

ACTIN ASSOCIATED WITH MEMBRANES FROM 3T3 MOUSE FIBROBLAST AND HeLa CELLS

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ABSTRACT

A protein component of membranes isolated from 3T3 mouse fibroblasts and HeLa cells has been identified as actin by peptide mapping. Extensive but apparently not total coincidence was found between the peptide maps of these two nonmuscle membrane-associated actins compared to chick skeletal muscle actin. Between 2 and 4% of the total membrane protein appears in the actin band on sodium dodecyl sulfate polyacrylamide gels of 3T3 membranes while about 4% of the membrane protein appears as the actin band from HeLa membranes. These values represent approximately the same proportion of actin to total protein found in the cell homogenates. Treatment of intact cells with levels of cytochalasin B sufficient to cause pronounced morphological changes did not change the amount of actin associated with the membrane in either 3T3 or HeLa cells. However, incubation of isolated membranes under conditions favoring conversion of actin from filamentous to monomeric form resulted in dissociation of approximately 80 and 60% of the actin from 3T3 and HeLa membranes, respectively. Thus, approximately 20% of 3T3 membrane actin and 40% of HeLa membrane actin remained associated with the membrane even under actin depolymerizing conditions.

Actin has now been identified as a component of essentially all eukaryotic animal cells in which it has been sought. In addition, myosin- and tropomyosin-like proteins have been identified in many of these nonmuscle cells. (An extensive review on the subject of contractile proteins of nonmuscle cells has recently appeared [29].) In several electron microscope preparations, apparent contact between plasma membrane and actin filaments (or thin filaments with the dimensions of actin) has been observed *in situ* (24, 36, 42, 44, 45) or in isolated plasma membranes (28). Biochemical evidence has also been presented that actin is associ-

ated with membranes of synaptosomes (3). It is, therefore, likely that actin filaments function at least in part via attachment to the plasma membrane of cells. Furthermore, control of the membrane-actin association seems a likely point for regulation of actin function as an effector of diverse cellular movements and morphological changes. We have, therefore, chosen as the initial phase of studies on the mechanisms of cellular movements to try to identify actin in membranes from 3T3 mouse fibroblasts and HeLa cells.

Most of the available methods for actin characterization seem inappropriate for the identification

of membrane-associated actin. The usual morphological method of heavy meromyosin binding might fail to reveal actin in or near the plane of the membrane, while actin activation of myosin ATPase would also be unsatisfactory because of high endogenous levels of membrane ATPases. Consequently, we decided to take advantage of the high degree of evolutionary conservation in the primary structure of actin, first shown in muscle actins by Carsten and Katz (8) and later by others for cytoplasmic actin (4, 15, 39), and to identify nonmuscle membrane actin by peptide mapping. We report now the identification of actin associated with the membranes of 3T3 and HeLa cells and a partial characterization of the nature of the actin-membrane association.

MATERIALS AND METHODS

Culture Conditions

3T3 cells obtained from Dr. Howard Green at Massachusetts Institute of Technology were grown on Falcon plastic plates in Dulbecco's modified Eagle medium supplemented with 10% calf serum. Penicillin and streptomycin were added to give 100 U/ml and 100 μ g/ml, respectively. HeLa cells were grown in spinner culture in Joklik modified minimal essential medium (MEM) with 3.5% calf serum and 3.5% fetal calf serum. Kanamycin was present at 10 μ g/ml to suppress growth of *Mycoplasma*. A doubling time of about 24 h was maintained by daily dilution of a suitable aliquot with an equal volume of fresh medium. HeLa cultures were tested once monthly for contamination with *Mycoplasma* by Flow Laboratories, Inc., Rockville, Md. and found to be uncontaminated.

