ABSTRACT

The directional polymerization of actin on the erythrocyte membrane has been examined at various concentrations of G-actin by thin-section electron microscopy. For this purpose, a new experimental system using single-layered erythrocyte membranes with the cytoplasmic surfaces freely exposed was developed. The preformed actin filaments did not bind with the cytoplasmic surface of the erythrocyte membranes. When the erythrocyte membranes were incubated at low concentrations (0.3 and 0.5 μM) of G-actin, >80% of polymerized actin filaments pointed toward the membranes mainly in an end-on fashion, as judged by arrowhead formation with heavy meromyosin. At higher concentrations (2 and 4 μM) of G-actin, about half of the polymerized actin filaments were directed with arrowheads pointing toward the membranes, while the rest of the filaments showed the opposite polarity pointing away from the membranes. The majority of polymerized actin filaments formed loops at the points of attachment to the membranes. In contrast, when G-actin (2 and 4 μM) in the presence of cytochalasin B was polymerized into filaments, ~70% showed the polarity pointing away from the membrane mainly in an end-on fashion. To check the treadmilling phenomena, the erythrocyte membranes with bidirectionally polymerized actin filaments were further incubated with G-actin at the overall critical concentration. In this case, almost all (90%) of actin filaments showed the polarity with arrowheads pointing toward the membranes. The results obtained are discussed with special reference to the mode of association of actin filaments with the plasma membrane in general.

Since actin-containing microfilaments were identified in situ in various cell types (1), special interest has been focused on the manner in which microfilaments are associated with the plasma membrane. In virtually all the cells examined, actin filaments attach to the plasma membrane in such a way that arrowheads with heavy meromyosin (HMM) point away from the membrane (2–5). This polarity is regarded as important for the bidirectional arrangement of actin filaments in the cytoplasm, which is well suited for construction of the contractile apparatus as exemplified in striated myofibrils (for reviews, see references 2 and 6). The question now arises of what factors are responsible for the polarized attachment of actin filaments to the membrane. In thin-section electron microscopy, actin filaments seem to attach to the membrane by way of the specially layered structures which we collectively designated as the plasmalemmal undercoat (7). However, the molecular mechanism of such attachment has not been elucidated. On the other hand, recent studies on the kinetics of actin polymerization in vitro have revealed the biased addition of G-actin to preformed F-actin; the F-actin can elongate mainly by adding G-actin at its barbed end (8–10). It is important to consider that the polarized polymerization of actin is related to the polarity of the actin filaments with respect to the plasma membrane.

It is widely accepted that spectrin, together with actin, constitutes the cytoskeletal network underlying the erythrocyte membrane and that this network is bound to the membrane proper through anchoring protein (ankyrin) (for a review, see reference 11). More recently, it has been strongly stressed that actin exists as short filaments with which spectrin molecules are connected (12–20). The actin filaments seem to play a key role in the formation of a continuous network of spectrin molecules. Hence, we consider the erythrocyte cytoskeleton as a unique system in which we can discuss at the molecular level the attachment of actin filaments to the plasma membrane. Within the erythrocyte cytoskeleton, short actin filaments are expected to serve as nuclei for actin polymerization. In the previous studies, all the actin filaments

Abbreviations used in this paper: HMM, heavy meromyosin.
grown at low concentrations of G-actin were polarized with arrowheads pointing toward the erythrocyte membrane, opposite in polarity to those in many other cells (12, 13, 20). This discrepancy has prompted us to investigate the regulation of the polarity of actin filaments in the erythrocyte cytoskeleton. Considering the kinetics of biased polymerization of actin in vitro, the previous studies may have dealt with only the polymerization at the barbed ends owing to the low concentration of G-actin. For the polymerization experiments at higher concentrations of G-actin, it is necessary to control the precise incubation time and to distinguish actin filaments directly grown on the erythrocyte cytoskeleton from freely formed actin filaments.

In the present study a new incubation system of the erythrocyte membrane has been developed to examine the polarity of actin filaments with the membrane at various concentrations of G-actin. Taking advantage of this system, the bidirectional polymerization of G-actin on the erythrocyte membrane was clearly demonstrated by thin-section electron microscopy. The results obtained have provided helpful information on the mechanism of association of actin filaments with the plasma membrane in general.

