

# Electron Microscopic Study of Reassociation of Spectrin and Actin with the Human Erythrocyte Membrane

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**ABSTRACT** Reassociation of spectrin and actin with human erythrocyte membranes was studied by stereoscopic electron microscopy of thin sections combined with tannic acid–glutaraldehyde fixation. Treatment of the erythrocyte membrane with 0.1 mM EDTA (pH 8.0) extracted >90% of the spectrin and actin and concomitantly removed filamentous meshworks underlying the membranes, followed by fragmentation into small inside-out vesicles. When such spectrin-depleted vesicles were incubated with the EDTA extract (crude spectrin), a filamentous meshwork, similar to those of the original membranes, was reformed on the cytoplasmic surface of the vesicles. The filamentous components, with a uniform thickness of 9 nm, took a tortuous course and joined one another often in an end-to-end fashion to form an irregular but continuous meshwork parallel to the membrane. Purified spectrin was also reassociated with the vesicles in a population density of filamentous components almost comparable to that of the crude spectrin-reassociated vesicles. However, the meshwork formation was much smaller in extent, showing many independent filamentous components closely applied to the vesicle surface. When muscle G-actin was added to the crude spectrin- or purified spectrin-reassociated vesicles under conditions which favor actin polymerization, actin filaments were seen to attach to the vesicles through the filamentous components. Two modes of association of actin filaments with the membrane were seen: end-to-membrane and side-to-membrane associations. In the end-to-membrane association, each actin filament was bound with several filamentous components exhibiting a spiderlike configuration, which was considered to be the unit of the filamentous meshwork of the original erythrocyte membrane.

The existence of the cytoskeletal network underlying the erythrocyte membrane is now well documented (for reviews, see references 23, 25, 42). Evidence has accumulated that the erythrocyte cytoskeleton is mainly constituted of spectrin, the major peripheral membrane protein (28, 47, 48). It is reasonably proposed that the erythrocyte cytoskeleton may play an important role in regulating the topography of intramembraneous proteins (11, 12, 29, 31, 38) and in determining erythrocyte shape and deformity (4, 16, 27, 35). In search of the supramolecular organization of the cytoskeleton, extensive studies have recently been directed to the structure (18, 33, 39) and chemical nature of spectrin (15, 24, 25, 41), including its binding with other proteins, such as erythrocyte actin, band 2.1, and band 4.1 (2, 14, 22, 45, 47, 49). Although the erythrocyte actin accompanied spectrin during extraction with low-salt EDTA,

at present it is not clear whether actin is an essential structural component of the cytoskeleton (5–10, 20, 30, 32, 37, 43).

Morphologically, the erythrocyte cytoskeleton was first demonstrated by scanning electron microscopy (17) and by negative staining in Triton X-treated ghost preparations (36, 48). More recently, we have successfully visualized the cytoskeleton in thin-section electron microscopy (44) by the use of tannic acid in the fixative (26). The cytoskeleton is resolved into two layers: a horizontally disposed, anastomosing meshwork of filamentous components is attached to the membrane proper through a layer of vertical components with a granular appearance. Taking advantage of tannic acid fixation, we will be able to analyze ultrastructurally the mode of association with the membrane and the dynamic aspects of the cytoskeleton.

Spectrin—in either crude or purified preparations—can be

reassociated with the spectrin-depleted erythrocyte membranes (1, 2, 22, 49). F-actin can bind to the spectrin-reassociated membranes (9, 10). Detailed ultrastructural study of such reassociation may lead to better understanding of the organization and function of the cytoskeleton. We describe here the reassociation of spectrin (in a form of filamentous components) and of actin (in the F-form) with the erythrocyte membranes, as revealed by stereoscopic electron microscopy of thin sections after tannic acid fixation.

## MATERIALS AND METHODS

### *Preparation of Erythrocyte Membrane*

Erythrocyte membranes were prepared from freshly drawn human blood according to the procedure of Fairbanks et al. (13).

### *Dissociation of Spectrin from the Erythrocyte Membrane*

Spectrin-depleted inside-out vesicles were prepared by EDTA extraction (13). By this treatment, >90% of the spectrin and actin were extracted from the membrane (see reference 44). The EDTA extract, which was comprised mainly of spectrin and actin, was used as crude spectrin for the reassociation experiments, after concentrated by ultrafiltration (Toyo UX-10 membranes, Toyo Roshi Co., Ltd., Tokyo).

### *Preparation of Purified Spectrin and Purified Actin*

Spectrin dimer and tetramer were prepared (44) basically according to the method of Marchesi (24) and Ungewickell and Gratzer (46). Erythrocyte actin was prepared by the method of Sheetz et al. (34) with slight modification, using gel filtration (Sephadex G-100, Pharmacia Fine Chemicals, Uppsala, Sweden) before polymerization and depolymerization. Muscle actin was prepared by the procedure of Spudich and Watt (40).