Membranes

Membranes were prepared from 3T3 and HeLa cells by the method of Brunette and Till (6) with the following modifications. 3T3 cells were washed free of serum and trypsinized for 1-3 min in 0.016 M sodium citrate, 0.01 M KCl, pH 7.6 containing 0.1% trypsin (33). Apparently, this brief treatment is important for successful application of the Brunette and Till protocol to cells grown on a surface rather than in suspension (personal communication from Dr. Rose Sheinin). Trypsinization was stopped by adding calf serum, and the cells were scraped off the substrate with a rubber policeman and centrifuged at low speed. After a wash in normal saline, the Brunette and Till procedure was initiated (6). The interfacial material from each two-phase separation was removed and resuspended in fresh upper and lower phase solutions. In some experiments $ZnCl_2$ was omitted from the upper and lower phases, a modification which seemed to reduce nuclear contamination.

For HeLa membrane preparations, the third centrifugation on the two-phase system was omitted because this step did not alter the degree of contamination with nuclei, unbroken cells, and fine debris, as judged by phase-contrast microscopy. In early experiments of this series it was sometimes found that after the first centrifugation the phases either did not separate or they did not separate into equal volumes. This problem was eliminated when the following two procedures were adopted. (a) Phases were routinely prepared, the day before use, from stock solutions stored frozen at $-20^\circ C$. (b) After collection of the crude ghost fraction by centrifugation at 1,400 rpm for 15 min, the supernatant fraction was decanted, the pellet was recentrifuged at 2,800 rpm (5 min, International 269 rotor, International Scientific Instruments Inc., Palo Alto, Calif.), and the resulting supernate was removed by aspiration.

Electron Microscopy

Membranes were fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 or Epon-Araldite. Thin sections were cut with a diamond knife, stained with uranyl acetate followed by lead citrate, and photographed.

Peptide Maps

The preparation of material for peptide mapping followed, with considerable modification, the method described by Fine and Bray (15) and by Bray (4). 3T3 cells were labeled with a mixture of [^{14}C]amino acids by replacing the normal growth medium with amino acid-free MEM containing serum and 1 mCi of amino acid mixture per 200-400 ml. After approximately 4 h, MEM amino acids were added to give between $\frac{1}{20}$ and $\frac{1}{5}$ the normal concentration and the incubation was continued for about 9 h. HeLa cells were labeled similarly except that serum was absent during the initial 4-h incubation with [^{14}C]amino acids. Serum was added at the beginning of the 9-h incubation period, but unlabeled amino acids were not added at all. Membranes were prepared from the labeled cells as described, and aliquots of the labeled membranes dissolved in sodium dodecyl sulfate (SDS) and mercaptoethanol were then analyzed by polyacrylamide gel electrophoresis according to Laemmli for 3T3 cells (22) or Neville for HeLa cells (25). Gels were stained with Coomassie Brilliant Blue and the band coelectrophoresing with the actin standard on a parallel gel was cut out with a razor blade. The excised band was cut into pieces about 1 mm³ and the stain was removed by shaking the fragments at 37°C in 1 ml of 50% methanol, 7.5% acetic acid. This wash was repeated until the gel pieces were free of blue color. Little or no radioactivity was removed during this procedure. The protein was then eluted from the gel by shaking the fragments at 37°C in 0.5 ml of 0.1% SDS, 0.05 M NaH_2PO_4 , pH 7.5, plus 1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis.

In addition, 0.25–0.50 mg of electrophoretically homogeneous, nonradioactive chick muscle actin was added as carrier protein. The solution was removed after 8–12 h and the elution was repeated identically except that no additional carrier actin was added. A third treatment generally eluted very little additional radioactivity from the gel and was discarded. The materials from the first and second elutions were combined in a 15-ml centrifuge tube and the protein was precipitated by addition of 50% trichloroacetic acid to a final concentration of 10%. The mixture was chilled on ice for 15 min and the precipitate was collected as a thin film adhering to the side of the centrifuge tube by centrifugation at low speed (approx. 1,000 g) for 1 min, then at high speed (approx. 10,000 g) for 5 min. The supernate, containing less than 10% of the total radioactivity, was discarded, and the precipitate washed once with 0.1 M HCl in acetone and once with acetone by gently adding and then removing the wash solutions with a Pasteur pipette. After lyophilization for at least 4 h to remove residual trichloroacetic acid, the protein was digested at 37°C in 0.25–0.50 ml of 0.1 M NH_4HCO_3 containing 5 μg of trypsin-L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK). An additional 5 μg of trypsin-TPCK was added after approximately 4 h and the digestion continued overnight.

