Membrane-associated Actin from the Microvillar Membranes of Ascites Tumor Cells

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ABSTRACT A membrane fraction (MF2) has been purified from isolated microvilli of the MAT-C1 subline of the 13762 rat mammary ascites adenocarcinoma under conditions which cause F-actin depolymerization. This membrane preparation contains actin as a major component, although no filamentous structures are observed by transmission electron microscopy. Membranes were extracted with a Triton X-100-containing actin-stabilizing buffer (S buffer) or actin-destabilizing buffer (D buffer). In D buffer > 90% of metabolically labeled protein and glycoprotein was extracted, and 80–90% of these labeled species was extracted in S buffer. When S buffer extracts of MF2 were fractionated by either gel filtration on Sepharose 6 B or rate-zonal sucrose density gradient centrifugation, most of the actin was found to be intermediate in size between G- and F-actin. In D buffer most of the MF2 actin behaved as G-actin. Extraction and gel filtration of intact microvilli in S buffer also showed the presence of the intermediate form of actin, indicating that it did not arise during membrane preparation. When [^{35}S]methionine-labeled G-actin from ascites cells was added to S buffer extracts of MF2 and chromatographed, all of the radioactivity chromatographed as G-actin, indicating that the intermediate form of actin did not result from an association of G-actin molecules during extraction or chromatography. The results of this study suggest that the microvillar membrane fraction is enriched in an intermediate form of actin smaller than F-actin and larger than G-actin.

Actin is a ubiquitous component of mammalian cells, involved in muscle contraction and implicated in cell shape (1) and motility (2) and cell surface organization (3) in nonmuscle cells. Actin is known to exist in two different states: G-actin, the globular, soluble form, and F-actin, the filamentous, polymerized form, which predominates in muscle. In nonmuscle cells G-actin is found in cell extracts and can be quantified by electrophoresis after sedimentation of insoluble actin (4) or by analysis of its inhibition of DNase (5). The insoluble actin exists in several forms, as bundles of filaments in stress fibers, cores of brush border microvilli, and as meshworks of filaments (6). However, actin is also found associated with membranes in an insoluble form (7–10). In the case of the erythrocyte, membrane actin is present as a significant component, but no F-actin-like filaments can be observed by electron microscopy (11). Based on the ability of actin-containing extracts to accelerate G-actin polymerization by nucleation (12), the postulation has been made that erythrocyte actin exists as oligomeric units (12, 13), possibly similar to short lengths of F-actin. Direct observation of the actin oligomers has not been possible, because the erythrocyte actin is present as a macromolecular complex with the erythrocyte cytoskeletal protein spectrin (14).

In attempting to understand the nature and roles of the membrane-cytoskeleton interaction, we have been studying microvilli isolated from ascites tumor cells (15). Membranes isolated from the microvilli under conditions which depolymerize F-actin still contain actin as a major component but do not have filamentous structures observable by electron microscopy (Carraway, Cerra, Bell, and Carraway. Manuscript submitted for publication.) In this communication we present biochemical evidence that much of the membrane-associated actin is present in a form intermediate in size between G- and F-actin.

MATERIALS AND METHODS

Preparation and Labeling of Cells and Microvilli

The 13762 MAT-C1 ascites rat mammary adenocarcinoma cells were maintained and metabolically labeled as previously described (16). For labeling in

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vivo 12.5 mCi of [^{14}C]glucosamine (50–60 mCi/mmol) or [^{3}H]leucine (100 mCi/mmol) were injected into the peritoneal cavity 16 h before isolation of the cells. Branched microvilli were isolated from these cells by a modification of the method previously described (15), using differential centrifugation (Carraway et al. Manuscript submitted for publication.) After shearing of microvilli from cells by passage through a syringe needle (15), the suspensions were centrifuged at 750 *g* for 5 min to remove cell bodies. Microvilli were then pelleted by centrifugation at 48,000 *g* for 40 min and washed twice. Morphological and biochemical examinations (15) indicated that these preparations were not significantly different from those isolated by Percoll centrifugation (15), except that there were more intact branched structures and the yield was greatly increased (Carraway et al. Manuscript submitted for publication.).

Preparation of Microvillar Membranes

For preparation of microvillar membranes the microvilli were incubated for 30 min at room temperature in 5 mM glycine, 1 mM EDTA, 5 mM mercaptoethanol (pH 9.5) to depolymerize actin filaments and extract actin and actin-binding proteins (9), then homogenized in a tight Dounce homogenizer (20–30 strokes). Residual microvilli and large fragments were removed by centrifugation at 10,000 *g* for 15 min. The membrane fraction (MF2) was isolated by centrifugation at 150,000 *g* for 60 min. Membranes could be further purified by density gradient centrifugation, the major fraction banding at a density corresponding to 45% sucrose. Additional extraction of MF2 with glycine-EDTA-mercaptoethanol, which produces leaky membranes, did not extract significant amounts of actin or other proteins.

