavage plane in membranes be resolved. One solution would be to label the outer or inner surface of the membrane with molecules of known structure which can be identified in the freeze-etch prepara-

tions. In this study, we have labeled the outer surface of the human red blood cell ghost with fibrous actin, and have performed freeze-cleaving and deep-etching experiments to determine where the fibrous actin is located.

MATERIALS AND METHODS

Globular actin was prepared from rabbit muscle essentially as described by Rees and Young (7). Globular actin was polymerized to form fibrous actin by the addition of 1 mM $MgCl_2$.

Red blood cell ghosts were prepared from whole human blood collected in acid-citrate-dextrose and washed three times in 0.9% saline. The red blood cells were lysed at 4°C in 0.005 M Tris-Cl, 0.001 M EDTA, using a 1:30 ratio of packed cells to lysing solution, and the ghosts were collected by centrifugation at 48,000 *g* for 15 min. The ghosts were re-suspended in cold 0.1% glutaraldehyde in phosphate-buffered 0.9% saline for 3 min, then were washed two times in cold distilled water. This short fixation of the ghosts in dilute glutaraldehyde was necessary to prevent the ghosts from breaking up when they were suspended in distilled water. This did not affect the subsequent freeze-cleavage characteristics of the ghosts.

1 ml of a concentrated suspension of the glutaraldehyde-fixed ghosts was mixed with 1 ml of a solution of F-actin, and small droplets of this mixture were placed on 3-mm copper planchets for immediate freezing in liquid Freon 22 (E. I. du Pont de Nemours Co., Wilmington, Del.) and subsequent storage in liquid N_2 . Alternatively, the ghost suspension in distilled water was mixed with an equal volume of G-actin, then 1 mM of $MgCl_2$ was added and polymerization was allowed to proceed for 1 hr at 4°C; the ghosts were then washed two times in cold distilled water before preparation of the specimens for freeze-etching.

The specimens were freeze-cleaved and etched and shadowed with platinum-carbon in a Balzers freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) according to the method of Moor and Mühlenthaler (1). Some specimens were freeze-cleaved and immediately replicated with platinum-carbon so that minimal etching was accomplished. Other specimens were deep-etched before replication by keeping the cleaved specimens at $-100^\circ C$ for 40 sec to 1 min, allowing water to sublime from the fractured face. The platinum-carbon replicas were examined in a Phillips 200 electron microscope.

RESULTS AND DISCUSSION

The ultrastructural appearance of a replica of a human red cell ghost suspended in frozen distilled water is shown in Fig. 1. This preparation was

freeze-cleaved and immediately replicated with platinum-carbon without allowing any deep-etching to take place. The entire cleaved face of the ghost is covered with particles which are approximately 85 Å in diameter. Fig. 2 shows the appearance of a red cell ghost suspended in distilled water which has been freeze-cleaved and then allowed to deep-etch for 40 sec at $-100^\circ C$ before shadowing. Water has been sublimed from the surface of the cleaved specimen. This removal of ice from around the embedded and freeze-cleaved ghost exposes another face which is relatively smooth and does not contain the 85-Å particles seen on the cleaved face. These features of deep-etched red cells ghosts have already been observed by Engstrom and Branton (8).

Electron micrographs of replicas showing freeze-cleaved and deep-etched red cell ghosts mixed with fibrous actin are shown in Figs. 3 and 4. The cleaved faces of the ghosts show the characteristic 85-Å particles, but no fibrous actin appears on this face. However, the smooth face revealed by deep-etching is covered by strands of F-actin which are sharply cut off at the junction of the smooth and particulate faces. The strands of F-actin do not extend out into the surrounding ice, but appear to be closely bound to the smooth face. The shadowed fibrous actin has a helical structure formed by the cross-over of the two F-actin filaments at approximately 360-Å intervals, and it shows a periodicity of approximately 50 Å, probably representing the G-actin subunits. These features are characteristic of the F-actin molecule (9), and make it a readily identifiable marker in freeze-etching studies. They also demonstrate the remarkable resolution that is possible with the freeze-etching technique.