An aliquot of the tryptic digest containing 75–120 μg of protein was applied to a cellulose thin-layer chromatography plate. Chromatography and electrophoresis were performed according to Gerday et al. (16) with the following modifications: reduction and alkylation were omitted and the origin was moved an additional 5 cm toward the anode to give better resolution of cathodically migrating peptides. The chromatogram was sprayed with Ninhydrin and the peptide map copied onto tracing paper 1 day later. An autoradiogram was then made using Kodak RP/R54 medical X-ray film (Eastman Kodak Co., Rochester, N. Y.). Adequate exposure of the film was generally achieved in 10 days if 10^4 dpm could be applied to the chromatogram. Coincidence of autoradiographic and Ninhydrin-sensitive peptides was then determined by laying the developed film either onto the chromatography plate or the tracing.

Assay of Actin Content

The actin content of membrane preparations was determined by three different assays. (a) A known amount of membrane protein (usually 0.1 mg) was analyzed on an SDS polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue and scanned at 550 nm. The actin peak on the scan was cut out and weighed and, from a standard curve of actin scan weights vs. milligrams of purified actin applied to the gel, the amount of actin protein in the membrane aliquot was determined. (b) The weight of the actin peak in the scan was compared to the weight of the entire scan. (c) The radioactivity recovered from the actin peak on an SDS gel was compared to the total radioactivity recovered

from the gel. All three methods gave comparable percent actin contents¹ and therefore method *b* was routinely used.

Chemical and Enzymatic Markers

Cellular RNA and DNA were labeled with radioactive uridine and thymidine, respectively, by adding the tracer to the normal growth medium during log phase growth and incubating for longer than 12 h. 5'-Nucleotidase, a plasma membrane marker, was assayed in 3T3 cells by the method of Avruch and Wallach (2) and in HeLa cells by the method of Heppel and Hilmoe (19). NADH-ferri-cyanide reductase was assayed according to Wallach and Kamat (38) and glyceraldehyde-3-phosphate dehydrogenase according to Velick (37), except that with HeLa cells the cysteine in the assay medium was replaced by 2-mercaptoethanol. Cytochrome oxidase was assayed according to Cooperstein and Lazarow (11).

Proteins

Actin from chick muscle was purified to electrophoretic homogeneity by the method of Spudich and Watt (35). Tubulin, which migrated as two electrophoretic components with molecular weights about 53,000 and 58,000 daltons, was purified from rat brain by the method of Weisenberg et al. (40). Protein concentrations were determined by the method of Lowry et al. (23).

Reagents

Media and sera for culture of 3T3 and HeLa cells were purchased from Grand Island Biological Co., Grand Island, N. Y. Acrylamide and bisacrylamide were electrophoretic grade and obtained from Bio-Rad Laboratories, Richmond, Calif. Coomassie Brilliant Blue was obtained from Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. All radioisotopes were purchased from New England Nuclear, Boston, Mass. Trypsin-TPCK was obtained from Worthington Biochemical Corp. Freehold, N. J. Cellulose thin-layer chromatography plates were CEL-300, 0.1 mm thick and obtained from Brinkmann Instruments, Inc., Westbury, N. Y. and phenylmethylsulfonyl fluoride was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

RESULTS

Characterization of Membrane Preparations

Electron microscopy showed that membranes prepared from 3T3 cells (Fig. 1) by the method of

¹ Wickus, G., E. Gruenstein, A. Rich, and P. Robbins. Alterations in actin associated with the membranes of fibroblasts following transformation. Manuscript in preparation.