Analytical Procedures

Quantification of G- and F-actin was performed by the DNase inhibition assay of Blikstad et al. (5). SDS PAGE was performed by the method of King and Laemmli (17) using 5–12% gradient gels. Chromatography was performed in 0.75 × 60 cm columns equilibrated with actin-stabilizing buffer (S buffer, 5 mM Tris, 0.15 M NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythritol, 0.5% Triton X-100 and 0.01 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.6) or actin-destabilizing buffer (D buffer, 0.75 M guanidine HCl, 0.5 M sodium acetate, 0.5 mM $CaCl_2$, 0.5 mM ATP, 0.5% Triton X-100, 0.01 mM PMSF, pH 7.6) (18). Sucrose density sedimentation was performed on a 5-20% linear sucrose gradient centrifuged at 130,000 *g* for 40 h. Two dimensional isoelectric focusing-SDS PAGE (IEF-SDS PAGE) was performed in the laboratory of Dr. Robert Rubin (University of Miami School of Medicine, Miami, FL) by a previously published procedure (19).

The myosin affinity technique was performed according to the procedure of Koch and Smith (20), using rabbit muscle myosin for precipitation and rabbit muscle F-actin for controls. For quantitative analysis cells labeled with [^{35}S]-methionine were used for preparation of MF2 fractions. The myosin precipitates were subjected to SDS PAGE, and the bands for actin and the 58,000-dalton component were excised and counted.

Preparation of [^{35}S]Methionine-labeled Actin and Incubation with S-Buffer Extracts

MAT-Cl cells were washed in McCoy's modified medium plus 20% calf serum and incubated with 350 mCi[^{35}S]methionine in 20 ml of medium for 1 h at 37°C. Microvilli were isolated and solubilized in D buffer and the extracts chromatographed over Sephadex G-150 in D buffer. Fractions containing predominantly actin were pooled, concentrated/dialyzed against S buffer, and added to MF2 solubilized in S buffer. The resulting solution was chromatographed on Sepharose 6B and fractions monitored for radioactivity and by SDS PAGE.

RESULTS

Membrane Preparation and Extraction

Microvilli can be isolated from 13762 MAT-Cl mammary ascites tumor cells by a gentle shearing procedure followed by centrifugation (15). Microvillar membranes (MF2) were isolated from microvilli by homogenization in glycine, EDTA, mercaptoethanol, pH 9.5 (GEM buffer), and centrifugation. This is a modification of the procedure used to extract spectrin-actin from erythrocyte membranes (11, 21) and actin and associated proteins from ascites cell surface envelopes (9) and was designed to solubilize the actin microfilaments which form the core of the microvilli. Transmission electron microscopy of thin sections of MF2 preparations indicated a population of vesicles heterogeneous in size. Although actin was the major component by SDS PAGE (Fig. 1) and 2D IEF SDS PAGE (data not shown) of the MF2 preparations, no structures resembling microfilaments were observed by electron microscopy (Carraway et al. Manuscript submitted for publication.). Even if small numbers of microfilaments were present and unobserved, they would be too few to account for the abundance of actin. However, filamentous actin is often destroyed by conditions used for preparation of membrane samples for electron microscopy (22). Therefore, we decided to use biochemical methods to investigate the nature of the MF2 actin.

Several experiments indicate that MF2 actin is not merely trapped in the vesicles. When MF2 preparations were treated with GEM buffer under conditions that make vesicles leaky, no release of actin was observed. More conclusively, when MF2 preparations were treated with 0.1–0.2% Triton X-100 in phosphate-buffered saline (PBS) or GEM buffer at 25°C under conditions that release >80% of the membrane glycoproteins (determined by glucosamine label), only 10–20% of the MF2 actin was released.

The second major component of the MF2 fraction was a 58,000-dalton polypeptide (58 kd) which is present in branched