MATERIALS AND METHODS

Materials: G-actin and HMM were kindly supplied by Dr. Masafumi Yano (University of Tokyo), who prepared G-actin basically by the method of Spudich and Watt (21). G-actin thus prepared was used within 4 d. For each experiment, G-actin was carefully pipetted out from the top layer of supernatants after centrifugation at 220,000 g for 1.5 h. For some experiments, G-actin was prepared by gel filtration on Sephadex G-150, showing no significant differences between the results obtained from non-gel filtered and filtered G-actin. For isolation of G-actin was catalyzed by the Biuret method (22) and corrected by the colorimetric micro-Kjeldahl analysis (23).

Preparation of the Single-layered Erythrocyte Membrane System: Polylysine-coated teflon disks 6 mm diam (or glass coverslips) were prepared by being evaporated with carbon and then coated with 0.1% poly-L-lysine. After excess poly-L-lysine was washed off with distilled water, the teflon disks were further rinsed with 150 mM NaCl/1 mM EDTA/5 mM sodium phosphate (pH 7.5) and used within 6 h. Human erythrocytes were obtained from freshly drawn human blood and washed 8–10 times with 150 mM NaCl/1 mM EDTA/5 mM sodium phosphate (pH 7.5). For isolation of G-actin was catalyzed by the Biuret method (22) and corrected by the colorimetric micro-Kjeldahl analysis (23).

Incubation of G-actin with the Glutaraldehyde-fixed Acto-HMM Complexes: Glutaraldehyde-fixed acto-HMM complexes were prepared as the nuclei for actin polymerization according to the method of Cooper and Pollard (24) and Isenberg et al. (25). G-actin (270 μg/ml) was incubated together with HMM in a molar ratio of HMM to actin of ~1:2 in 150 mM KCl/5 mM MgSO4/20 mM sodium phosphate (pH 7.5) at 20°C for 30 min. 2.6 ml of this solution was then fixed by adding 2 ml of 0.1% glutaraldehyde in 150 mM KCl/5 mM MgSO4/20 mM sodium phosphate (pH 7.5) on ice. After 3 min, free aldehyde groups were quenched for 5 min by the addition of 3 ml of 0.1 M ethanolamine/150 mM KCl/5 mM MgSO4/20 mM sodium phosphate (pH 7.5). These glutaraldehyde-fixed acto-HMM complexes were dialyzed against the polymerization buffer. Using the glutaraldehyde-fixed acto-HMM complexes, the polymerization of actin filaments was carried out at 22°C in the polymerization buffer according to the method described by Pollard and Mooseker (10). The complexes were incubated with various concentrations of G-actin of 1–6 μM for 10–40 s for control samples, and 30–60 s for samples with 10 μM cytochalasin B. The length of actin filaments elongated from both barbed and pointed ends of the complexes was measured on negatively stained preparations (50 measurements for each incubation condition).

RESULTS

Ultrastructure of Erythrocyte Membranes Attached to Teflon Disks

In sections cut parallel to teflon disks (or glass coverslips), the surface of the disk was seen to be covered almost completely with single-layered erythrocyte membranes, though the edge of the erythrocyte membrane was occasionally curled up (Fig. 1). Transverse sections revealed a single layer of the plasma membranes of trilaminar structures. On the free surface of the membranes was observed the characteristic fuzzy layer which we previously described in thin-section electron microscopy as the cytoskeletal network underlying the erythrocyte membrane (Fig. 1). Clearly, the cytoplasmic surfaces of all the erythrocyte membranes were exposed and faced upward. SDS PAGE of these membrane preparations showed almost the same polypeptide pattern as those of isolated erythrocyte membranes, except for a slight decrease in amount of the band 6 protein (data not shown).

When such membrane preparations were incubated with preformed F-actin at 22°C and then washed with the polymerization buffer, actin filaments attached exclusively on the gap regions between the membranes, but not on the membranes, as revealed in the transverse and tangential sections (Fig. 1 C). This attachment property was not changed after HMM (1 mg/ml in the polymerization buffer) at 4°C for 7 min. After being washed with the polymerization buffer, the teflon disks were processed for thin-section electron microscopy. In some experiments, the HMM treatment was omitted.