### *Reassociation of Crude Spectrin*

Reassociation was carried out by incubation for 90 min at 4°C after mixing the concentrated crude spectrin (0.16, 0.48, and 1.4 mg of total protein) with the spectrin-depleted inside-out vesicles (0.47 mg of membrane protein) in 20 ml of 0.5 mM sodium phosphate buffer (adjusted to final pH 8.0) containing different concentrations of KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.05 mM EDTA, and 4% sucrose, all at final concentrations essentially as described by Bennett and Branton (1). Protein concentrations were estimated by the method of Lowry et al. (21). After incubation, the vesicles were collected by centrifugation for 15 min at 34,000 × g and washed with 5 mM sodium phosphate (pH 8.0). The degree of reassociation was estimated from the relative peak areas of spectrin and actin to band 3 protein on the densitometric scans of sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.

### *Reassociation of Purified Spectrin and Actin*

Purified spectrin (dimer or tetramer) with or without purified erythrocyte G-actin was incubated for 90 min at 4°C with the spectrin-depleted vesicles (0.47 mg of membrane protein) in 20 ml of 0.5 mM sodium phosphate buffer (adjusted to final pH 8.0), containing 150 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.05 mM EDTA, and 4% sucrose, all at final concentrations. Different mixing ratios of purified spectrin to purified actin were used: 1.24 mg spectrin/no actin, 1.24 mg spectrin/0.16 mg actin, 1.24 mg spectrin/0.48 mg actin, and no spectrin/0.16 mg actin. The ratios used were determined on the basis of the ratio of proteins contained in the crude spectrin.

After the purified spectrin(dimer)-reassociated vesicles were washed twice in 5 mM sodium phosphate buffer, pH 8.0, muscle G-actin (40 μg/ml) was incubated with the vesicles (0.3 mg of membrane protein/ml) at 30°C for 1 h in 20 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM ATP, 0.75 mM mercaptoethanol, 5 mM sodium phosphate buffer, pH 6.5, as used by Cohen et al. (7). The crude spectrin-reassociated vesicles and the fragmented ghosts (7) were incubated with muscle G-actin in the above condition, except that the incubation was done at 4°C. After incubation, the vesicles were collected by centrifugation at 34,000 g for 15 min and washed three times. A part of the sample was washed in 100 mM KCl and

10 mM sodium phosphate buffer, pH 7.5, and incubated with heavy meromyosin subfragment-1 (1mg/ml) at 4°C for 1 h.

### *Electron Microscopy*

All the membrane samples were processed for electron microscopy as previously described (44). Sections, either 100- or 50-nm thick, were examined in a Hitachi HU-12 electron microscope equipped with gonio-stage operated at 100 kV. Printed micrographs of the stereo pairs (±10°) were viewed and examined under a Sokkisha stereoscope MS-27 (Sokkisha Co., Ltd., Tokyo).

### *SDS-Polyacrylamide Gel Electrophoresis*

The samples were subjected to electrophoresis in both 10% polyacrylamide slab gels by the method of Laemmli (19) and 5.6% polyacrylamide disc gels by the method of Fairbanks et al. (13). Gels were stained with Coomassie Brilliant Blue R-250. Gel densitometry was made by densitometric scans at 570 nm through a 0.2-mm slit (Fujiox densitometer, Fuji Riken Co., Ltd., Tokyo) on SDS-polyacrylamide slab gels stained after electrophoresis.

## RESULTS

### *Cytoskeletal Network of Erythrocyte Membrane*

Under the electron microscope, thin sections of the human erythrocyte membranes fixed with tannic acid–glutaraldehyde provided a direct view of the anastomosing meshwork of filamentous components of the cytoskeleton in tangentially or obliquely cut regions of the membranes (41). Stereoscopic examination of stereo pair electron micrographs was very helpful not only in providing three-dimensional images of the cytoskeleton but also in following more accurately the entire length of the filamentous components (Fig. 1). The filamentous components of a relatively uniform thickness of ~9 nm were seen to take a tortuous course and to join one another in an end-to-end fashion to form a meshwork. Several (mainly 4–6) filamentous components converged into a junction point with a spotlike configuration, showing a spiderlike unit (Fig. 1). Thus, the meshwork as a whole appeared to be a combination of such spiderlike repeating units.

### *Spectrin-depleted Inside-out Vesicles*

When the erythrocyte membranes were treated with 0.1 mM EDTA, the membranes fragmented into small, inside-out vesicles of various sizes up to 1 μm in diameter (Fig. 2). Formation of such inside-out vesicles was advantageous to the spectrin-reassociation experiments. In addition, such vesicles were usually not closed. In thin-section electron microscopy, no filamentous component was detected on the vesicles, leaving only granular components (Fig. 2), many of which constituted the vertical components of the cytoskeleton, as described in a previous paper (44).