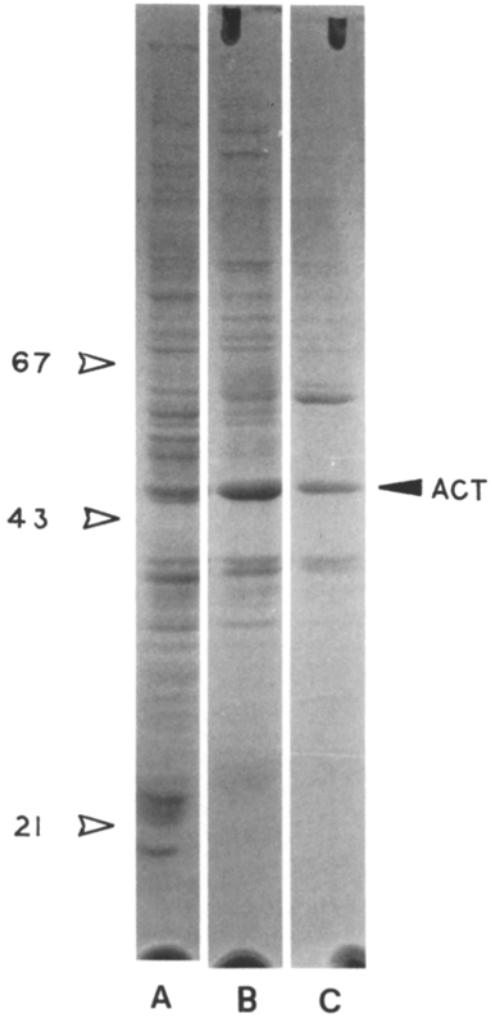

FIGURE 1 Dodecyl sulfate PAGE of MAT-Cl intact cells (A), microvilli (B), and MF2 (C) showing actin (*ACT*) as a major component of MF2 along with the 58,000 dalton polypeptide. Lanes A and B contain ~40 μg protein; lane C, 20 μg. Molecular weights of standard proteins are shown to the left of the gel (× 10^3).

microvilli of the MAT-Cl ascites subline of this mammary tumor. This protein is essentially completely insoluble when microvilli are extracted with 0.2% Triton in phosphate-buffered saline (Carraway et al. Manuscript submitted for publication.). A number of other more minor polypeptide components are present in the MF2 fraction, but are not readily observable unless gels are more heavily loaded than that in Fig. 1. These include the major microvillar glycoproteins ASGP-1 and ASGP-2, which stain poorly with Coomassie Blue (23).

When the DNase inhibition assay of Blikstad et al. (5) was used to assess the state of the actin in MF2, the results predicted a ratio of F-actin to G-actin of 1.1–1.6. However, since neither F- nor G-actin had been observed in these preparations, we examined the actin by fractionation procedures to determine the size of the membrane-associated actin. For the fractionation attempts we chose the buffer systems used by Strauch, et al. (18). The S buffer was designed to prevent interconversion of G- and F-actin, and the D buffer depolymerizes F-actin to give G-actin (5). Both buffers contain Triton X-100 for membrane solubilization.

When MF2 was extracted under these conditions, >90% of metabolically labeled leucine- or glucosamine-containing moieties were extracted from the membranes in D buffer and 80–95% in S buffer with >90% recoveries of label for both buffers. Electrophoretic analysis on dodecyl sulfate gels indicate that >80% of the MF2 actin was solubilized (not sedimented at 150,000 *g* for 1 h) in S buffer and >90% in D buffer. In contrast, a substantial fraction of actin sedimented from intact microvilli treated with S buffer but not D buffer, results which are consistent with the observation of microfilaments in microvilli but not in MF2. The 58-kd polypeptide was not solubilized by the S buffer, but was completely soluble in D buffer. These results indicate that both S and D buffers release actin in a soluble form, in contrast to the behavior with Triton in PBS. Since S buffer is supposed to retard depolymerization of F-actin, the extracts were examined by fractionation techniques to determine the nature of the solubilized actin.

Fractionation of Extracts

MF2 preparations solubilized in S and D buffers were fractionated by rate-zonal ultracentrifugation on sucrose density gradients. The radioactivity profiles from the gradients were monitored (Fig. 2), and fractions from the gradients were analyzed by SDS PAGE. Rabbit muscle G- and F-actin were ultracentrifuged on similar gradients in S buffer, and rabbit muscle G-actin was run in D buffer. In S buffer essentially all of the rabbit muscle G-actin was found near the top of the gradient in fractions 1 and 2 (Fig. 2*A*). The F-actin in S buffer was all found at the bottom of the gradient. None was observed in other fractions. In contrast, the MF2 actin in S buffer was found primarily in fractions *3* and *4* within the gradient (Fig. 3*A*). These results suggest that the MF2 actin in S buffer is intermediate in size between G and F-actin. In D buffer the rabbit muscle G-actin is found in fractions *1* and *2* of the gradient. The MF2 actin in D buffer is also found primarily in fractions *1* and *2* (Fig. 3*B*), indicating that MF2 actin is converted to G-actin in D buffer. There is some trailing into the gradient, however, suggesting that depolymerization may not be complete under the conditions used.

In S buffer the 58-kd polypeptide was found near the bottom of the gradient, together with a small amount of actin (10–20% of total). In D buffer 58 kd was soluble and was found in the first two gradient fractions.