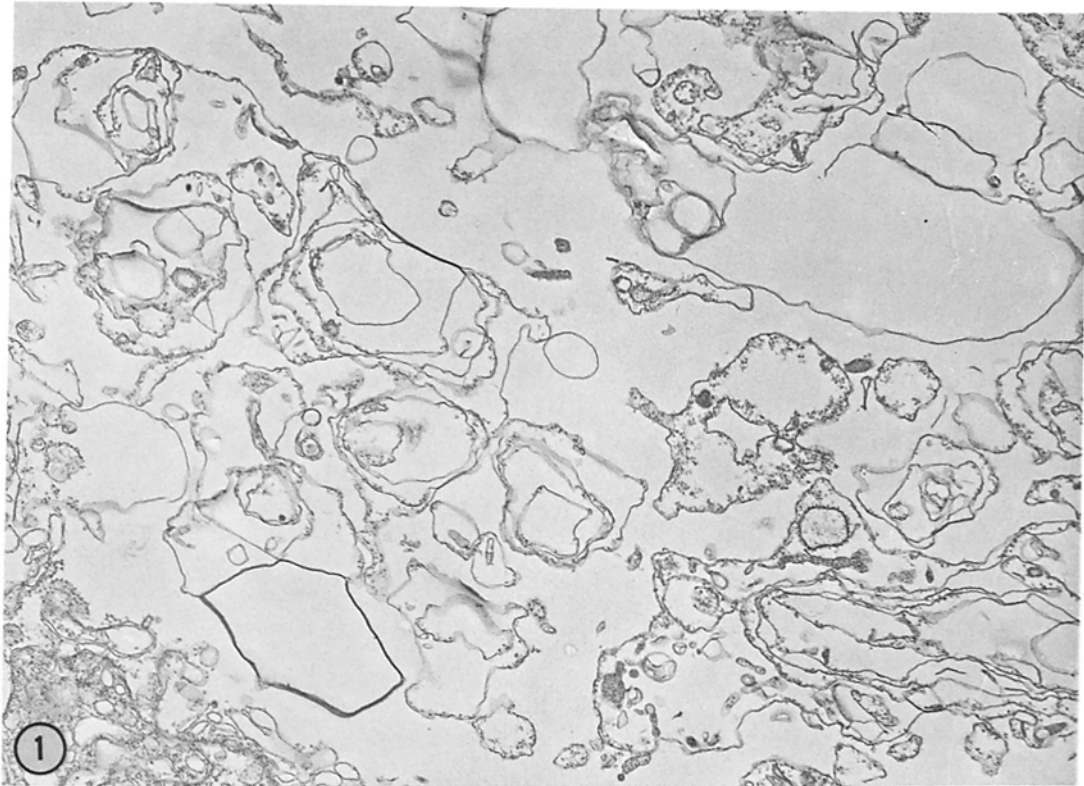


FIGURE 1 Electron micrograph of 3T3 membranes prepared by the method of Brunette and Till (6). Sheets of membrane lined on one surface with fibrogranular material are the major feature. Smooth-surfaced, irregular vesicles are also commonly seen along with occasional badly swollen mitochondria. $\times 15,000$.

Brunette and Till (6) consisted largely of long, rolled sheets which were characteristically smooth on one side and lined with fibrogranular material on the other. Swollen mitochondria and smooth-surfaced irregular vesicles were also commonly seen. Occasional intact nuclei were present and were visible under phase-contrast light microscopy as well. Morphological data and enzymatic data (see Table I) for the 3T3 membranes indicate the presence of considerable amounts of microsomes and mitochondria. For this reason we have been reluctant to refer to these preparations as *plasma membranes*.

Data for HeLa membranes, on the other hand, suggested that these membranes are derived primarily from plasma membrane. Electron microscopy (Fig. 2) showed that the membranes from HeLa cells were primarily rolled sheets lined on one surface with fibrogranular material. A few closed, smooth-surfaced vesicles were also present.

Phase microscopy (not illustrated) showed that some of these membranes formed large sheets resembling intact cells with a hole through which cytoplasmic contents had been extruded. More frequently, large, spindle-shaped fragments were noted, and these probably correspond to the rolled sheets of membrane seen in electron micrographs. Variable but small amounts of fine debris and granular aggregates, probably representing cytoplasmic debris, were also seen together with a few free nuclei, nuclei enclosed in membranes, and unruptured cells.

