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The Polymeric State of Actin in the Human Erythrocyte Cytoskeleton

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Reports on the polymeric state of actin in the red cell have been diverse. We have used phalloidin to stabilize the actin in erythrocyte ghosts prior to extraction in low ionic strength media. A mild proteolytic digestion and Sepharose 4B gel filtration enable an F-actin polymer to be isolated in pure form [1]. Detailed size analysis of this polymer in a range of experiments suggests that actin exists in the erythrocyte principally as a polymer of 100 nm length composed of 30 monomers in a double helical chain 15 monomers long with an estimated molecular weight of 1.3×10^6 daltons.

Key words: actin, cytoskeleton, red cell, erythrocyte, size distribution

Reports on the state of actin in the human erythrocyte have been diverse. The early work of Tilney and Detmers [2] suggested the presence of G-actin in the erythrocyte although more recently the work of Pinder et al [3], Lin and Lin [4], Cohen and Branton [5], and Brenner and Korn [6] has led to a consensus that actin is present as short oligomers of F-actin that are associated with spectrin in a high molecular weight complex.

Since erythrocyte ghosts are routinely prepared by lysis in hypotonic solutions and spectrin extraction entails incubation under conditions of low ionic strength in the absence of divalent cations, actin in these extracts should exist principally in the G-form, yet the presence of short oligomeric filaments of actin has been inferred by the fact that these extracts have an accelerating effect on F-actin polymerization [6].

In attempting to extract erythrocyte actin in what may be its physiologic form, the major problem has been that the F to G transformation of actin is sensitive to the type of conditions used to extract it. To overcome this we have used phalloidin to stabilize the F-actin in the erythrocyte ghost. Phalloidin is a toxin isolated from the mushroom Amanita phalloides [7] that has been reported [8] to both stabilize the filamentous form of actin and to increase the rate of the oligomerization of G to F. Phalloidin seems to bind to F-actin throughout its length, stabilizing the F form, and effectively reducing the G-actin critical concentration in equilibrium with the F polymer. While a recent report [9] has presented some evidence that phalloidin can cause a significant enhancement of polymerization under conditions where actin would normally either not polymerize or only partially polymerize, the conditions to achieve this are stringent and it should be possible to select suitable conditions such that the actin F:G equilibrium is "frozen."

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METHODS

Extraction and Purification of Actin

Human erythrocyte ghosts were prepared by hypotonic lysis at 4°C as previously described [10] and were shaken gently overnight on ice in a solution of 10^{-6} phalloidin (Boehringer) in 5 mM phosphate (pH 8.0) containing 0.03 mM phenylmethane sulphonyl fluoride (PMSF). These ghosts were extracted by incubation at 37°C in 5 volumes of extraction buffer (0.4 mM sodium phosphate, 0.1 mM EDTA, 0.03 mM PMSF at pH 8.5) [10] with the addition of 10^{-6} M phalloidin. Vesicles were removed from this solution by centrifugation at 300,000 g for 1 hr and the supernatant concentrated in the extraction buffer by ultrafiltration through an Amicon PM30 membrane at 4°C to 1-2 mg/ml. Tris-HCl (pH 8.0) was added to this solution to give a final concentration of 10 mM Tris and an ice cold 1 mg/ml solution of trypsin (Worthington) in 10 mM Tris-HCl (pH 8.0) was added to the solution at an enzyme:substrate ratio of 1:20 and the mixture incubated on ice for 60 min before the reaction was stopped by the addition of diisopropylfluorophosphate (DFP) (Aldrich Chemical Co.) at a final concentration of 1 mM.

The F-actin was further purified on a preparative scale by gel filtration on a 90 × 2.5 cm column of Sepharose 4B (Pharmacia Ltd.) with elution by a buffer of 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 0.5 mM β-mercaptoethanol, 0.03 mM PMSF, 10^{-7} M phalloidoin, and 0.05% NaN_3. The elution profile and SDS gel electrophoresis of a range of fractions (Fig. 4) show that actin is present in the void volume of this column. The void fractions were concentrated by ultrafiltration through an Amicon PM30 membrane to approximately 0.5 mg/ml and stored in the elution buffer in the presence of 1 mM DFP.

Polyacrylamide Gel Electrophoresis

Nondenaturing electrophoresis was performed as previously described [11]. Second dimension and slab gels were 10% acrylamide gels containing 0.1% $NaDodSO_4$ as described by Laemmli [12].

Two-Dimensional Peptide Maps

Chymotryptic maps were prepared from Coomassie blue stained protein bands by the method of Elder et al [13] with modifications as described by Speicher et al [14].

Determination of Protein Concentration

Protein concentrations were determined by the method of Lowry [15].

Rotary-Shadow Replication

Samples were prepared for rotary shadow replication by spraying onto freshly cleaved mica in a solution of 70% glycerol [16,17]. Where applicable the phalloidin concentration was maintained at 10^{-7} M.