### *Reassociation of Crude Spectrin*

**PROTEIN ASSAY:** The amount of reassociation of the crude spectrin depended on the concentration of KCl and the mixing ratio of crude spectrin to membrane protein. When the spectrin-depleted inside-out vesicles were incubated for 90 min in the presence of 150 mM KCl and at 3:1 in the mixing ratio by weight of crude spectrin to membrane protein, the vesicles seemed to be saturated, showing the reassociation value of 68% of the extractable amount of spectrin and actin of the original erythrocyte membrane. Interestingly, the ratio of actin to spectrin was invariably 0.15–0.20 by weight, at almost the same value in different preparations: original erythrocyte mem-

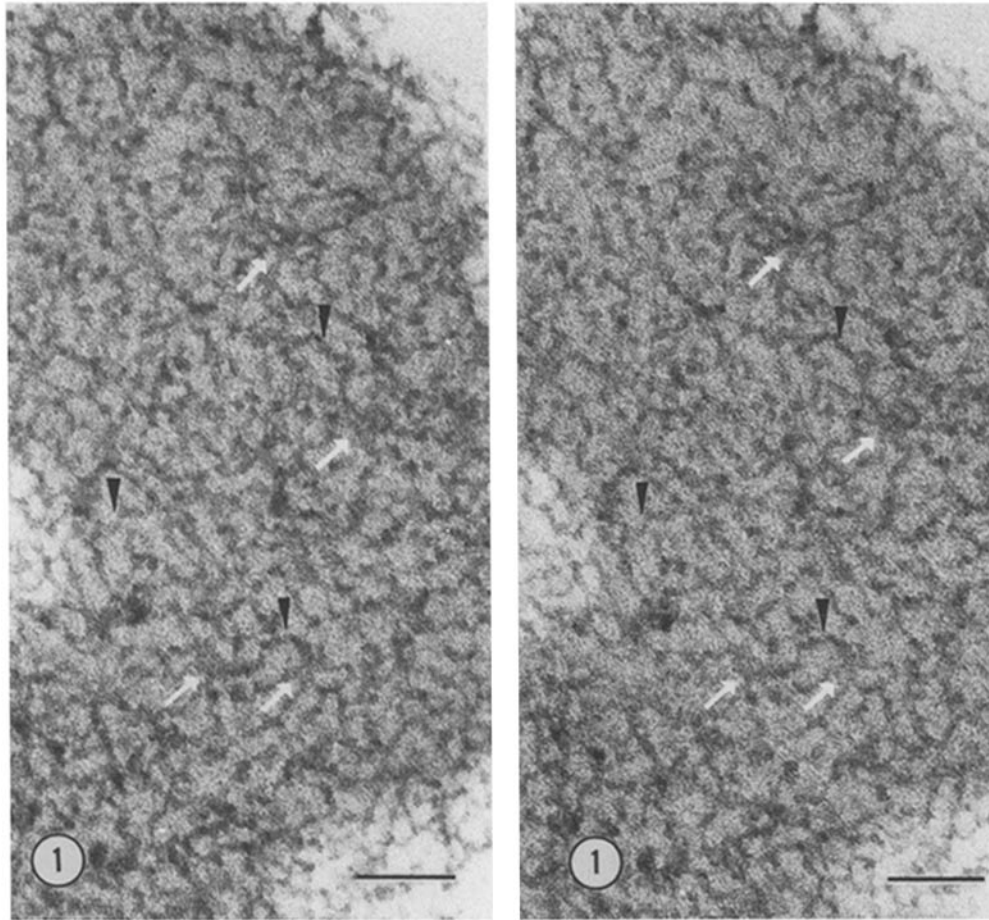


FIGURE 1 Stereo image of the cytoskeletal network on the cytoplasmic surface of the human erythrocyte membranes. Stereoscopic examination is useful in following the entire length of the filamentous components, which correspond to spectrin molecules (black spearheads). The filamentous components  $\sim 9$ -nm thick are seen to form a meshwork. Several filamentous components often converge into a junction point (white arrows). Bar,  $0.1 \mu\text{m}$ .  $\times 130,000$ .