The membrane preparations were further characterized using enzymatic and chemical markers (Table I). Certain markers behaved similarly in both membrane preparations. The specific activity of 5'-nucleotidase, an enzyme commonly used as a plasma membrane marker, was significantly increased in both membrane preparations relative to the total cell homogenates. On the other hand,

TABLE I
Enzymatic Data for the 3T3 and HeLa Membranes

Marker	Organelle	Purification factor (spec act membranes / sp act total cell homogenate)	
		3T3	HeLa
5'-Nucleotidase	Plasma membranes	4.8	8.5
Glyceraldehyde-3-phosphate dehydrogenase	"Soluble" cytoplasm	0	0.014
Cytochrome <i>c</i> oxidase	Mitochondria	1.5	—
NADH-ferricyanide reductase	Microsomes + mitochondria	1.9	0.43
Uridine (^{14}C) or (^3H)	RNA	0.44	0.76
(^3H)Thymidine	DNA	0.19	0.13

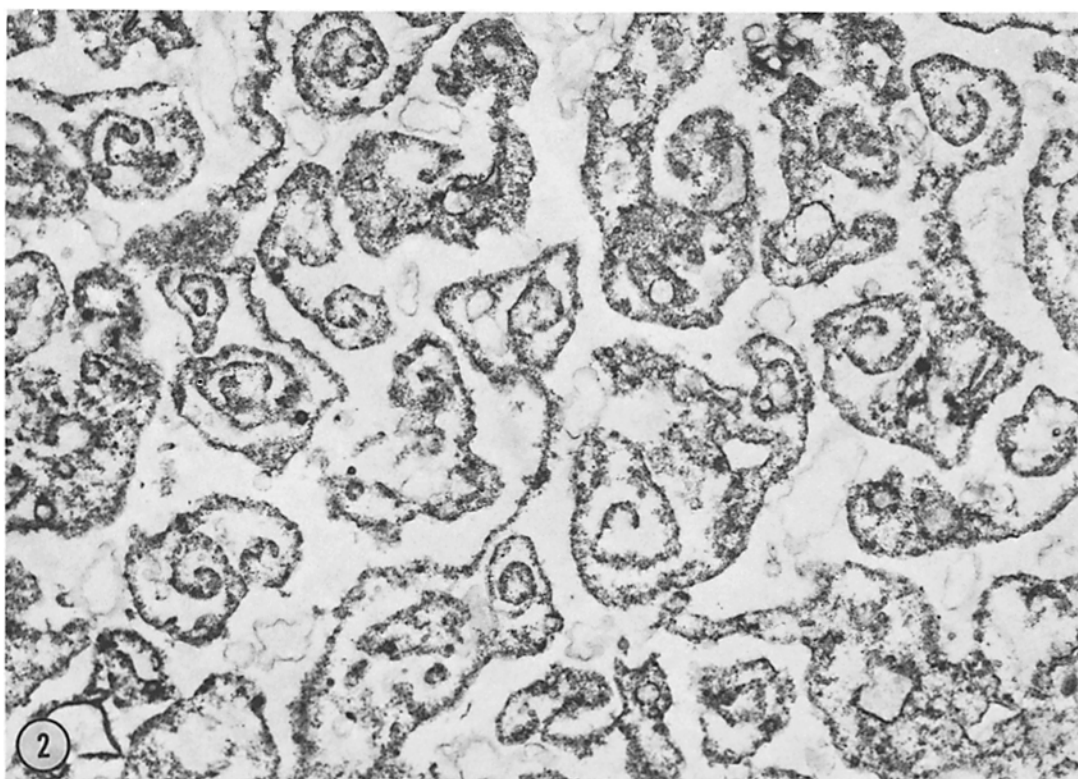


FIGURE 2 Electron micrograph of HeLa membranes prepared by the method of Brunette and Till (6). Long sheets or "jelly rolls" of membrane lined on one surface with fibrogranular material are the dominant feature. Occasional smooth-surfaced vesicles are visible while other morphologically identifiable organelles are generally absent. $\times 7,900$.

glyceraldehyde-3-phosphate dehydrogenase, a soluble cytoplasmic enzyme, was almost totally removed. The nearly complete absence of this enzyme from the membranes was not the result of destruction of the enzyme because the enzyme

activity was completely recovered in the other fractions. Nuclei, as judged by DNA content, have also been largely but not completely removed from both membrane preparations.