To examine the effect of treadmilling of actin filaments, we incubated some teflon disks that had been incubated with 4 μM G-actin solution as described above in a solution containing both F- and G-actins at the steady state at 4°C for 10 h. To check the nonspecific binding of F-actin to the erythrocyte membranes, teflon disks were incubated with F-actin (4 μM) at 22°C for 10 min.

Thin-section Electron Microscopy: Erythrocyte membranes on the teflon disks were fixed with 2.5% glutaraldehyde/1 mM sodium cacodylate (pH 7.4) containing 1% tannic acid at 4°C overnight. After two rinses in 0.1 M sodium cacodylate (pH 7.4), the membranes were fixed in 1% OsO4/0.1 M sodium cacodylate (pH 7.4) on ice for 2 h. The samples were washed with distilled water and stained en bloc with 0.5% aqueous uranyl acetate for 2 h at room temperature. They were then dehydrated in graded concentrations of ethanol and embedded in Epon 812. After polymerization of Epon, the single-layered membranes were easily separated from the disks at the level of carbon film. Thin sections were cut transverse (~50 nm thick) or tangential (~100–200 nm thick) to the erythrocyte membranes and picked up on the Formvar-filmed grids. Transverse sections were observed in a Hitachi H-700 electron microscope operated at 75 kV, and tangential sections were stereoscopically examined in a Hitachi H-700 electron microscope equipped with a gonioscope at 200 kV.
**Figure 1** Electron micrographs of single-layered erythrocyte membrane preparations before (A and B) and after (C and D) incubation with F-actin. (A) Transverse section of the erythrocyte membrane preparation reveals a single layer of the plasma membrane with a typical trilaminar structure (TI) on a poly-L-lysine-coated teflon disk (Tf). The characteristic fuzzy layer (f) of the cytoskeletal network is situated on the free surface of the membrane. Note the edge of the membrane (arrowhead). Bar, 0.1 μm. x 122,000. (B) In a section cut parallel to the membranes, the surface of the disk is seen to be covered almost completely with single-layered erythrocyte membranes (M). Bar, 1 μm. x 5,500. (C and D) As seen in a section cut parallel to the membranes, when the membranes are incubated with preformed F-actin, actin filaments (arrows) are found exclusively in the gap regions between the membranes, but not on the membranes (M) either before (C) or after (D) HMM treatment. Bar, 0.1 μm. x 32,000.

HMM decoration (Fig. 1D). This indicated that the preformed F-actin nonspecifically bound to the polylysine-coated teflon; the binding of F-actin to the cytoplasmic surfaces of the erythrocyte membrane was almost negligible in our incubation conditions. Thus, the single-layered membrane preparations appeared to offer a good system for analyzing the mode of actin polymerization on the cytoplasmic surfaces of the erythrocyte membrane.

**Evaluation of the Polymerization of G-actin**

The rates of G-actin polymerization were estimated at both ends of the glutaraldehyde-fixed acto-HMM complexes. The mild glutaraldehyde fixation was reported to inhibit the dissociation of HMM from the acto-HMM complexes upon addition of ATP without any effects on the elongation from both ends of the complexes (24, 25). Therefore, we used the
complexes as nuclei to measure the polymerization rate of G-actin in the presence of ATP. Since the elongation of actin filaments did not occur at all ends of the complexes, we chose the complexes with the elongated actin filaments for estimation of the polymerization rates. As a result, the relationship between the elongation rate of actin filaments and the concentrations of G-actin was obtained at both ends of the complexes, as shown in Fig. 2. This is quite consistent with the relationship described by Pollard and Mooseker (10). At any concentration of G-actin the rate of polymerization was greater at the barbed ends than at the pointed ends, indicating that actin filaments grew faster at the barbed ends and that the critical concentrations of actin polymerization were different at the two ends. From this relationship, the overall critical concentration at which the net addition of G-actin at the barbed ends was well balanced by the net loss of G-actin at the pointed ends was estimated to be ~0.6 μM. In the presence of 10 μM cytochalasin B, the elongation of actin filaments was largely blocked at the barbed ends, while the growth rate at the pointed ends was reduced by ~50%. Since it was shown that the critical concentration at the barbed ends was ~0.12 μM, we chose three lower concentrations (0.1, 0.3, 0.5 μM) and two higher concentrations (2.0, 4.0 μM) of G-actin for incubation of the erythrocyte membranes.