Negative Stained Samples

Samples were stained with a mixture of 2% phosphotungstic acid containing 20 μg/ml bacitracin (pH 7.3), by diluting 1 volume of sample into 9 volumes of stain to give a final protein concentration of 10 μg/ml [18]. Glow discharged

carbon films were floated onto the samples and picked up with clean 200 mesh grids, excess sample was drawn off the film, and the sample allowed to dry (J. Albert, personal communication).

Both shadowed replicas and negative stained preparations were examined on a Phillips EM-300 electron microscope.

Size Analysis

F-actin filaments in electron micrographs were measured and the measurements analyzed using the Videoplan Image Analysis System (Carl Zeiss, West Germany). Data were stored on floppy discs and measurements from different micrographs were combined to produce size distributions and statistical analyses.

RESULTS

Erythrocyte ghosts treated with phalloidin were morphologically indistinguishable from normal erythrocyte ghosts by phase-contrast microscopy and could be extracted by low ionic strength solutions of EDTA at alkaline pH to yield a supernatant containing spectrin, actin, and band 4.1 in yields and proportions comparable to those obtained from normal ghosts (Fig. 1a,c and Table I).

The two dimensional electrophoretic analysis of the extracts of normal (Fig. 1a) and phalloidin (Fig. 1c) ghosts indicate that the ghosts contain actin in two forms—monomeric G-actin and a form associated with spectrin in a high molecular weight complex. Spectrin is present in these gels in dimer (S_2) and tetramer (S_4), forms, both of which appear to be largely actin free, as well as in a high molecular weight complex with actin (complex). If the extracts are first analyzed in lower percentage acrylamide nondenaturing gels, higher order spectrin oligomers can be resolved which appear to be actin-free as well (results not shown) [19].

It is not clear from this analysis whether the actin associated with spectrin in the high molecular weight complex is in the F or the G form. Chymotryptic peptide maps prepared from stained protein bands excised from this type of gel indicate that the actin associated with the complex is identical to that which migrates in the G form. The peptide map of erythrocyte actin (Fig. 2b) shows the same pattern of iodinated peptides as that of human platelet actin (Fig. 2c) and rabbit muscle actin (Fig. 2a). Corresponding radiolabeled peptides show identical electrophoretic mobility in the first dimension in all three samples but there are characteristic differences in chromatographic mobility in the second dimension of some peptides reflecting changes in uncharged amino acids in the primary sequence.

When tryptic digests of the extracts of both normal and phalloidin treated ghosts were compared on the two-dimensional, nondenaturing SDS gel electrophoresis system (Figs. 1b,d) the pattern of Coomassie blue staining spots appeared to be almost identical but with one striking difference: A high molecular weight form of actin was present in the digest of the phalloidin treated ghost extract. deVries and Wieland [20] have reported that treatment of F-actin with phalloidin confers a measure of resistance to proteolysis by subtilisin upon the molecule and it seems likely that this high molecular weight form might represent a similarly protected F-actin polymer.