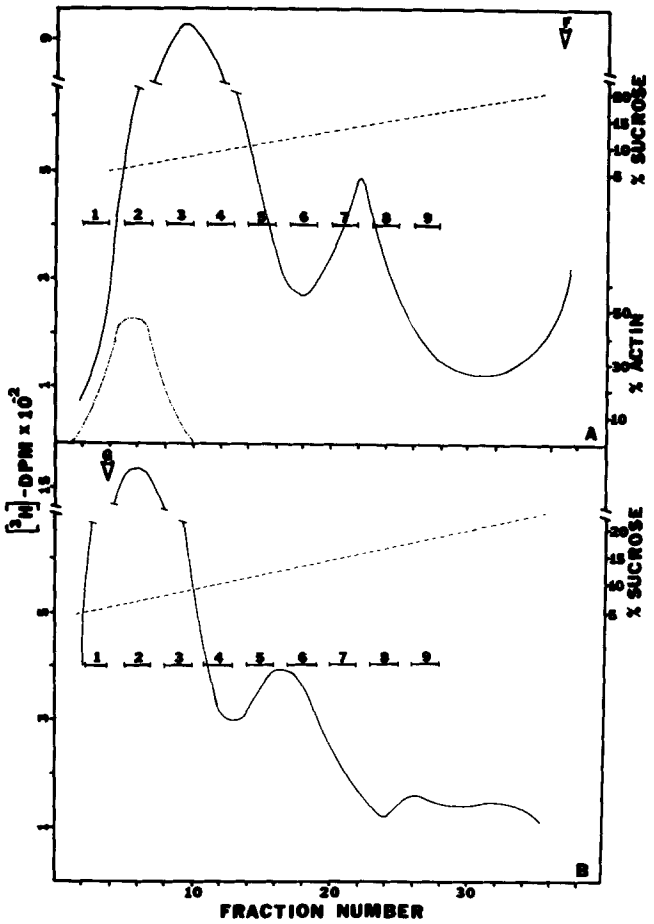

FIGURE 2 Sucrose density gradient centrifugation of leucine-labeled MF2 solubilized in S buffer (A) or D buffer (B). MF2 (0.5 mg protein per ml) was extracted for 15 min at room temperature in S or D buffer, applied to a 5–20% sucrose gradient and centrifuged at 22,000 rpm for 40 h in a SW 27.1 rotor (Beckman Instruments, Richmond, CA). Fractions were counted, pooled as shown, dialyzed, and electrophoresed. The numbers on the bars on A and B indicate fractions applied to the corresponding numbered gel lanes in Fig. 3 A and B, respectively. Rabbit muscle G-actin in S buffer (—●—) was run as a standard on a gradient similar to A. The standard gradient was monitored by electrophoresis and gel scanning. Each experimental gradient was run three times with the same results. The dashed lines indicate sucrose concentration. The open arrows show the migration positions on the gradient for rabbit muscle G- and F-actin in separate experiments. The F- and G-actin were not detected in other regions of the gradient, indicating that they did not change during the incubation in S buffer or centrifugation.

The results, from gradient fractionation of solubilized MF2, suggest the presence in S buffer of a form of actin intermediate in size between G- and F-actin. To confirm this result, extracts were chromatographed on Sepharose 6B and monitored by counting (data not shown) and SDS PAGE. The gels were scanned and the elution profiles for actin and 58 kd plotted from the results (Fig. 4). Rabbit muscle G- and F-actin were analyzed under identical conditions (Fig. 4). Most of the MF2 actin in S buffer eluted at a volume intermediate between G- and F-actin. About 30% of the actin coeluted with G-actin. Since this is a greater amount than found by ultracentrifugation, some of the intermediate actin may be breaking down during the time required for chromatography. Rabbit muscle F-actin was eluted at the void volume in S buffer. No actin was observed included within the column in this experiment,

