CYTOSKELETAL PROTEINS ASSOCIATED WITH CELL SURFACE ENVELOPES FROM SARCOMA 180 ASCITES TUMOR CELLS

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Summary: Membrane envelopes prepared from Zn^{++}-treated Sarcoma 180 cells contain polypeptides which appear to be related to the putative cellular cytoskeletal elements responsible for control of cell shape and motility. These include actin, myosin, α-actinin and a large polypeptide (mol wt 250,000) with some similarities to spectrin of the erythrocyte membrane. If the envelopes are vesiculated by extraction with alkaline EDTA solutions at low ionic strength, four major polypeptides are released, including the actin and spectrin-like materials; myosin is not extracted. The stabilized envelopes offer a useful source of material for the characterization of cytoskeletal elements and for the investigation of their associations with the membrane.

Cell shape and motility are vital cellular functions which are probably controlled by cytoskeletal elements attached to the plasma membrane (1, 2). The molecular nature of these substances and their attachment sites is still obscure, but actin- and myosin-like proteins have been demonstrated in a number of cell types (1), and actin has been found in association with isolated plasma membranes (3). Other candidates for cytoskeletal elements have recently been isolated from extracts of chicken gizzard (4) and polymorphonuclear leukocytes (5). These have molecular weights of about 250,000 compared to 210,000 for myosin and 43,000 for actin. Some of their properties, e.g. solubility and actin-binding, are reminiscent of those of the erythrocyte structural protein spectrin. Immunofluorescence staining of normal fibroblasts shows cable-like structures extending along the cell for each of these three types of proteins (4, 6, 7). Transformed cells do not show the actin cables (8) by this method, and they also have, at least in some cases, smaller amounts of actin associated with isolated plasma membranes than do their normal counterparts (3).

We have previously demonstrated that plasma membrane envelopes prepared

from Sarcoma 180 ascites cells contain a set of high molecular weight polypeptides which are virtually absent from plasma membrane vesicles prepared from the same cell type (9). We now report that extraction of the envelopes with EDTA at low ionic strength converts the envelopes to vesicles with concomitant extraction of a unique set of proteins, including actin- and spectrin-like polypeptides, but not myosin. These are the same conditions which extract spectrin and actin from erythrocyte ghosts with concomitant vesiculation (10, 11).

Methods: Sarcoma 180 ascites cells were grown in the peritoneal cavity of mice and isolated as previously described (9). Cell surface membrane envelopes were prepared by procedures described previously (9, 12, 13). Washed membranes were extracted at a final concentration of 1 mg/ml in 5 mM glycine, 1 mM EDTA and 5 mM mercaptoethanol, pH 9.5, by stirring gently in the cold for 8-10 hrs. Vesicles were pelleted at 23,500 x g for 35 minutes. The supernatant was recovered for subsequent fractionation of proteins on a Sepharose 4B column in 50 mM Tris, 5 mM glycine, 1 mM EDTA and 5 mM mercaptoethanol, pH 8.5. The membrane pellet from the EDTA extraction was extracted further with 0.45 M KH_2PO_4 - KCl, pH 6.5, at a final concentration of 0.5 mg protein/ml for 12-18 hr at 4°C. The supernatant was collected after centrifugation at 23,500 x g for 35 min and diluted 1:20 with ice cold distilled water. Precipitated myosin was recovered by centrifugation and further purified by repeated depolymerization-polymerization (14).

Protein concentrations were determined by the procedure of Lowry et al. (15). Polyacrylamide gels were run in the presence of sodium dodecyl sulfate (SDS) using acrylamide and bis-acrylamide concentrations of 5% and 0.025% respectively. Membrane samples were dissolved as previously described (9). Myosin and actomyosin ATPase activities were determined by the assays described by Bárány and Bárány (16). Inorganic phosphate released was measured by a modification of the method of Taussky and Shorr (17). Partially purified protein components were obtained from gel chromatography fractions and further separated by polyacrylamide gel electrophoresis in dodecyl sulfate. The protein bands were stained with Coomassie blue for localization and identification and then excised for hydrolysis. The gel slices were soaked in distilled water and hydrolyzed in 6N HCl containing 0.2% phenol and 0.1% thioglycolic acid for 22 hrs at 110° (18). A sample of acrylamide gel containing no protein was hydrolyzed as a blank. The amino acid composition was obtained on a microanalyzer designed and built by Dr. T. H. Liao (19).