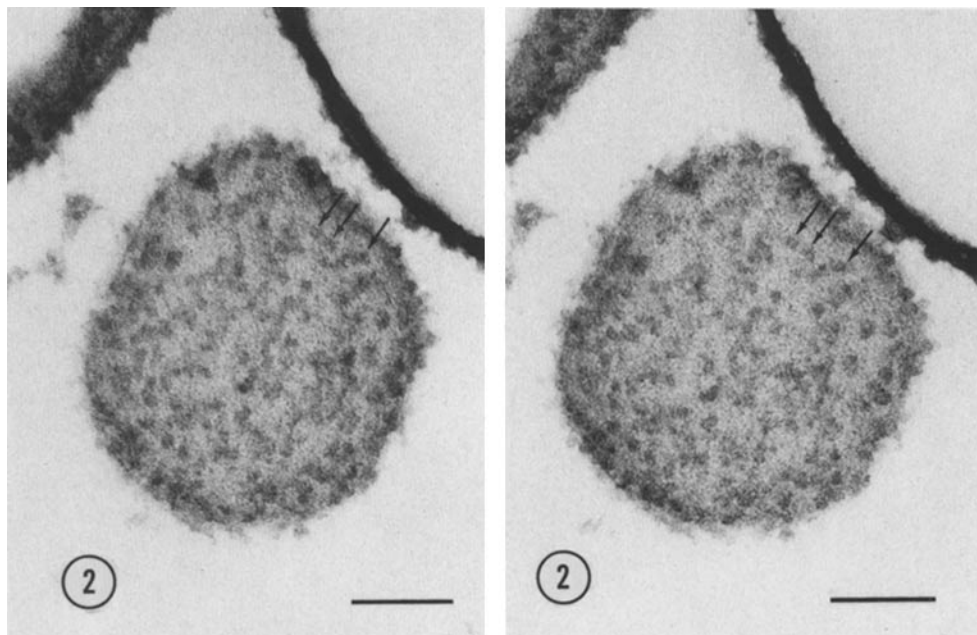


FIGURE 2 Stereo image of spectrin-depleted inside-out vesicles. When  $>90\%$  of the spectrin and actin are extracted from the membrane, no filamentous components are observed on the resultant vesicular forms of the erythrocyte membrane. Only granular components are detected on the outer cytoplasmic surface of the vesicles (black arrows). Bar,  $0.1 \mu\text{m}$ .  $\times 130,000$ .

branes, EDTA extract (crude spectrin), and crude spectrin-reassociated vesicles.

**ELECTRON MICROSCOPY:** The membrane vesicles that reassociated with crude spectrin in the saturation condition, as described above, were examined by thin-section electron microscopy. The crude spectrin-reassociated vesicles were characterized by the formation of filamentous meshworks on the outer, cytoplasmic surface of the inside-out vesicles (Fig. 3). The meshworks were very similar to those seen in the original erythrocyte membranes, though they were not exactly comparable in meshwork density and continuity. Neither filamentous meshwork nor filamentous component was applied to the other side of the membranes. The construction of the meshwork was more clearly recognized by stereoscopic examination of the electron micrographs (Fig. 4). The filamentous components  $\sim 9$ -nm thick took a highly tortuous course, and joined each other often in an end-to-end fashion to form an irregular meshwork. The number of filamentous components that converged into a junction point was usually 2–5, as compared to 4–7 in the original cytoskeleton. In the reassociation, the filamentous meshworks were invariably formed all over the vesicles, disposing parallel to the membrane proper (Fig. 4). Note that individual filamentous components were never seen to extend freely from off the membrane. The filamentous units of  $\sim 100$  nm in length were discernible as unit structures; thus, two units double the length. They did not always join adjacent filaments, but occasionally terminated freely at their ends. The filamentous components appeared to attach to the membrane at irregular intervals along their entire length, being partially raised from the membrane in a scalloped or wavy form. Furthermore, vertical granular components tended to be associated with the filamentous components, so that only a few granular components were left freely in the interstices of the filamentous meshwork.