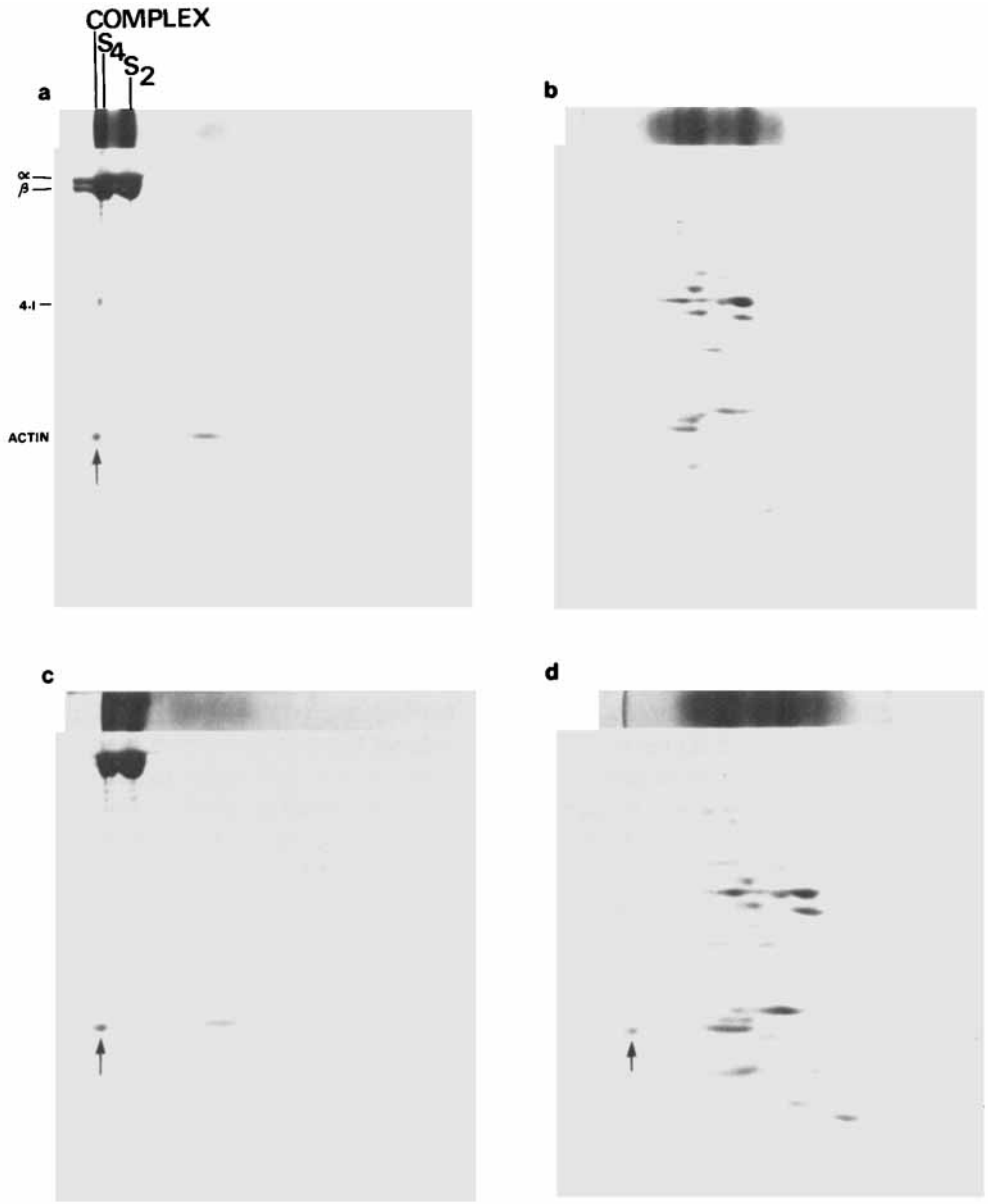

Fig. 1. Two dimensional nondenaturing/$NaDodSO_4$ gel electrophoresis of ghost low ionic strength extracts before and after digestion. Erythrocyte ghosts were prepared as described in Methods and were then shaken overnight in 5 mM phosphate (pH 8) with or without 10^{-6} M phalloidin on ice. Both samples were then incubated as described to extract the membrane cytoskeletal components. This extracted material was analyzed before and after digestion with trypsin by electrophoresis under nondenaturing conditions in the first dimension in 4% acrylamide gels (left to right) followed by $NaDodSO_4$ gel electrophoresis into a 10% gel in the second dimension (top to bottom). (a) Extract of normal ghosts; (b) digest of normal ghost extract; (c) extract of phalloidin-stabilized ghosts; (d) digest of phalloidin-stabilized ghost extact.

TABLE I. Protein Yields

	Normal		With phalloidin	
	Total protein (mg)	(%)	Total protein (mg)	(%)
Ghosts	114	100	225.7	100
Vesicles	81.9	72	162.5	72
Extract	23.9	21	47.5	21
Purified F-actin	–	–	4.9	2

Rotary shadowed samples of the phalloidin treated ghost extract digest (Fig. 3c) show structures similar to those seen in samples of muscle F-actin (Fig. 3a), which supports the hypothesis that this digest contains protected F-actin filaments.

This high molecular weight form of actin can be purified by gel filtration on Sepharose 4B as described in the Methods section. $NaDodSO_4$ gel electrophoresis of fractions obtained from such a gel filtration (Fig. 4) indicate that the material eluting in the void volume of this column is composed of highly purified actin. The identity of this material as F-actin was confirmed by its morphology in electron micrographs of both rotary shadowed replicas and negative stained preparations as well as by peptide mapping.

The yields of material at various steps of this purification were calculated from estimates of the amount of protein and are given in Table I.

Size Distribution of Erythrocyte F-Actin

The size distribution of F-actin filaments in electron micrographs was analyzed and the results are summarized in histograms in Figure 5 and in statistical form in Table II. The rotary shadowed replicas of erythrocyte F-actin show a narrow size distribution (Fig. 5a).

The absence of long F-actin filaments in these preparations could indicate that the size distributions are an artifact of the stress forces created during the spraying stage of the preparation of the material for rotary shadow replication [17], although it should be borne in mind that phalloidin does tend to stabilize F-actin to breakage by shear stress [8]. Tyler and Branton [17] have reported filaments of up to 2 μm in length in the absence of phalloidin, much longer than the longest filaments visible here.