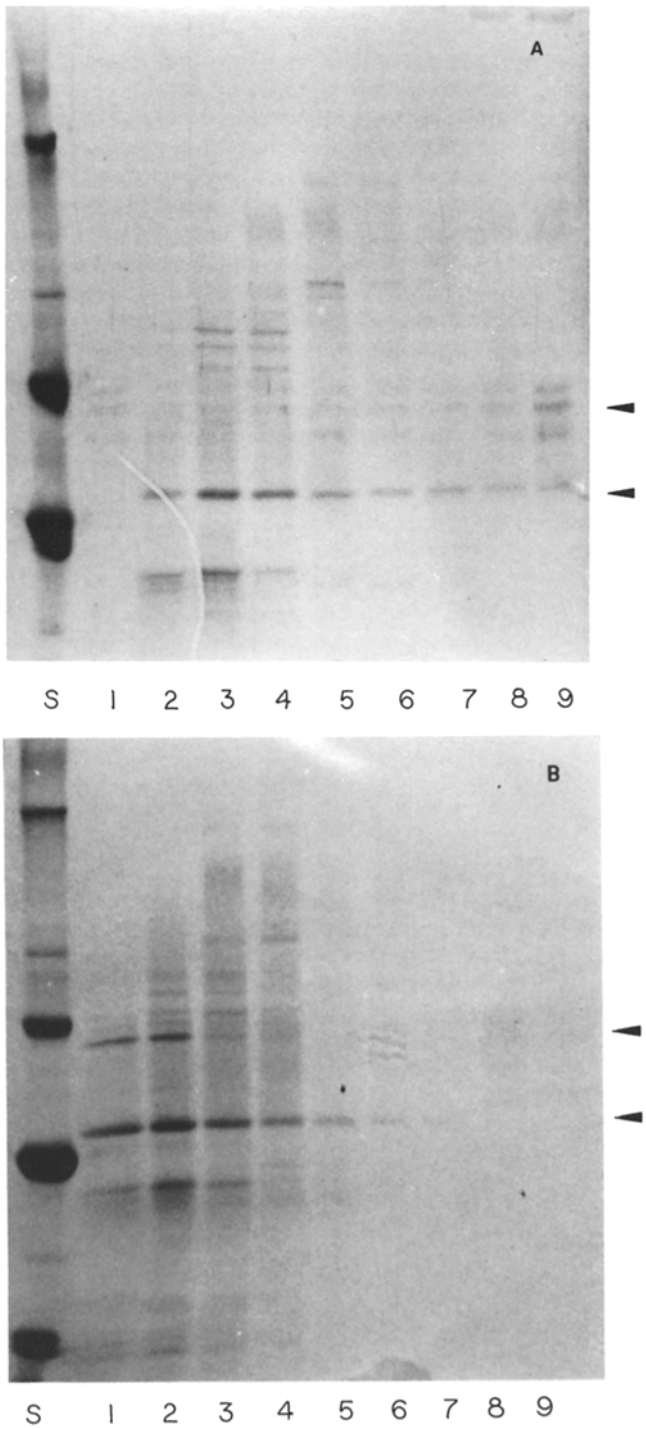

FIGURE 3 Dodecyl sulfate polyacrylamide gel electrophoresis of fractions from the gradient in Fig. 2 of samples centrifuged in S buffer (A) or D buffer (B). Aliquots from gradient fractions were dialyzed into dodecyl sulfate in buffer before applying to gels. The lanes with the heaviest stain contain ~20 μg protein. Fractions after fraction 9 from the elution profile in Fig. 2 did not contain detectable proteins other than small amounts of actin. The arrowheads to the right of the gel mark the positions of actin (lower) and the 58,000 dalton polypeptide (upper); S indicates a lane containing standards. The two heaviest bands are ovalbumin (lower) and bovine serum albumin (upper).

indicating that no substantial breakdown of F-actin is occurring under the conditions used. A small amount of MF2 actin (~10%) was observed together with 58 kd eluting slightly after the void volume of the column. It is not shown on the profile because of difficulties in quantitating the small amounts present across the peak.

When MF2 was solubilized and chromatographed in D buffer (Fig. 4 *B*), the actin eluted at a volume corresponding to that of rabbit muscle G-actin in D buffer (Fig. 4 *B*) as would be expected from the previous observations on the depolymerization of actin in this buffer (5). These results are consistent with the findings of Strauch et al. (18) concerning the stability of G- and F-actin in S buffer.

To assure that the presence of the intermediate actin did not result from degradation of F-actin during the membrane preparation in GEM buffer, microvilli were subjected to gel filtration in S buffer. As shown in Fig. 5, F-actin is observed as a substantial component of the microvilli, as expected, along with the intermediate form of actin and some G-actin.