**Polymerization of G-actin on the Erythrocyte Membranes**

The single-layered erythrocyte membranes on the teflon disks were incubated with various concentrations of G-actin at 22°C for 10 min in the presence or absence of cytochalasin B. At lower concentrations of G-actin (0.3 and 0.5 μM), the end-to-membrane association was predominant, while at the higher concentrations of G-actin (2.0 and 4.0 μM), many actin filaments attached to the membranes in a side-to-membrane fashion, forming loops at the points of attachment to the membranes (Fig. 3). There were no fundamental differences in the manner of association of actin filaments with the membranes between HMM-decorated and undecorated actin filaments. Hence, in this study, the polarity of polymerized actin filaments with respect to the membranes was analyzed after HMM decoration both in transverse thin section and tangential semi-thin sections. The analysis in transverse sections was made only on the actin filaments that were directly attached to the cytoplasmic surfaces of the erythrocyte membranes. To avoid subconsciously biased results, we scored all the actin filaments directly attached to the membranes. The tangential sections were used to analyze the spatial relationship between actin filaments and membranes in combination with stereoscopic electron microscopy.

**Incubation with 0.1–0.5 μM G-actin:** Judging from Fig. 2b, no growth of actin filaments was expected on both ends at 0.1 μM of G-actin, while the growth of actin filaments was expected only at the barbed ends at 0.3 and 0.5 μM of G-actin. As expected, virtually no actin filaments were observed on the cytoplasmic surfaces of the membranes when the membranes were incubated with 0.1 μM G-actin. In contrast, many short actin filaments were found to grow in continuity with the cytoskeletal network of the erythrocyte membranes at the concentrations of 0.3 and 0.5 μM. Interestingly, after HMM treatment 95 and 83% of polymerized actin filaments pointed toward the membranes at 0.3 and 0.5 μM of G-actin, respectively (Fig. 4A and see Fig. 8). The exact lengths of all filaments were not measurable in thin sections.
FIGURE 4  Electron micrographs of transverse sections of the single-layered erythrocyte membranes incubated with low (A) and higher (B) concentrations of G-actin, and incubated at the steady state after incubation with higher concentrations of G-actin (C), followed by HMM decoration. (A) At the G-actin concentration of 0.5 μM, actin filaments grow in continuity with the cytoskeletal network of the membranes, and most of filaments are directed with arrowheads of HMM pointing toward the membranes (double arrowheads). (B) At the G-actin concentration of 2 μM, about half of the polymerized filaments are directed with arrowheads pointing toward the membranes, while the rest of filaments showed the opposite polarity, pointing away from the membranes (double arrowheads in the upper row). Some filaments form loops at the points of attachments to the membranes (curved arrows in the lower row). (C) When the erythrocyte membranes with bidirectionally polymerized actin filaments (at 4 μM G-actin concentration) are incubated at the steady state, almost all actin filaments are directed with arrowheads pointing toward the membranes (double arrowheads). Bar, 0.1 μm, × 92,000.
The stereoscopic observation on tangential sections clearly demonstrated that actin filaments were attached to the membranes in an end-on fashion (Fig. 5A).