Moreover negative stained preparations of digests of extracts of phalloidin treated ghosts show F-actin filaments with a similar size distribution to those of the rotary shadowed replicas of purified F-actin. More detailed analysis of the micrographs of negatively stained, purified erythrocyte F-actin (Fig. 5b) indicates a similar size distribution to that of the rotary shadowed replicas (with a slight increase in longer filaments) supporting the view that the rotary shadowed replicas are not artifactual. In these experiments the red cell actin has been stabilized by phalloidin under "depolymerizing" ionic conditions. However it has been recently reported [9] that muscle actin under conditions that would normally cause only partial polymerization will show a significant enhancement of polymerization in the presence of equimolar phalloidin. This result is attributed to the ability of phalloidin to stabilize actin filaments, thereby diminishing the rate of depolymerization and to its ability to bind to G-actin monomers. The latter are believed to exist in the "F-actin tertiary conformation" and are thus able to initiate polymer formation.

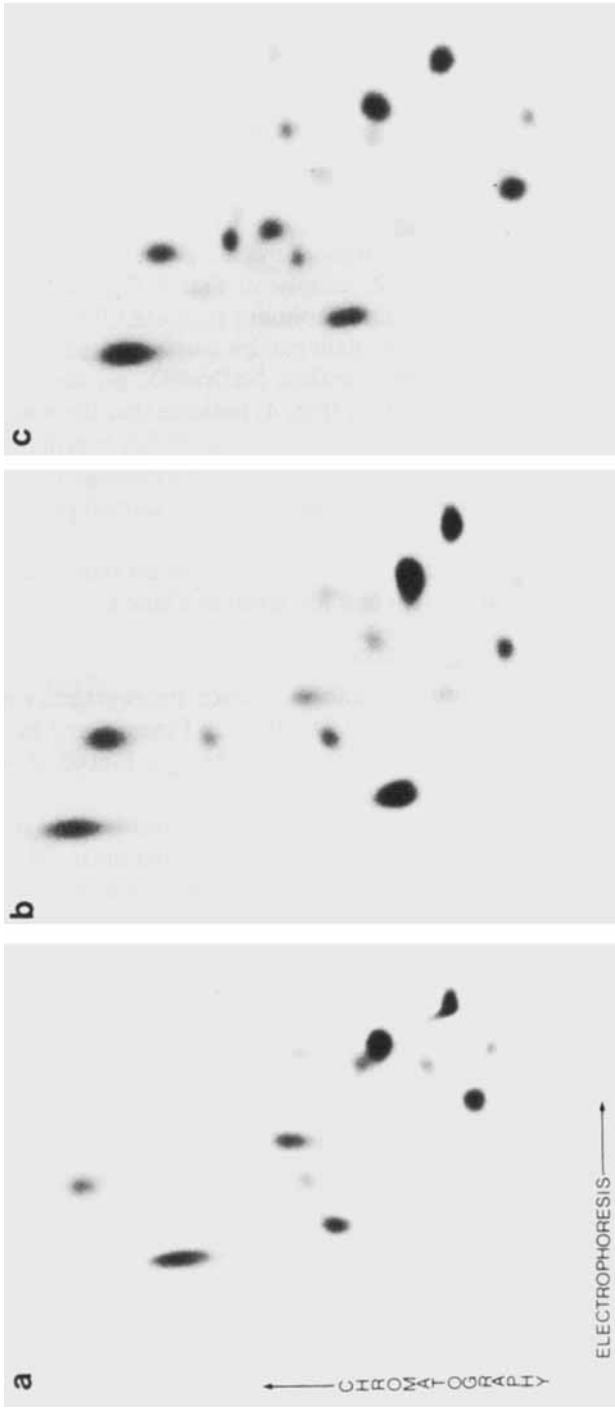

Fig. 2. Two dimensional chymotryptic peptide maps of actin samples. Coomassie blue stained actin bands were excised from polyacrylamide gels and labeled with ^{125}I, digested with chymotrypsin, and mapped as described in Methods. (a) Rabbit skeletal muscle actin; (b) human erythrocyte actin; (c) human platelet actin.