We conclude from these experiments that the smooth, nonparticulate face of the red cell ghost is the actual outer surface of the red cell membrane, and that the face containing the 85-Å particles is an internal layer of the membrane. This conclusion supports the view originally suggested by Branton (2).

As an alternative to this interpretation, it has been suggested that the smooth face of membranes which is exposed by deep-etching is a "pseudo-membrane" made up of residual nonvolatile material deposited on the membrane face during sublimation of the overlying ice (10) or a layer of non-sublimable water bound to the membrane. Since the actin fibers present on the deep-etched surface of the membranes show the expected 50 Å periodic-

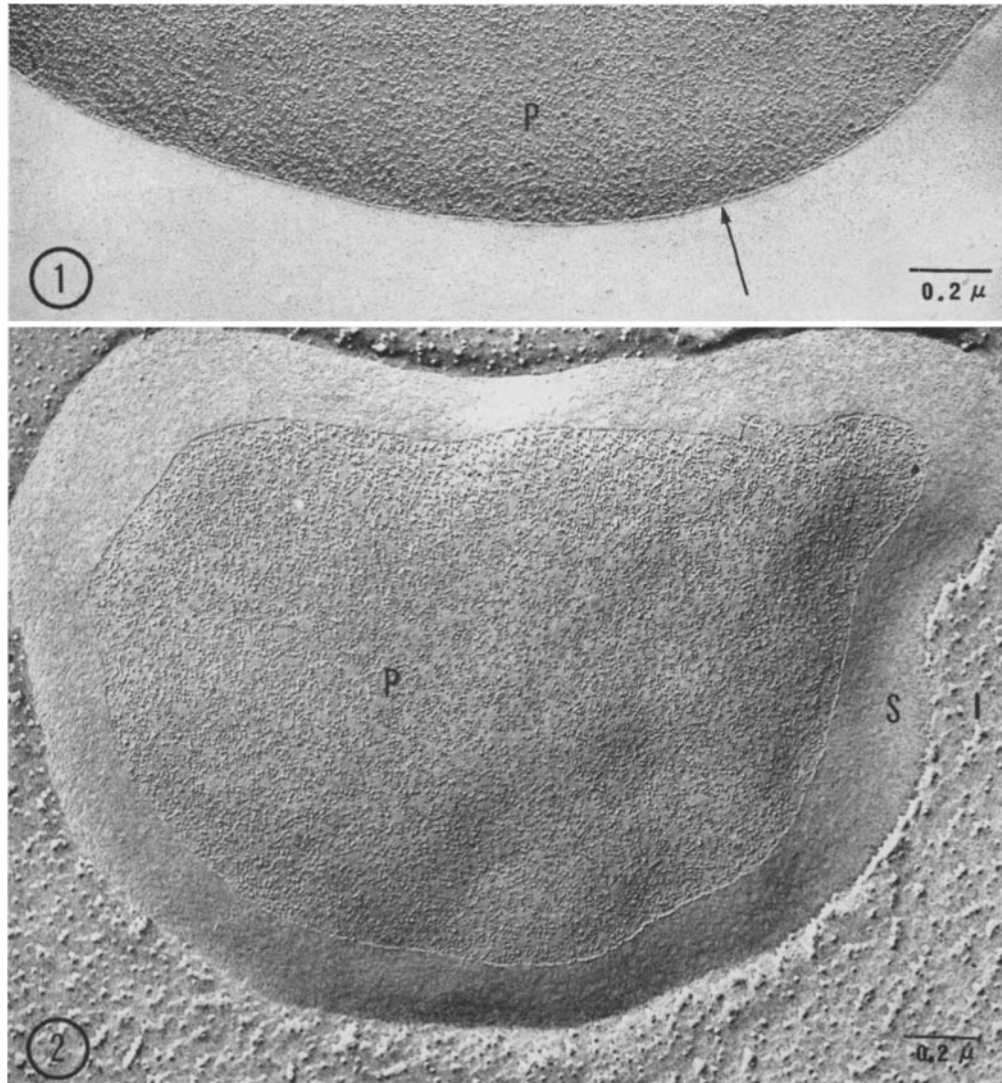


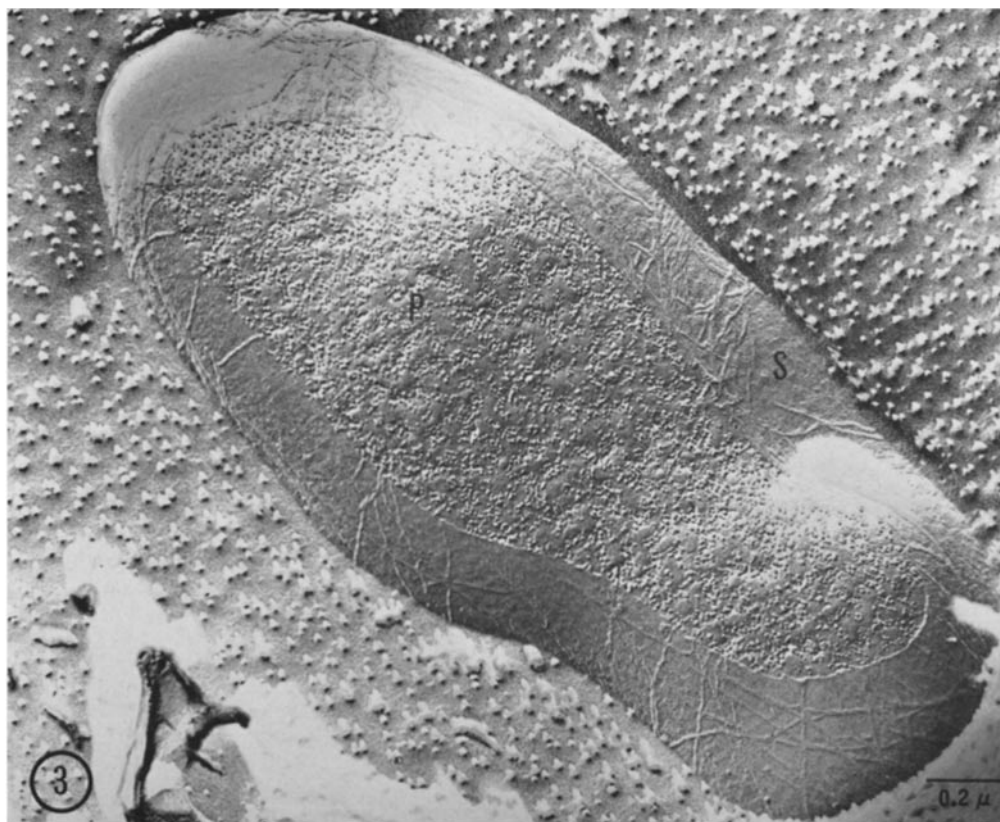
FIGURE 1 Platinum-carbon replica of part of a freeze-cleaved human red cell ghost suspended in frozen distilled water. The cleaved convex face of the ghost (*P*) is covered with 85-A particles. A small ridge (arrow) is present at the junction of the particulate face of the ghost with the surrounding ice. Deep-etching was not done in this preparation. $\times 50,000$.

FIGURE 2 Platinum-carbon replica of a freeze-cleaved and deep-etched human red cell ghost suspended in frozen distilled water. The cleaved convex face of the ghost (*P*) is covered with 85-A particles. Sublimation of the ice (*I*) from the surface of the cleaved specimen during deep-etching has exposed a smooth face (*S*) which is sharply demarcated from the face containing the particles. $\times 50,000$.

ity, it is clear that such a layer of nonsublammable material is not substantial enough to obscure the 85-A particles.