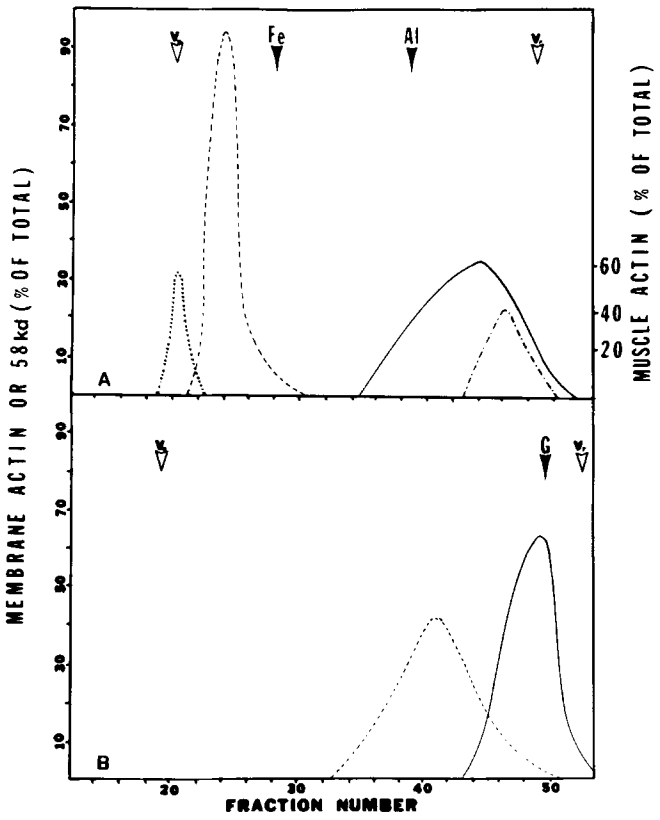

FIGURE 4 Elution profile from Sepharose 6B of actin (——) and the 58 dalton polypeptide (- - -) chromatographed in S buffer (A) or D buffer (B). MF2 (0.5 mg protein per ml) was solubilized as for Fig. 2 and applied directly to a Sepharose 6B column. Column fractions were monitored by dodecyl sulfate PAGE. Elution profiles for rabbit muscle F-actin (····) and G-actin (—·—) in S buffer are shown as standards for comparisons in A. These samples were run in S buffer with ~0.1 mg/ml actin applied to each column. The columns were monitored by dodecyl sulfate electrophoresis and the gels were scanned. The protein eluted in each fraction is plotted as arbitrary units for each chromatographic run. Since actin represents ~20% of the protein in the membranes, the amount of actin in the standards is approximately the same as the actin in the chromatographed, solubilized membranes. This equivalence was borne out by the similarity of staining densities (data not shown) on dodecyl sulfate gels of the membranes and standard actin bands. The open arrows in A show the void (*Vo*) and retained (*Vr*) volumes. The closed arrows show elution volumes for ferritin (*Fe*) and aldolase (A). The open arrows in B show the void (*Vo*) and retained (*Vr*) volumes. The closed arrow shows the elution volume of G-actin in D buffer.

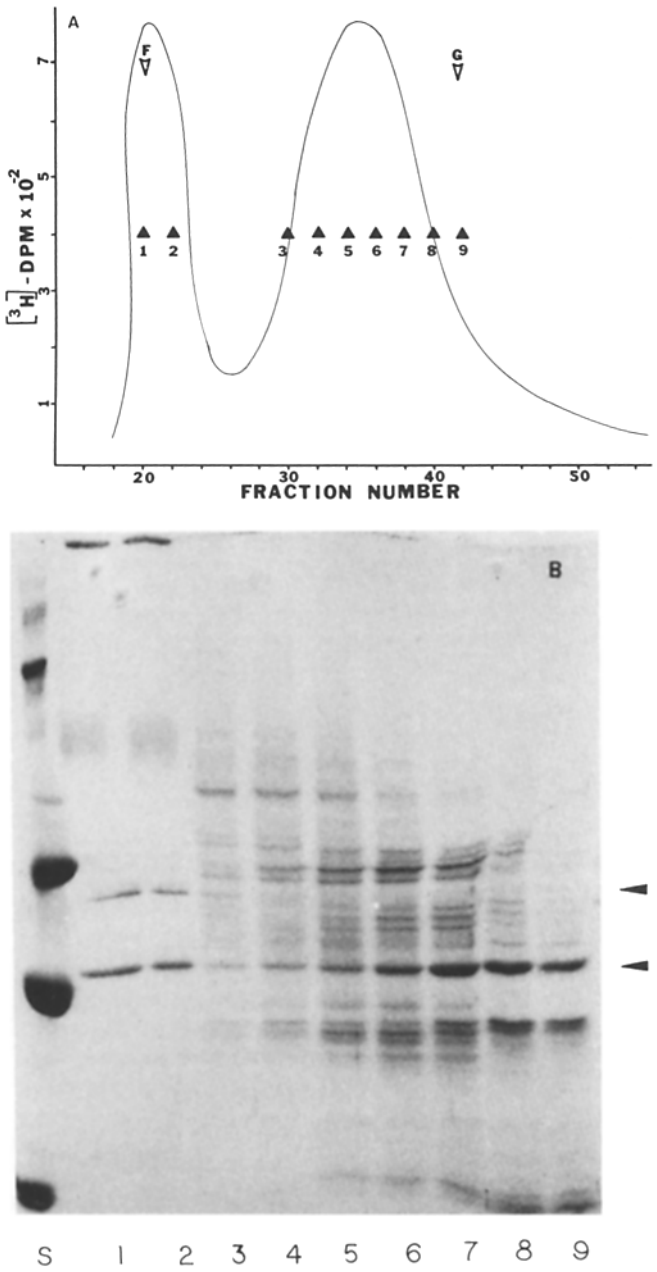

FIGURE 5 Gel filtration of leucine-labeled microvilli in S buffer. Fractions marked 1-9 in the profile (A) correspond to gel lanes 1-9 (B). Arrowheads marked by F and G in the profile correspond to the elution volumes of rabbit muscle F- and G-actin, respectively. The experimental protocol was the same as used for Fig. 4, except that microvilli were used rather than MF2.