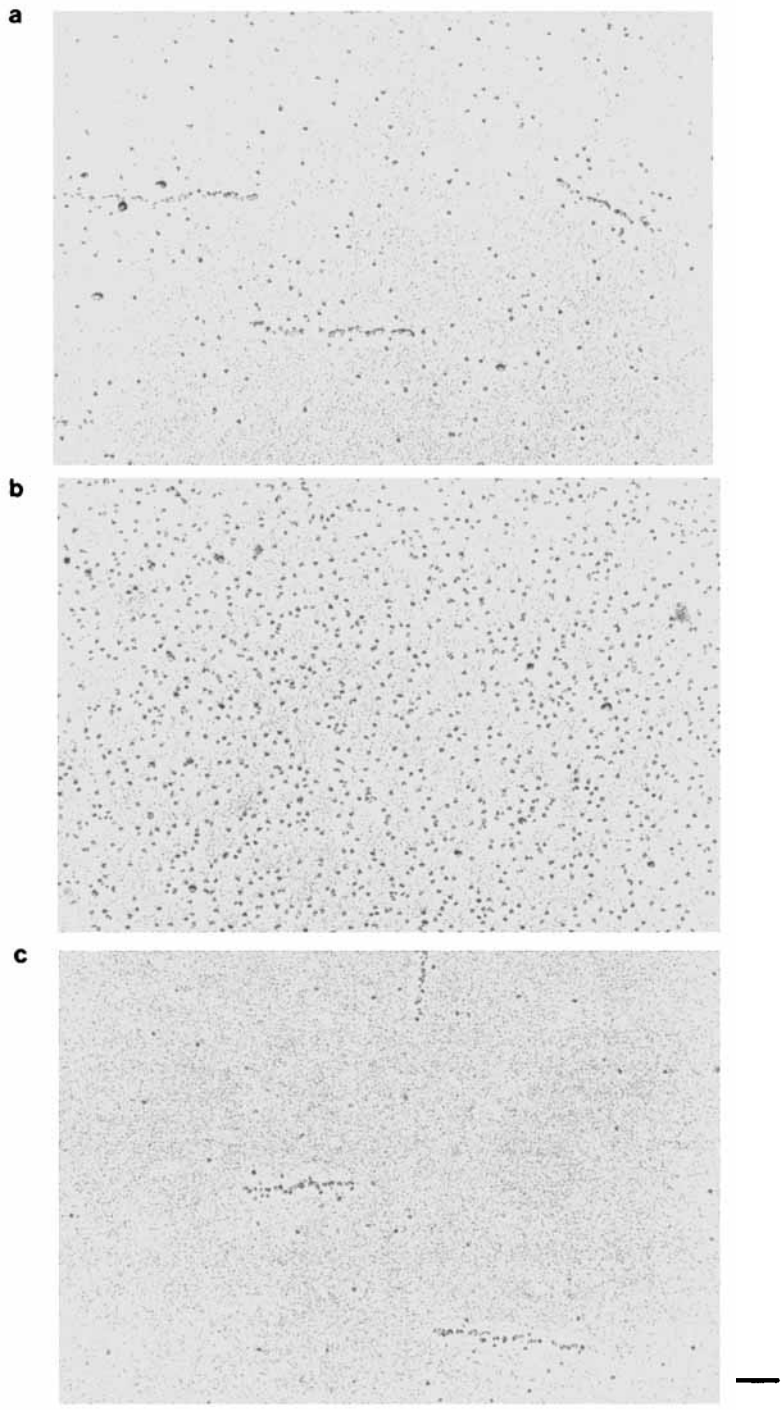

Fig. 3. Rotary shadowed replicas of actin samples. Samples of actin were replicated as described in Methods. (a) Rabbit skeletal muscle F-actin; (b) rabbit skeletal muscle G-actin incubated overnight on ice in 10^{-6} M phalloidin, 5 mM phosphate (ph 8.0); (c) purified human erythrocyte F-actin. Bar represents 100 nm.