**INCUBATION WITH 2 AND 4 μM G-ACTIN:** At the G-actin concentrations of 2.0 and 4.0 μM, polymerization of G-actin was expected to occur at both barbed and pointed ends at different rates (see Fig. 2b). When the single-layered erythrocyte membranes were incubated at these concentrations of G-actin, a large number of actin filaments were observed to be associated with the cytoplasmic surfaces of the membranes. In transverse sections, ~55% of these filaments were directed with arrowheads pointing toward the membranes, while the rest of the filaments showed the opposite polarity, pointing away from the membranes (Fig. 4B and see Fig. 8). One of the most interesting observations was that the majority of polymerized actin filaments formed loops at the points of attachment to the membranes, as clearly revealed in tangential semi-thin sections (Fig. 5B). The looped filaments often seem

**FIGURE 5** Stereo pairs of electron micrographs of the tangential semi-thin sections of the single-layered erythrocyte membranes incubated with low (A) and higher (B) concentrations of G-actin, and incubated at the steady state after incubation with higher concentrations of G-actin (C). (A) When the membranes were incubated at 0.5 μM of G-actin, actin filaments are seen to attach to the membranes in an end-on fashion and the most of filaments are directed with arrowheads of HMM pointing toward the membranes (double arrowheads). (B) When the membranes are incubated at 2 μM G-actin, many polymerized actin filaments are associated with the membranes in a loop-forming fashion (curved arrows). (C) When the membranes with bidirectional polymerized actin filaments (at 4 μM G-actin concentration) are incubated at the steady state, the filaments attach to the membranes in an end-on fashion and most of the filaments are directed with arrowheads pointing toward the membranes. Bar, 0.1 μm. × 75,000.
to graze the membrane surfaces, being connected by the filamentous components. However, a small number of filaments were associated with the membranes in an end-on fashion. Some long filaments attached to the membrane at more than two attaching points, possibly due to annealing of two actin filaments. It should be mentioned that for estimation of the polarity of actin filaments on the membranes, each loop-forming filament was counted as two filaments with opposite polarities.

To check whether the treadmilling phenomena occurs, we incubated some of the erythrocyte membrane preparations with polymerized actin filaments with the polymerization buffer containing G- and F-actin at the steady state (0.6 μM in G-actin) at 4°C for 10 h. Judging from the inorganic phosphate concentration, the amount of ATP in the medium did not remarkably decrease during this incubation. The transverse sections of such preparations showed that almost all actin filaments (90%) were directed with arrowheads pointing toward the membranes (Fig. 4C and see Fig. 8). Tangential semi-thin sections demonstrated more clearly that long actin filaments attached to the membranes mainly in an end-on fashion (Fig. 5C).

**INCUBATION WITH 2 AND 4 μM G-ACTIN IN THE PRESENCE OF CYTOCHALASIN B:** When the single-layered erythrocyte membranes were incubated with 2 or 4 μM G-actin in the presence of 4 or 10 μM cytochalasin B, most of the actin filaments were observed to attach to the membranes in an end-on fashion. About 70% of actin filaments showed the polarity with arrowheads pointing away from the membranes (Fig. 6 and see Fig. 8). Such end-on association was confirmed in the tangential semi-thin sections (Fig. 7). Similar results were obtained when the pretreatment of the membranes with cytochalasin B was omitted.

**SUMMARY:** The results are summarized in Fig. 8. The
centrations of G-actin (data not shown).

In this study, we have examined the bidirectional polymerization of G-actin on the erythrocyte membranes in order to better understand the mode of association of actin filaments with the plasma membrane in general. For this examination, we have developed a new experimental system using single-layered erythrocyte membranes with the cytoplasmic surface freely exposed. There are several advantages in using this system: (a) the system is very easily handled in all steps of the experiments; (b) the polymerization time can be precisely determined; (c) the membranes can be easily washed so that nonspecific binding of free actin filaments or HMM may be largely avoided; and (d) the concentrations of G-actin can be well maintained during incubation, minimizing the decrease of G-actin with time. Indeed, the preformed actin filaments did not bind with the cytoplasmic surface of the erythrocyte membrane, whereas they firmly adhered to the polylysine-coated surfaces of teflon disks. The only inevitable disadvantage in this approach was that it is not possible in thin-section electron microscopy to estimate the length of actin filaments grown on the membranes, making it difficult to quantitatively analyze the rate of actin polymerization. However, this disadvantage was not such a serious problem here because our present analysis has been focused on the polarity of actin filaments with respect to the membranes.