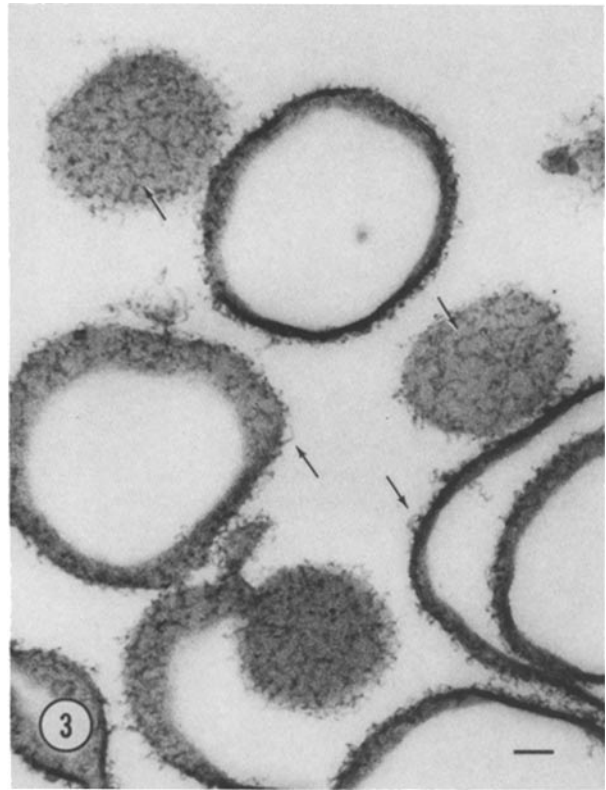


FIGURE 3 Electron micrograph of crude spectrin-reassociated vesicles. When spectrin-depleted vesicles are incubated with crude spectrin, the filamentous meshwork is reformed exclusively on the outer cytoplasmic surface (arrows). Note that the reformed filamentous network resemble that of the original erythrocyte membrane (Fig. 1). Bar,  $0.1 \mu\text{m}$ .  $\times 50,000$ .

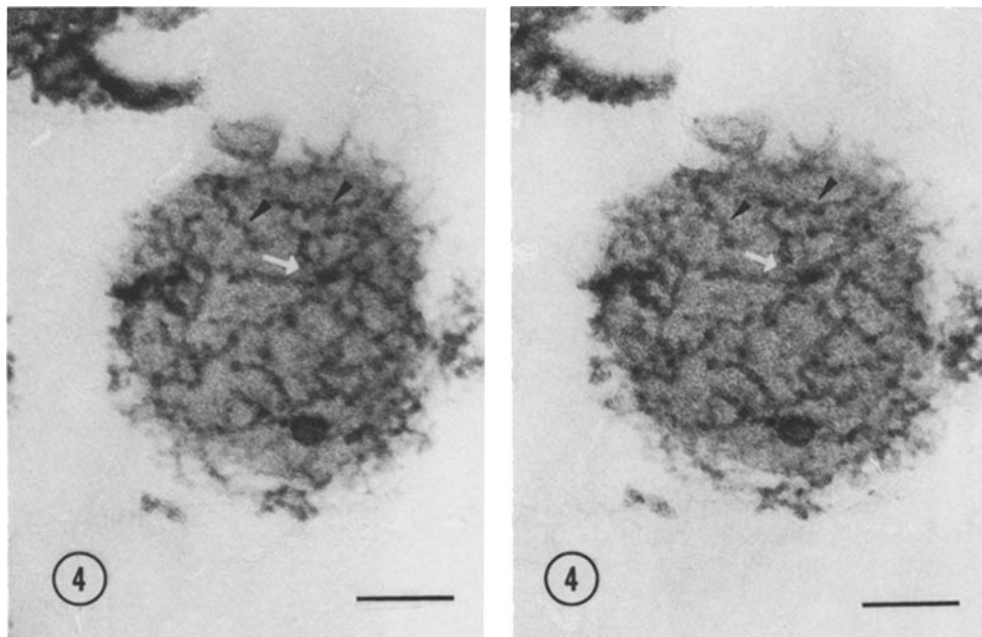


FIGURE 4 Stereo image of the crude spectrin-reassociated vesicles. The filamentous components  $\sim 9$ -nm thick take a highly tortuous course (black spearheads), and join one another often in an end-to-end fashion to form an irregular meshwork, similar to that of the original membrane (see Fig. 1). Some filamentous structures are converged into a junctional point (white arrows). In the reassociation, the filamentous meshworks are formed all over the vesicles, parallel to the membrane. No filamentous component is ever seen to extend freely from off the membrane. Bar,  $0.1 \mu\text{m}$ .  $\times 130,000$ .

### Reassociation of Purified Spectrin

**PROTEIN ASSAY:** Purified spectrin (1.24 mg of total protein) was incubated with inside-out, spectrin-depleted vesicles in the presence of 150 mM KCl. After 90 min of incubation at 4°C, 60–70% of the extractable amount of spectrin of the original erythrocyte membrane was reassociated. This value was almost equivalent to that in the reassociation with crude spectrin.

**ELECTRON MICROSCOPY:** The overall appearance of the inside-out vesicles reassociated with purified spectrin was similar to that of the crude spectrin-reassociated ones (Fig. 5). The filamentous structures similar to those of the crude spectrin-reassociated vesicles were found applied all over the outer, cytoplasmic surface of the vesicles. The filamentous components showed a uniform thickness of 9 nm with a highly tortuous course, as seen in the filamentous meshwork of the original erythrocyte membrane and the crude spectrin-reassociated vesicle. A careful comparison with the crude spectrin-reassociated vesicle indicated that the meshwork formation in the purified spectrin-reassociated one was much smaller in extent, with most filamentous components exhibiting free ends. When the spectrin dimer was reassociated, the filamentous components were usually 100 nm in length, whereas, in the case of tetramer reassociation, those components were estimated to be ~200-nm long. It should be noted, however, that the reassociated filaments were closely applied to the membranes, with few extending freely from off the membrane. The granular components also appeared to be associated with the filaments, though more granules were found freely in the interstices of the filamentous meshwork than in those on the crude spectrin-reassociated vesicles. No filamentous component was found on the other side of the vesicles.