It is also unlikely that the freeze-cleaving process

occurred along the outer membrane surface and completely removed actin from the particulate face of the ghosts. No actin was ever seen on the particulate faces of the membranes. The actin



FIGURES 3 and 4 Platinum-carbon replicas of freeze-cleaved and deep-etched human red cell ghosts covered with fibrous actin. The cleaved convex faces of the ghosts (*P*) contain the usual 85-A particles. The smooth faces (*S*) exposed by sublimation of water from the surface of the replica during deep-etching are covered by strands of F-actin. The strands are sharply cut off at the junction of the particulate and smooth faces. The fibrous actin shows a periodicity of approximately 50 Å (arrow). Fig. 3, $\times 50,000$. Fig. 4, $\times 75,000$.

fibers were tightly bound to the red cell membrane since repeated washings of the coated ghosts with distilled water did not remove the actin. This is consistent with previous studies which have shown that various proteins can be conjugated directly to the surface of glutaraldehyde-treated red cells (11). A further indication that the actin is bound to the membrane and is not simply deposited on the membrane surfaces during sublimation of water is that actin fibers are not seen extending onto the ice surrounding the exposed membrane surface.

The chemical nature of the 85-A particles seen on freeze-cleaved membrane faces remains to be elucidated. However, since the particles are not present on the outer surface of the cell, it is unlikely that they represent red blood cell antigenic sites.

Experiments attempting to visualize antibodies to red cell membrane antigens on the smooth surfaces exposed by deep-etching are underway.

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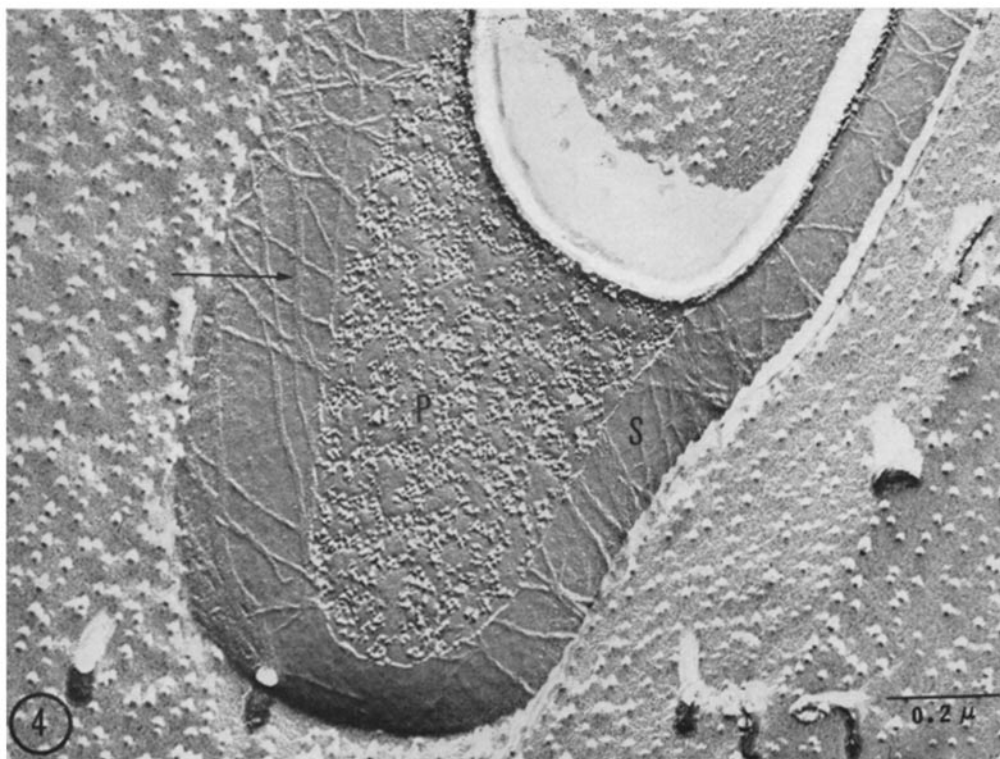


FIGURE 4 See legend under Fig. 3.

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