Results: The recent emphasis on the role of cytoskeletal components in cell surface behavior has led us to pursue further investigations of possible cytoskeletal elements associated with cell surface envelopes. There are five different polypeptides which are of interest in this regard and which represent a significant fraction of the "membrane" protein. These are shown as

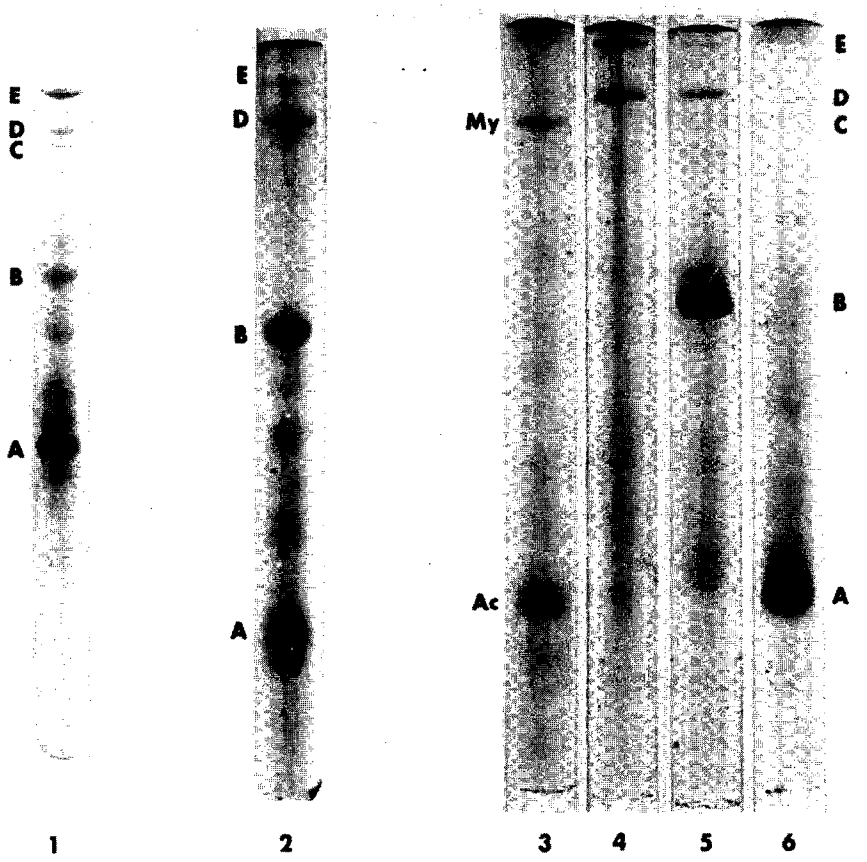

Fig. 1 - SDS polyacrylamide electrophoresis of putative cytoskeletal proteins. Gel 1 shows isolated membrane envelopes prepared as previously described (9). Gel 2 shows EDTA extract of the membranes. Gel 3 shows purified rabbit muscle actomyosin, with the myosin heavy chain labeled My and the actin chain labeled Ac. Gels 4-6 show chromatographic fractions of the extracted proteins separated on a Sepharose 4B column in Tris-glycine-EDTA. The individual gels were not run under identical conditions so that direct comparisons of migration distances for 1, 2 and 3-6 are not meaningful.

bands A-E on an SDS acrylamide gel (Fig. 1, gel 1). If membrane envelopes are extracted with EDTA at low ionic strength, they fragment into vesicles (phase contrast and electron microscopy) with the release of four of these polypeptides, shown on gel 2 of Fig. 1. This supernatant, after concentration, can be separated by column chromatography on Sepharose 4B in Tris-EDTA into three fractions, containing predominantly (in order of elution) poly-

peptides E and D, polypeptide B and polypeptide A. Experiments on the purified fractions, the whole membranes and the extracted membranes permit tentative identification of the polypeptides as discussed below.

Band A is a non-muscle actin or actin-like protein. It has an apparent molecular weight of 43,000 and runs coincident with purified rabbit muscle actin on both SDS and non-SDS acrylamide gels. Sarcoma actin obtained from SDS gels of the Sepharose 4B column eluates shows an amino acid analysis (Table 1), that is very similar to that of other actins (1, 20) using criteria previously established for comparison (21, 22).