### Association of Actin

**PROTEIN ASSAY:** When the purified erythrocyte or muscle G-actin (0, 8, 24  $\mu\text{g}/\text{ml}$ ) was incubated with the spectrin-

depleted vesicles or the purified spectrin-reassociated vesicles at 4°C, there was no detectable reassociation of actin. In contrast, when G-actin was incubated with the purified spectrin-reassociated vesicles under conditions which favor actin polymerization (at 30°C, with the addition of MgATP), actin was demonstrated to bind to the purified spectrin-reassociated vesicles in the F-form (see reference 9).

**ELECTRON MICROSCOPY:** When G-actin was added to the erythrocyte membrane preparations under the conditions which favor actin polymerization, F-actin was formed in close association with the membranes. The polymerization conditions of G-actin in the presence of membranes were slightly different in different kinds of the membrane preparations; that is, warming (at 30°C) was required for purified spectrin-reassociated vesicles, whereas it was not necessary for the crude spectrin-reassociated vesicles and fragmented ghosts (see references 8, 9). Actin filaments thus formed were seen to attach exclusively to the cytoplasmic surfaces of the membranes through the filamentous components of the cytoskeleton.

In the fragmented ghosts, which usually formed right-side-out vesicles, the construction of the cytoskeleton was basically the same as that in the original erythrocyte membrane. Actin filaments were confined to the vesicles and attached to the membrane in an end-to-membrane fashion at various angles. Their attaching ends were often found to connect with the junction points of filamentous components of the cytoskeleton. When treated with heavy meromyosin (HMM) S-1, the actin filaments were decorated with the arrowheads pointing toward the membrane.

In the crude spectrin-reassociated vesicles, two modes of association of actin filaments with the membranes were seen: end-to-membrane and side-to-membrane. In the end-to-membrane association, each actin filament was connected with several filamentous components of the cytoskeleton. Such association sites often corresponded to the junction points of the

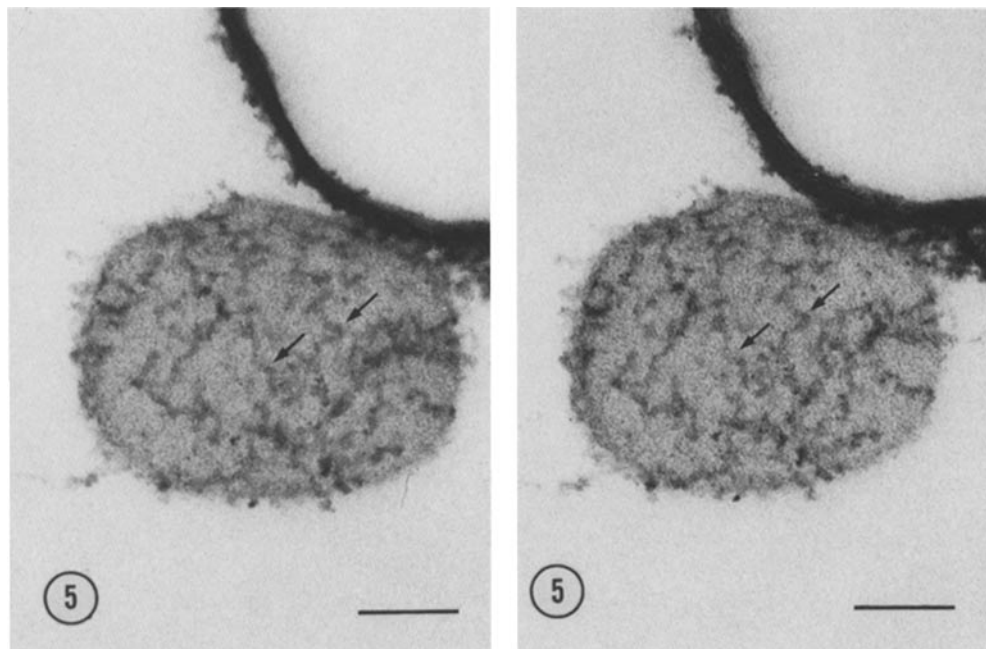


FIGURE 5 Stereo image of the purified spectrin-reassociated vesicles. When purified spectrin is reassociated with the spectrin-depleted inside-out vesicles, the filamentous structures similar to those of the crude spectrin-reassociated vesicles are found applied all over the outer surface of the vesicles. The filamentous components show a tortuous course parallel to the membrane (arrows), with few extending freely from off the membrane. The meshwork formation is much smaller in extent, with most filamentous components exhibiting free ends. Bar, 0.1  $\mu\text{m}$ .  $\times 130,000$ .

filamentous meshwork. The actin filaments were arranged so that the arrowheads with HMM S-1 pointed toward the membranes (see reference 7). In the side-to-membrane association, which occurred less frequently, actin filaments were applied in various lengths to the membrane. The filamentous components extended from the membrane and attached end-on to the actin filament.