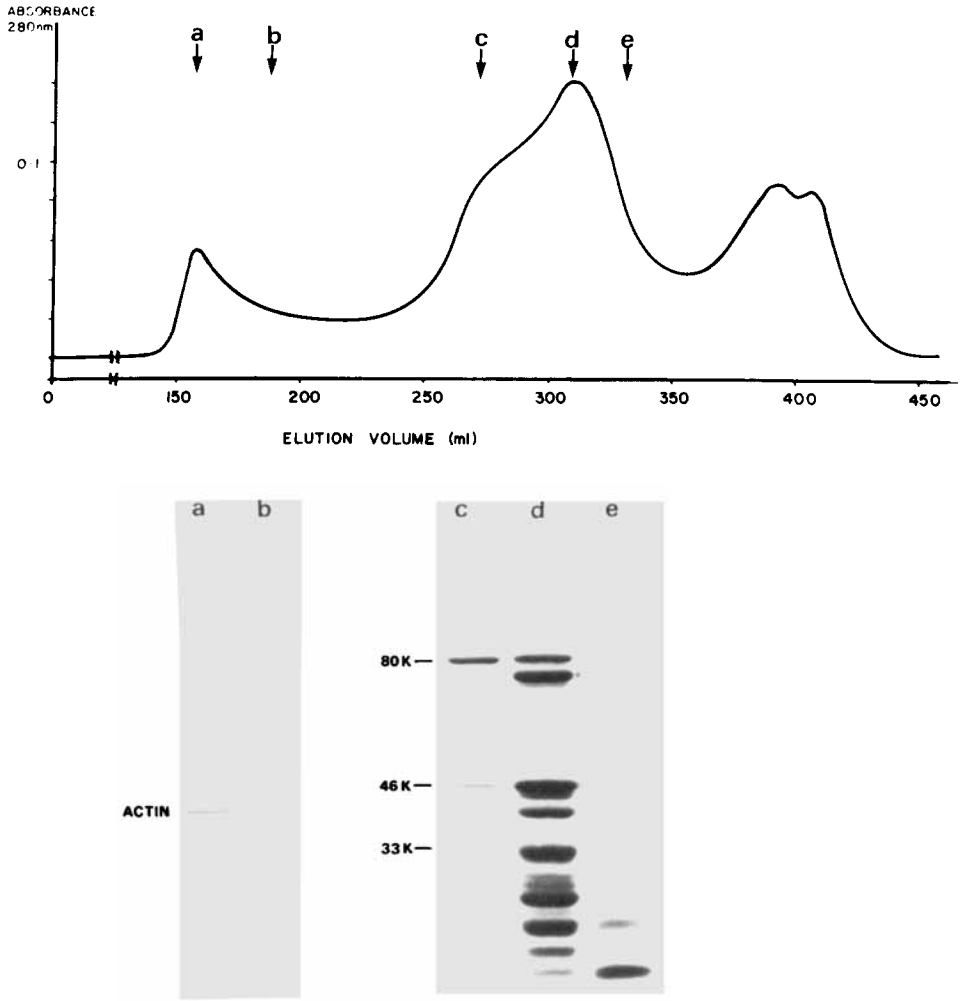

Fig. 4. Sepharose 4B gel filtration of partially digested extract of phalloidin-stabilized ghosts. The digest prepared as described in Methods was gel filtered on a 90 × 2.5 cm column of Sepharose 4B. Elution with a buffer of 10 mM tris (pH 7.6), 0.1 mM EDTA, 0.5 mM β-mercaptoethanol, 0.03 mM PMSF, 10^{-7} M phalloidin, and 0.05% NaN_3. Absorbance at 280 nm was monitored and fractions analyzed by electrophoresis in a $NaDodSO_4$ 10% polyacrylamide gel using the buffer system of Laemmli [12].

To test whether phalloidin treatment of ghosts causes a change in the F:G ratio or in the F-actin size distribution we have incubated rabbit muscle G-actin overnight in 5 mM phosphate buffer (ph 8.0) in the absence of ATP and divalent cations (the same conditions used for ghost incubation) and have observed no polymerization (Fig. 3b). However the rate limiting step in the polymerization of G-actin seems to be a change in conformation of the monomer and the formation of short F-actin oligomers that act as nuclei for the addition of further G-actin monomers. Since such nuclei may be present in the material extracted at low ionic strength from erythrocyte ghosts [4,5,6], it is conceivable the phalloidin could cause their associa-

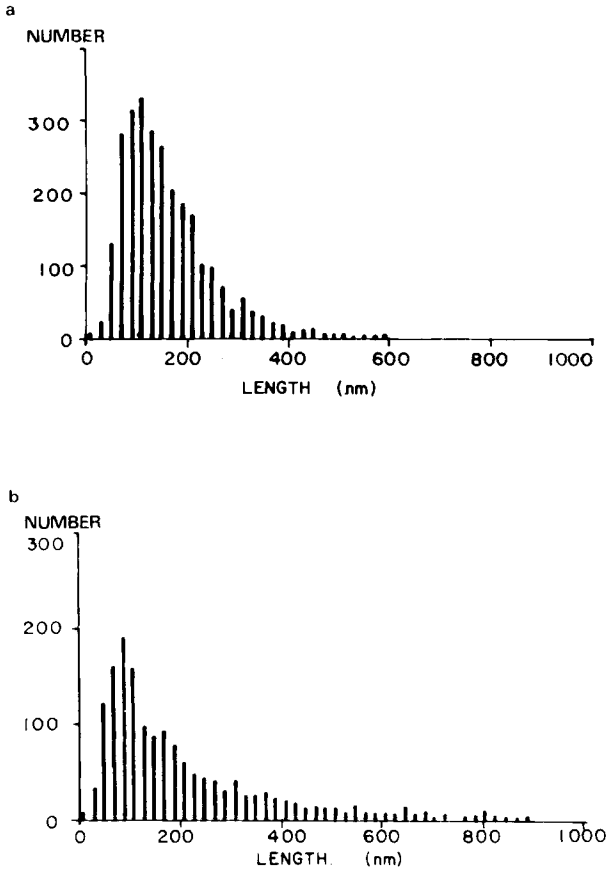

Fig. 5. Histograms of F-actin size distributions. (a) Rotary shadow replicas of human erythrocyte F-actin after purification as described in Methods. (b) Negatively stained samples of human erythrocyte actin after purification as described in Methods.