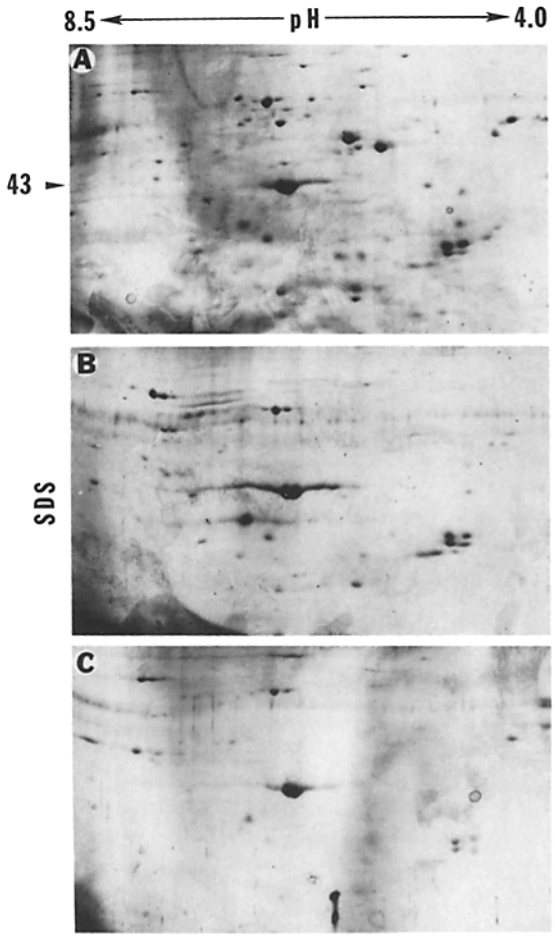

FIGURE 6 Analysis of gradient fractions by 2-dimensional isoelectric focusing dodecyl sulfate electrophoresis for identification of actin. Gels A, B, and C show fractions corresponding to lanes 2, 3, and 4 of Fig. 3A. The doublet in the center of the gel corresponds exactly to actin isolated from these cells and to actin observed in other nonmuscle cells.

Identification of Actin

To establish that the 43,000 dalton polypeptide from the gradients and columns was actually actin, samples from the appropriate sucrose density gradients and gel filtration eluates were examined by 2-D IEF-SDS PAGE. A first dimension doublet of molecular weight 43,000, presumably β- and γ-actins, was observed as the predominant polypeptide in all instances (Fig. 6 is shown as an example). This doublet was identical to the actin doublet in intact ascites cells. Although a number of minor polypeptides were found in fractions containing intermediate actin, none of the major species were observed to coincide with intermediate actin in both elution behavior on gel filtration and migration on the gradient. Some of them showed different behavior relative to actin on the gradient and column. This is not too surprising, since both gel filtration and sedimentation are complex functions of the sizes and shapes of the molecules. An example of this behavior is shown by 58 kd, which behaves in D buffer as if it is slightly larger than actin by gel filtration and slightly smaller by sedimentation.

The polypeptides observed in MF2 fractions with intermediate actin were also observed in microvilli. Thus, it is unlikely that they are proteolytic fragments generated during the preparations. We have not observed any polypeptide in the appropriate quantity and with the appropriate behavior in S buffer to suggest a direct association with the intermediate actin. However, to establish unequivocally the presence or absence of other actin binding proteins, particularly small polypeptides, will require purification of the intermediate actin.

Recombination with [^{35}S]G-Actin

Another possible explanation for the presence of an intermediate form of actin is that monomeric actin has become associated into oligomers during extraction or fractionation, possibly under the influence of some factor in the extract. To test this possibility G-actin was prepared from microvilli of cells labeled with [^{35}S]methionine. The actin was purified by

gel filtration in D buffer on Sephadex G-150. It was shown by sedimentation analysis to be capable of polymerization in KCl. The radioactive actin was dialyzed into S buffer, mixed with MF2 extract in S buffer and chromatographed on Sepharose 6B in the same buffer. The eluate was analyzed by electrophoresis to identify the components of the radioactive peaks. The radioactivity was eluted almost entirely at a volume corresponding to G-actin. Essentially none was found at the volume of elution of the intermediate form of actin. A small amount of actin radioactivity was present near the void volume, eluting with the 58-kd polypeptide, suggesting a possible association of actin with this polypeptide. The failure of actin to polymerize in S buffer under our extraction conditions is consistent with the observed critical concentration of rabbit muscle actin in S buffer (18).