In the purified spectrin-reassociated vesicles, the attachment of actin filaments with the membrane was similar to that in the crude spectrin-reassociated vesicles (Figs. 6 and 7). In the end-

to-membrane association, the actin filament was bound with several filamentous components exhibiting a spiderlike configuration, which was considered to be the unit of the continuous filamentous meshwork (Fig. 7 *a* and *b*). The side-to-membrane association was predominant in this preparation (Fig. 6). Through this association, the actin filaments occasionally formed a bridge between two vesicles. Some of the actin filaments were decorated irregularly with free filamentous components. This may be explained by the detachment of actin filaments from the vesicles after their association. The treat-

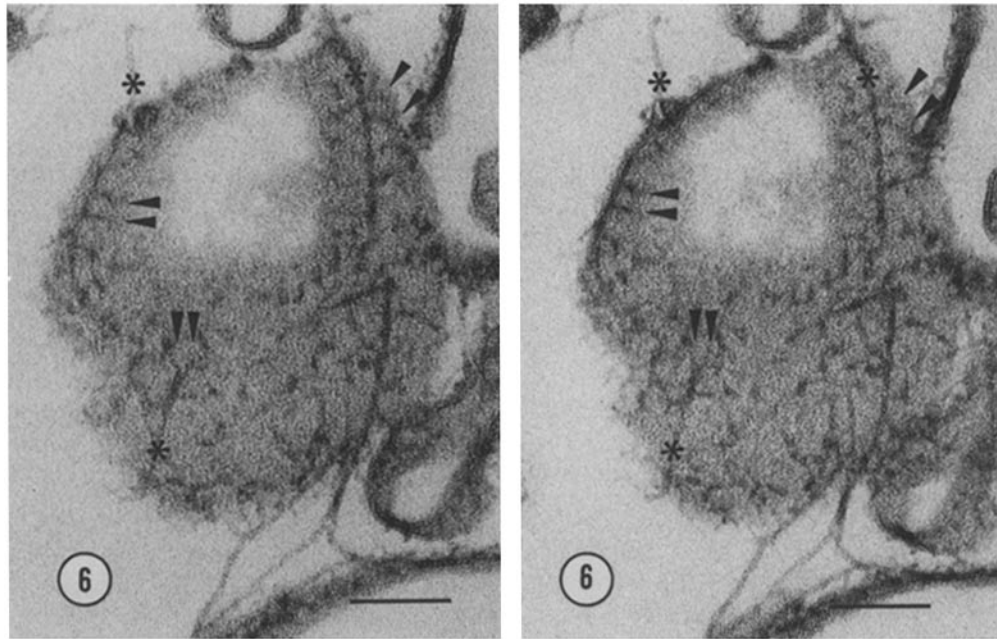


FIGURE 6 Stereo image of the purified spectrin-reassociated vesicle associated with actin filaments. The actin filaments (asterisks) are disposed parallel to the membrane in an end-to-membrane association. The filamentous components (spearheads) extend from the membrane and attach end-on to the actin filaments. Bar, 0.1  $\mu\text{m}$ .  $\times 130,000$ .