TABLE II. Statistical Analysis of F-Actin Size Distribution

	Rotary shadowed RBC	Negatively stained RBC
Total count	2753	1594
Sum	445532 nm	338564 nm
Mean	161.8 nm	212.4 nm
Median	139.9 nm	146.1 nm
Mode (20 nm class interval)	109.3 nm	89.8 nm

tion to form longer polymers or the addition of further monomers to them even under conditions that would not normally favor such conversions. We have investigated this possibility by polymerizing rabbit muscle G-actin in the presence of KCl, Mg^{2+}, ATP, and phalloidin, and then dialyzing the F-actin/G-actin mixture into the 5 mM phosphate (pH 8.0) ghost incubation buffer containing phalloidin. The mixture was then sonicated to an extent sufficient to break up the F-actin (Phalloidin

stabilized F-actin is much more resistant to such treatment than untreated F-actin). The sonicated material was then incubated overnight on ice under the same conditions employed for the extraction of ghosts and any increase in size of the actin fragments was looked for. Since we were unable to find any increase in length of F-actin filaments formed under these conditions (results not shown) it seems unlikely that phalloidin-induced G- or F-actin self association occurs to any extent during the phalloidin treatment of red cell ghosts.

DISCUSSION

The work of Pinder et al [3], Lin and Lin [4], Cohen and Branton [5], and Brenner and Korn [6] has implied the presence of F-actin polymers, able to promote polymerization of G-actin, in low ionic strength extracts of erythrocyte ghosts. The presence of these polymers in a high molecular weight complex with spectrin has led to two principal conclusions: (i) since F-actin is not visible in rotary shadowed replicas of these extracts it is likely to be present as short oligomers that act as nucleation sites for further G-actin polymerization; and (ii) the actin may be involved in cross-linking spectrin tetramers to form an extensive two dimensional cytoskeleton [22].

Recent results from this laboratory [19] have demonstrated that such a network can be derived from spectrin alone without the assistance of actin or any other protein.

It is paradoxical that actin filaments are not evident in rotary shadowed replicas of extracts of phalloidin-stabilized ghosts (Fig. 6a), but they are evident once purified (Fig. 6c). We feel that the most likely explanation for this is that at the approximately modal size of 100 nm the filaments would be similar in length to that of a spectrin dimer and as such would be difficult to find in a field of spectrin dimers in which they would be present at a ratio of less than 1 actin filament to 15 spectrin dimers. However, if negatively stained preparations are studied, then actin is indeed seen in both extracts and purified actin samples of phalloidin-stabilized ghosts (Fig. 6b,d). Negative staining is a high resolution technique since the negative stain penetrates to the surface of the molecule outlining surface topography. On the other hand the shadowing technique builds up a larger Pt/C replica of the molecule with a loss of surface resolution. Since spectrin is very poorly resolved in negatively stained preparations, one would expect that F-actin would be much more evident in these preparations.

Erythrocyte F-actin appears to be stable during ghost preparation but the stabilized F-actin is much more resistant to such treatment than untreated F-actin). presence of phalloidin. So far we have been unable to isolate visible actin filaments from extracts of normal ghosts that have been phalloidin stabilized after extraction but prior to digestion. We feel that the most likely explanation of this change in susceptibility to depolymerization is that the F-actin filaments are partially stabilized by spectrin and it is only after trypsin digestion that they become completely susceptible to depolymerization. Thus extracts of normal ghosts could contain short F-actin oligomers that are stabilized by being bound to spectrin and can act as polymerization nuclei in the presence of exogeneous actin under suitable conditions.