Myosin Affinity Analysis

Because of the possibility that MF2 polymeric actin might be breaking down to oligomers during the chromatography or density gradient centrifugation, the myosin affinity technique was used to assay for F-actin in S buffer-solubilized, methionine-labeled MF2. Supernates of MF2, solubilized in S buffer, contained ~80% of the total MF2 actin, but only 10% of the supernate actin was precipitated by myosin. In contrast, the pellet from MF2 in S buffer contained 20% of the MF2 actin, 70% of which was precipitated by myosin. When unfractionated MF2 in S buffer was assayed after only a 15-min incubation in the buffer, ~30% of the actin was precipitated. Interestingly, the 58-kd polypeptide was precipitated with the myosin from both the unfractionated MF2 in S buffer and the pellet in S buffer, suggesting that it is associated with the actin.

DISCUSSION

Actin is one of the two major protein components of the microvillar membranes of the MAT-Cl ascites tumor cells. The inability to release the actin by hypotonic or detergent treatments which render the membranes soluble or leaky indicates that the actin is not simply soluble actin trapped in membrane vesicles. Several lines of evidence indicate that most of the membrane-associated actin is not F-actin. (*a*) No microfilaments have been observed in thin sections of the membranes by transmission electron microscopy. (*b*) Membrane actin solubilized in S buffer is eluted predominantly within the retarded volume of the column, far from the elution volume of rabbit muscle F-actin. (*c*) The soluble membrane actin did not sediment with rabbit muscle F-actin upon rate-zonal density gradient centrifugation. (*d*) Rabbit muscle F-actin was stable in S buffer under the chromatography and centrifugation conditions used. (*e*) The soluble membrane actin did not bind to myosin in the myosin affinity assay.

Several additional lines of evidence indicate that the membrane-associated actin is not formed as a result of self-association of monomeric actin in S buffer. (*a*) Although some of the actin fractionated by gel filtration or ultracentrifugation appears to be monomeric in S buffer, most of the actin appears larger. (*b*) Rabbit muscle G-actin is stable under the fractionation conditions in S buffer. (*c*) The critical concentration for polymerization of G-actin in S buffer (18) is substantially higher than the concentration of actin used in our experiments. (*d*) Mixing experiments with [^{35}S]G-actin showed no evidence of oligomerization. It is clear that the intermediate form of actin does not arise as a consequence of the membrane isolation, since it is present in microvilli, which are obtained in sealed form by gentle shearing of the cells (Carraway et al. Manuscript submitted for publication.).

Several explanations can be offered for the observed intermediate size of actin: association of monomeric actin with other proteins; an alteration in the conformation of monomeric actin; or the existence of the actin in an oligomeric state smaller than F-actin. Association of actin with other proteins is an unlikely explanation, because no other polypeptides are observed by electrophoresis in sufficient amount to account for the increase in size by such an association. An alteration in the conformation of actin to produce a molecule with an expanded Stokes radius also seems unlikely, since the intermediate form of actin is also observed by sucrose density gradient centrifugation. Moreover, it would be expected that actin would be more likely to assume an expanded conformation in D buffer, which contains guanidine hydrochloride, than in S buffer. Thus, we feel that the most likely explanation for the actin behavior is the presence of oligomers. It is not possible to state unequivocally that the actin associated with the membrane is the same size as the oligomers observed by fractionation, since multimeric membrane actin might be less stable than F-actin to S buffer. However, it is clear that most, if not all, of the membrane actin is different from F-actin or G-actin.

The postulation of oligomers explains the results in the DNase assay, if it is assumed that oligomers inhibit DNase, but to a lesser extent than G-actin. However, such behavior raises questions about the ability of the DNase assay to determine G- or F-actin in samples containing membranes or any oligomeric actin. The presence of oligomeric actin would confound the results, making the interpretations of G/F ratios unreliable, as noted for the microvillar membranes.

Our results raise questions about the exact nature of membrane-associated actin and how it is associated with the membrane. The actin is very resistant to extraction under conditions which remove F-actin and extract actin from erythrocyte membranes. It is interesting to note that the conditions (D buffer) which converted the actin to a monomeric form also solubilized the 58-kd polypeptide, suggesting that these proteins may be associated. Moreover, the actin that is not solubilized by S buffer is eluted near the void volume of the Sepharose 6B column with the 58-kd component. The nature of this actin is also uncertain. Additional studies are underway to define the properties of the 58-kd component, its association with the membrane and its relationship to actin. Further studies on the intermediate form of actin are in progress to determine its physical and chemical properties as well as its relationship to the membrane and to the actin microfilaments of the microvilli.

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