The behavior of the other markers is different in

the two membrane preparations. In HeLa membranes, the specific activity of NADH-ferricyanide reductase, a nonspecific marker for electron transport in mitochondria and endoplasmic reticulum, was reduced to about 40% of the value for the total cell homogenate. However, significant amounts of RNA (probably largely ribosomal RNA) remained with the HeLa membranes because the specific activity of the RNA was 75% of that of the total cell homogenate. While these values indicate incomplete, selective removal of mitochondria and endoplasmic reticulum from the HeLa membrane preparations, the purification factors for cytochrome *c* oxidase, NADH-ferricyanide reductase, and RNA in 3T3 membranes suggest that one or both of these organelles have been selectively purified along with the plasma membrane. As a caveat, it should be noted that while many of the markers chosen are characteristic for the indicated organelles in other tissues such as liver (12), the assumption of strict localization of these markers in the same organelles of 3T3 and HeLa cells remains an extrapolation. With this caveat in mind, the data are consistent with the predominant presence of plasma membranes in the HeLa membrane preparations and with plasma membranes plus mitochondria and endoplasmic reticulum as the major species in the 3T3 membrane preparations.

Actin Identification

The gel electrophoretic patterns of membranes, unfractionated cells, and standards are shown in Fig. 3. A band which co-migrates with actin is a prominent component of both membranes and whole cells. This component typically accounts for about 2-4% of the stain in 3T3 membranes, 3% in the unfractionated 3T3 cells, and about 4% in both HeLa membranes and unfractionated HeLa cells.

The electrophoretic pattern of the membranes is distinct from the pattern of the total cell homogenate (Fig. 3). In both 3T3 and HeLa membranes, a set of components with a molecular weight of around 200,000 daltons is characteristically enriched with respect to unfractionated cells. Erythrocyte ghosts also contain components (spectrin) in this molecular weight range (14), and the molecular weight of myosin heavy chain is 200,000 (17), but the relation, if any, between spectrin or myosin heavy chain and the high molecular weight components associated with 3T3 and HeLa membranes cannot be determined from the present data.

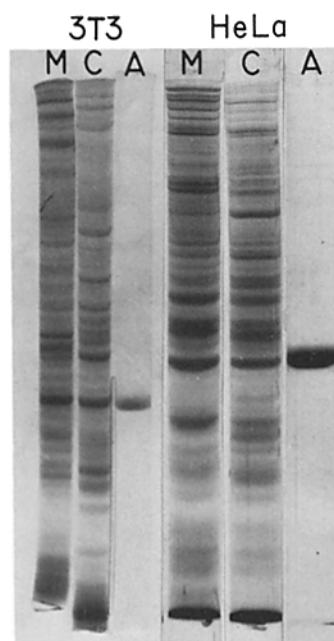


FIGURE 3 SDS polyacrylamide electrophoresis gels of 3T3 and HeLa total cell homogenates (C) and membranes (M). Gels marked (A) are chick skeletal muscle actin standards. The anode is at the bottom.

Identification of the presumptive actin band was performed as described in Materials and Methods. Presumptive actin from an electrophoretic gel of the membranes prepared from [¹⁴C]amino acid-labeled 3T3 or HeLa cells was cut out of the gel and the protein eluted, mixed with carrier chick muscle actin, and digested with trypsin. An aliquot of the tryptic digest was applied to a cellulose thin-layer chromatography plate and the peptides were separated by chromatography in the first dimension, followed by electrophoresis in the second dimension. Peptides of the carrier chick muscle actin were then located with Ninhydrin stain and the presumptive nonmuscle membrane actin peptides were located by autoradiography.