Band D material, isolated by column chromatography, shows properties similar to human erythrocyte spectrin. It has an apparent molecular weight of 250,000 , migrating slightly slower than the upper band of spectrin on SDS acrylamide gels. It tends to be insoluble at low pH and forms insoluble aggregates upon storage at 4°C. Bands D and E may have a monomer-polymer relationship, since E appears to form at the expense of D upon storage of membranes. The properties and molecular weight of D are also reminiscent of two proteins which have been purified recently from cytoplasmic extracts of macrophages (5) and chicken gizzard (4). The former (actin-binding protein) induces gel formation by actin (23). The latter (filamin) is extracted with smooth muscle myosin and appears by immunofluorescent staining to be present in filamentous structures in cultured fibroblasts. We suggest that these two proteins and polypeptide D are probably quite similar in their structures and functions. The fact that D is associated with the plasma membrane and is released together with actin may offer a clue to its role in cell structure.

Polypeptide C (apparent molecular weight 210,000) migrates slightly faster than the lower polypeptide of erythrocyte spectrin and coincident with rabbit muscle myosin. It is not extracted at low ionic strength, under conditions where myosin would not be soluble. Membrane envelopes show

ATPase activity when assayed by procedures used for actomyosin or myosin (16). The ATPase activity is extractable with high salt under conditions which release the 210,000 mol wt polypeptide along with several other polypeptides.

Polypeptide B has an apparent molecular weight of 100,000. If it is part of the cytoskeletal structure, it is probably related to α-actinin, a protein involved in filament structure in the Z-band in muscle (24). Immunofluorescent studies by Lazarides (25) suggest the presence of α-actinin or a similar protein in the cable-like structures associated with fibroblasts. Further support for the identification of band B as α-actinin is obtained from amino acid analysis (Table I) of B isolated from acrylamide gels, which indicates strong similarities between B and purified α-actinin (24) using previously established criteria for comparisons (21, 22).

Table 1

Amino acid composition of EDTA-extractable components isolated from SA-180 membranes.[a]

Amino Acid	Band A	Band B
Asp	10.8	12.3
Thr	6.5	5.5
Ser	7.0	5.9
Glu	13.9	15.6
Pro	3.4	3.1
Gly	9.7	7.4
Ala	8.8	8.6
Val	4.0	2.7
Met	2.6	2.3
Ile	4.6	5.4
Leu	9.2	10.4
Tyr	2.3	2.1
Phe	3.7	3.8
His	2.4	2.7
Lys	7,1	7.3
Arg	5.2	6.1

[a]"Relatedness coefficients" were calculated by the method of Marchaloni and Weltman. Values of 18 and 28 were obtained for bands A and B when compared to human platelet actin and pig muscle α-actinin, respectively. Any value less than 50 is considered indicative of strong similarities or homology between the proteins being compared.

Discussion. The results of this investigation indicate that putative cytoskeletal proteins are associated with cell surface membranes prepared from cells which have been "fixed" with Zn^{++} before isolation of the membranes. Some of these proteins apparently play a role in stabilization of the membrane envelope. This role may involve a complex set of interactions among the contributing species. The presence of actin alone is not sufficient for envelope stabilization, since actin can be found in both envelopes and isolated vesicles (3, 9). Myosin alone is likewise not sufficient, since it is present in the vesicles obtained after EDTA extraction. The band D polypeptide may play a key role in this respect, possibly by acting as a bridge between other filaments. It is noteworthy that this polypeptide is virtually absent from plasma membranes of Sarcoma cells which have been treated with trypsin under conditions where the cells become rounded (12). This does not necessarily mean that this polypeptide is accessible to the exterior of the cell, since it could be lost during membrane preparation as a result of altered membrane interactions caused by trypsin perturbations of membrane structure (12). This polypeptide is not the same as the "large external transformation-sensitive" polypeptide described by Hynes (26, 27) and others (28-30), in spite of a similar apparent molecular weight. It is not labeled by lactoperoxidase in either whole cells or membranes (9) and shows no evidence of carbohydrate by either periodate-Schiff staining or amino acid analysis (which should detect significant amounts of amino sugar).

The results presented here suggest that "stabilized" membrane envelopes offer a useful source for the investigation of cytoskeletal elements and for studying associations between these elements and the cell membrane. However, it should be noted that there is no evidence to indicate that the associations in the stabilized membranes exist in their native form. It is also possible that Zn^{++} produces artificial associations, e.g. precipitation of cytoskeletal elements or complexes, which fortuitously stabilize the membrane envelope. Delineation of the associations of membrane and cytoskeletal

components in both stabilized membranes and intact cells is obviously of considerable interest.

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