Using this system, it has become clear that actin filaments can grow bidirectionally from the cytoplasmic surfaces of the erythrocyte membranes depending on the incubation conditions (Fig. 8). In interpreting the present results combined with the recent biochemical data (12–20), the most likely explanation is that actin exists as short filaments with both ends free in the erythrocyte cytoskeletal network. In this scheme, actin can be polymerized on the barbed ends of the short actin filaments at low concentrations of G-actin and the growing filaments are polarized with the arrowheads of HMM pointing toward the membrane, while actin can be polymerized on the both ends at high concentrations of G-actin so that the filaments can grow bidirectionally on the membranes. In the presence of cytochalasin B, a barbed-end capping reagent (10), actin is polymerized on the pointed ends at high concentrations of G-actin, resulting in the filaments polarized with the arrowheads pointing away from the membranes. Although our results favor the above scheme, other possibilities cannot be ruled out—that actin filaments might be capped with other proteins at one or both ends. If this is the case, some or all capping proteins may be removed in our experimental preparations. Most recently, Pinder and Gratzer have reported the interesting biochemical finding that the pointed ends are capped (27). However, we consider that this conclusion still remains tentative, for they used G-actin at around the overall critical concentration in their experiment. The pointed end-capping effect should be examined using the G-actin concentrations above the critical concentration which is required for polymerization at the pointed ends. In the erythrocyte membrane cytoskeleton, only short segments of actin filaments in the cytoskeletal network seem to be stabilized against depolymerization through binding with spectrin and/or other proteins such as band 4.1. Indeed, when the erythrocyte membranes with bidirectional actin filaments were further incubated with G-actin at the overall critical concentration, almost all actin filaments showed the polarity with arrowheads pointing toward the membranes. These observations may indicate that dissociation of G-actin from the pointed ends during treadmilling is blocked in the very segments of actin filaments within the cytoskeletal network. This interpretation is consistent with the recent biochemical results by Pinder and Gratzer (27).

**DISCUSSION**

In this study, we have examined the bidirectional polymerization of G-actin on the erythrocyte membranes in order to...
It is widely accepted in a variety of cell types that actin filaments are associated with the plasma membrane in such a way that arrowheads with HMM point away from the membrane in situ (2–4). In the present study the same polarity of actin filaments with respect to the membranes was reproduced experimentally using cytochalasin B. Without cytochalasin B, actin filaments grew bidirectionally from the erythrocyte membranes. The finding of the polymerization of actin filaments in the pointed direction in the presence of cytochalasin B is significant, because it suggests that the erythrocyte membrane is not unique among membranes and that similar structures may exist for the attachment of actin filaments to the plasma membrane. Recently, spectrin-like proteins have been shown by immunofluorescence to occur in the cell periphery in various cell types (28–30). Furthermore, spectrin-like actin-binding proteins have been isolated from brain and intestinal brush border (29, 31–34). Such proteins, with actin, may form the cytoskeletal network underlying the plasma membrane, as is the case with the erythrocyte membrane. It is interesting to speculate that short actin filaments on the membranes with the barbed ends capped may serve as nuclei for unidirectional polymerization of actin in the pointed direction in cells. Indeed, several kinds of proteins that block polymerization at the barbed ends of actin filaments have been isolated from many cells (for a review, see reference 35). Such capping proteins may play an important role in the polarized attachment of actin filaments to the membrane.

The present paper is the first experimental demonstration using the plasma membrane that the polarized attachment of actin filaments to the membrane in situ in cells can be explained by the pointed-end growth. A similar result was reported from the experiment using the membranes of chromaffin granules isolated from bovine adrenal medulla (36). On the other hand, our results show a sharp contrast with those obtained from the observations on the actin filaments in the microvilli of the intestinal epithelial cell and in the acrosomal process in Limulus (37, 38). In the case of the microvilli and the acrosomal processes, the actin filaments appear to be elongated by inserting G-actin between the membranes and the barbed membrane-associated ends of actin filaments. However, in these systems it is not so clear at the molecular level in what manner actin filaments bind to the membranes. Therefore it is still premature to further compare the mechanism of growth of actin filaments with respect to the membranes in different cell types. Still, we believe that the erythrocyte membrane will continue to provide the basic information of how actin filaments are associated with the plasma membrane.

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