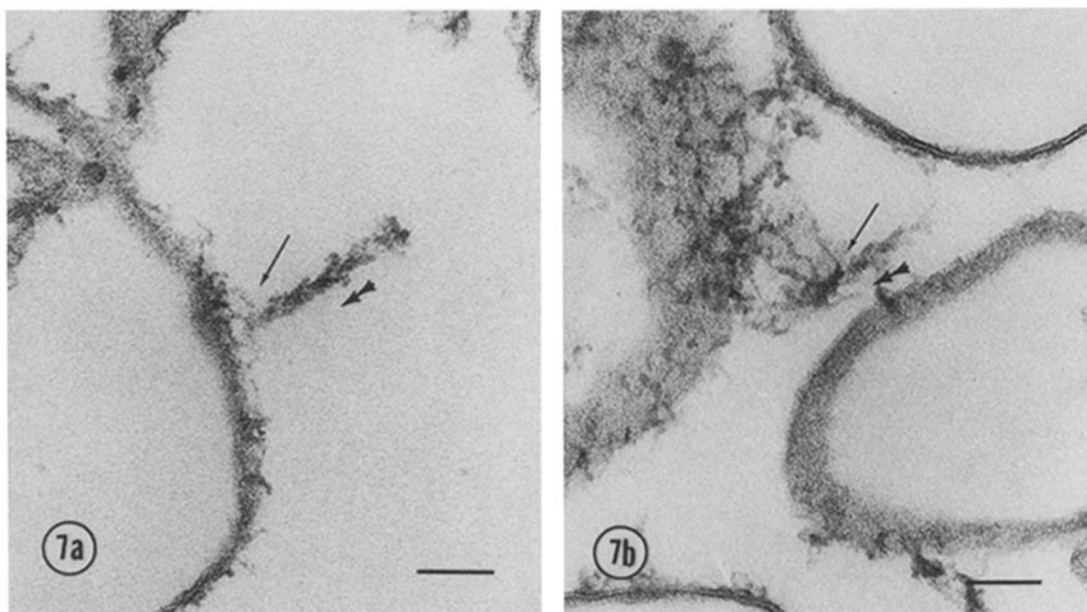


FIGURE 7 Electron micrographs of the purified spectrin-reassociated vesicles associated with actin filaments. The end portion of each actin filament (double spearheads) is bound with several filamentous components exhibiting a spiderlike configuration (arrows). The actin filaments are arranged so that the arrowheads with HMM S-1 point toward the membranes (double spearheads). Bar 0.1  $\mu\text{m}$ .  $\times 97,000$ .

ment with HMM S-1 did not seem to release such an actin-membrane association.

Under the conditions used for the present reassociation experiments, no actin filament was detected on the vesicles which were incubated at low temperature with crude spectrin, with purified spectrin alone, or with a mixture of purified spectrin and G-actin at a ratio the same as that in the crude spectrin. There was no fundamental difference in ultrastructure between the meshwork formed with purified spectrin alone and that formed with the purified spectrin-G-actin mixture (data not shown).

## DISCUSSION

These results revealed that the filamentous meshworks reformed with crude spectrin resembled those of the original erythrocyte cytoskeleton. Purified spectrin in the presence or absence of purified G-actin was also reassociated with the spectrin-depleted vesicles in a degree comparable to the reassociation with the crude spectrin; but morphologically it formed much less elaborated meshworks of the filamentous components. In shadow-casting electron microscopy, the purified spectrin in a dimer form appears as a flexible rod ~100-nm long (39). Two spectrin dimers can bind each other in a head-to-head fashion to form a tetramer rod 200-nm long. In thin sections, the reconstituted filamentous meshwork exhibited a characteristic appearance and dimensions similar to those of purified spectrin in either dimers or tetramers. From all the evidence available, we conclude that the filamentous components of the erythrocyte cytoskeleton indeed represent spectrin molecules and, thus, that the filamentous meshwork is constructed mainly of spectrin.

Spectrin can attach to the membrane through its binding with 2.1 and band 4.1 proteins (1-3, 14, 22, 45, 47, 49), which are not easily extractable in the EDTA treatment. In our results, the filamentous components in either meshwork or free form were disposed parallel to the surface of the vesicles, even the vesicles with larger curvature, in a scalloping or multiple looping manner along their entire course. In addition, the granular components that remained in the spectrin-depleted vesicles tended to be associated with the filamentous components, leaving few granules free in the interstices of the meshwork. These findings suggest that the interaction between spectrin molecules and the membrane may not be restricted to their end portions.

The role of actin in construction of the erythrocyte cytoskeleton is an important problem to be solved. The cytoskeleton left after Triton X-100 treatment disintegrates when actin is selectively removed by DNase I (37). A careful morphological comparison between the crude spectrin-reassociated vesicles and the purified spectrin-reassociated ones may provide information on the contribution of actin to the cytoskeleton. Indeed, the meshwork formation was much smaller in extent in the purified spectrin-reassociated vesicles, showing many independent filamentous components. Experimentally, F-actin, but not G-actin, can interact with spectrin (10). In our observations, actin was associated with the erythrocyte membrane only in the F-form through the filamentous components. The mode of such association on the membrane apparently resembles that of the spectrin-actin interaction *in vitro* (10).

In the fragmented ghosts and the crude spectrin-reassociated vesicles, G-actin was polymerized into F-actin more easily than in the purified spectrin-reassociated vesicles. Furthermore, actin filaments were seen to attach to the membrane often at the

junction points of the filamentous components. In the purified spectrin-reassociated vesicles, the end portion of each actin filament occasionally appeared to serve as a junction point to which several filamentous components converged to attach in an end-on fashion. In the decoration with HMM S-1, these actin filaments showed polarity, with arrowheads pointing toward the membrane (8). These findings favor the notion that in the erythrocyte cytoskeleton actin exists as a short polarized filament with which spectrin molecules are connected (8, see also reference 23). In the experiments on the actin association, such short actin filaments may serve as the nucleation points. It remains to determine how the length of F-actin is controlled in the intact erythrocyte cytoskeleton.

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