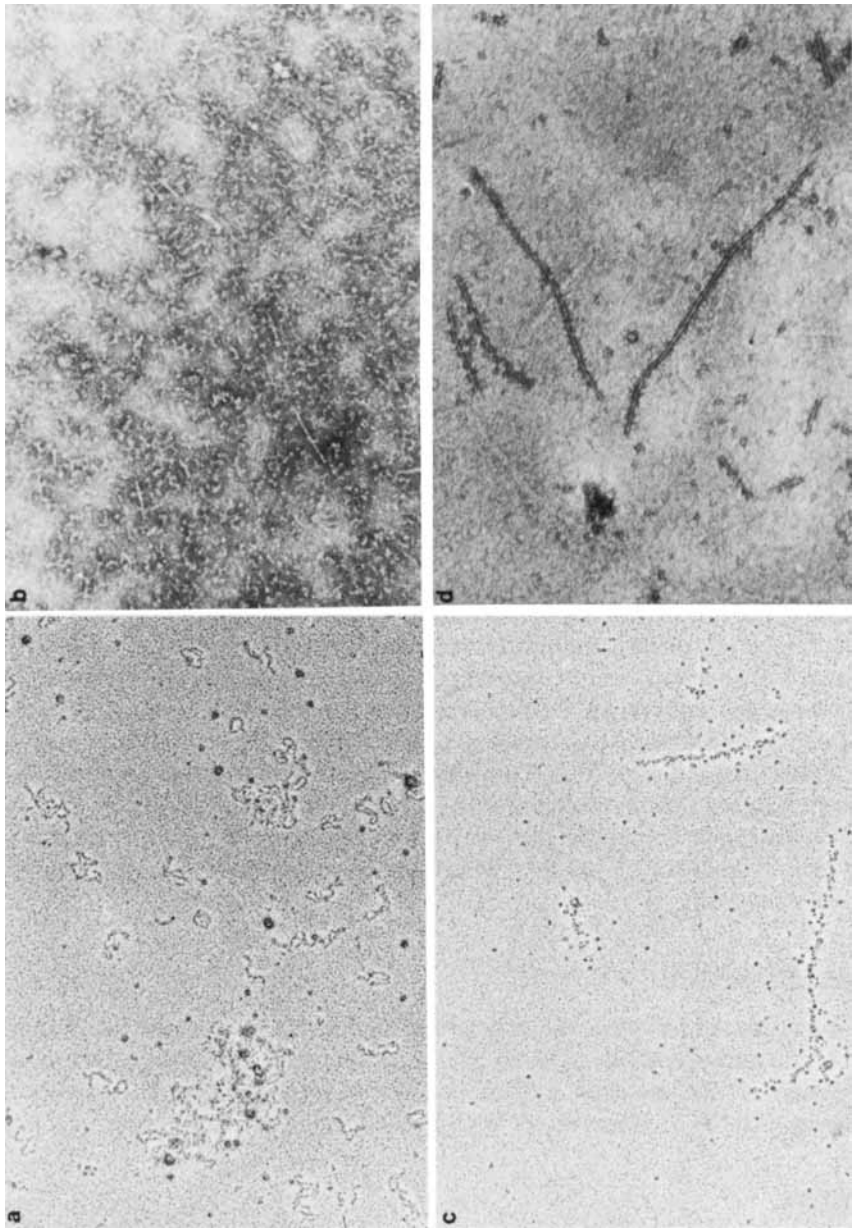

Fig. 6. Electron micrographs of extracted material and purified F-actin from human erythrocyte ghosts. (a) Rotary shadowed replicas of extract of phalloidin-stabilized ghosts; (b) negatively stained extracts of phalloidin-stabilized ghosts; (c) rotary shadowed replicas of purified phalloidin-stabilized human erythrocyte F-actin; (d) negatively stained, purified, phalloidin-stabilized human erythrocyte F-actin. Bar represents 100 nm.

The physiologic significance of erythrocyte actin remains speculative; it seems to play some role in nuclear extrusion [27] but much of the actin is extruded with the nucleus while spectrin remains almost completely with the developing red cell. This residual actin does not seem to be absolutely required to form an extensive cytoskeleton. One may speculate that the spectrin molecules form a horizontal network under the membrane while the F-actin filaments lie perpendicular to this plane. This may explain the recent results of Tsukita et al [23]. Brenner and Korn's [24] work suggests that spectrin may bind to the end of the F-actin filament opposite to that of cytochalasin D, as it decreases the critical concentration and tends to block the depolymerization effects of cytochalasin D. This binding of spectrin would be similar to that proposed for β-actinin in muscle cells. Our results suggest that red cell actin does not depolymerize rapidly until the spectrin–actin complex is dissociated, as red cell ghosts may be prepared in the absence of phalloidin without conversion of F-actin to G-actin. However when the cytoskeleton is broken up by the extraction in low ionic strength media, then either a loosening of the spectrin–actin association or the dissociation of another component results in depolymerization of the F-actin in the absence of phalloidin. Smaller oligomers of F-actin that remain in close association with spectrin may be more resistant to depolymerization under these conditions.

It is interesting that we have been able to visualize F-actin extracted from Triton shells [25] by trypsin digestion following phalloidin stabilization (results not shown) and that the integrity of such shells is sensitive to bovine pancreatic DNaseI [26] that will cause actin depolymerization by binding to G-actin, thereby effectively "reducing" its concentration.

Whatever the function of erythrocyte actin may be, our results suggest a method whereby erythrocyte actin may be prepared in reasonable amounts that could be used for a reevaluation of spectrin–actin and band 4.1-actin interactions using human red cell actin rather than actin derived from the muscle of a different species. Moreover the actin size distribution reported here with a modal length of 100 nm indicates that a double helical polymer of two chains each composed of 15 monomers yielding an F-actin molecule with an approximate molecular weight of 1.3×10^6 daltons, probably represents the predominant form in vivo and the size of these F-actin filaments may prove to be defined by interactions with spectrin oligomers and band 4.1.

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