Fig. 4 shows the results obtained for the presumptive actin associated with 3T3 membranes. There are 30 major Ninhydrin spots of which all but four have precisely coincident autoradiographic spots. In addition, many of the faint Ninhydrin spots also have coincident autoradiographic spots. There are nine autoradiographic spots with no associated Ninhydrin. We wish to draw three conclusions from these data. First, the high degree of coincidence of radioactive spots and Ninhydrin-reactive spots shows that the presump-

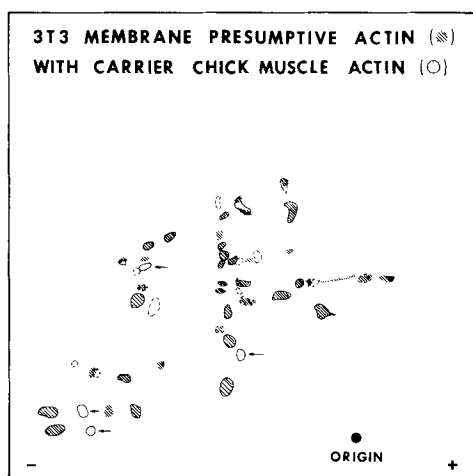


FIGURE 4 Tryptic peptide map of radioactively labeled presumptive membrane actin from 3T3 cells co-mapped with carrier chick muscle actin. Cross-hatched areas represent autoradiographic spots. Solid outlines represent major ninhydrin-staining peptides, and dashed outlines show very faint Ninhydrin spots.

tive actin associated with 3T3 membranes is indeed actin. Second, 3T3 actin may not be identical to chick muscle actin. This is apparent from the presence of major Ninhydrin spots with no associated radioactivity. Because the carrier chick muscle actin is highly purified ($\geq 99\%$ pure), every Ninhydrin-reactive spot must be derived from chick muscle actin and not from a contaminating protein. If the 3T3 actin were identical to chick muscle actin, every Ninhydrin-reactive spot should have radioactivity associated with it provided that all the 3T3 actin peptides are labeled. We expect that all the tryptic peptides from 3T3 actin were labeled because a mixture of [^{14}C]amino acids (including both lysine and arginine) was used to label the cells. Therefore, the observation of Ninhydrin-reactive spots without coincident radioactivity is consistent with the possibility that certain regions of 3T3 and chick muscle actin differ in primary structure. Third, the presence of radioactive peptides with no associated Ninhydrin spots could be caused by the presence of an impurity in the 3T3 actin band or by the occurrence in 3T3 actin of peptides not found in chick muscle actin, or by Ninhydrin-insensitive peptides from chick muscle actin. Although we consider it unlikely that the latter possibility accounts for all of the non-coincident autoradiographic spots, none of these choices can be ruled out on the basis of our present data.

Comparable results for HeLa membrane presumptive actin are shown in Fig. 5. 24 out of a total of 32 major Ninhydrin spots have coincident autoradiographic spots. Furthermore, all but two of the faint Ninhydrin spots have coincident or overlapping radioactivity. Once again, there are a number of radioactive spots not associated with Ninhydrin-reactive spots, and again we conclude that the presumptive actin associated with HeLa membranes is indeed actin but is probably not identical to chick muscle actin and may contain some impurities.

To test the specificity of the peptide mapping technique, nonidentical proteins were mapped together. Two of these controls are shown in Figs. 6 and 7. Radioactive actin from a gel of 3T3 membranes was mixed with carrier tubulin purified from rat brain, and a peptide map was prepared (Fig. 6). Only two Ninhydrin spots had coincident radioactivity, and a few others displayed partial overlap. Comparison of a radioactive nonactin band from a gel of HeLa membranes (for this map we chose a darkly staining band which co-migrates with glyceraldehyde-3-phosphate dehydrogenase) with carrier chick muscle actin is shown in Fig. 7. While eight Ninhydrin spots have coincident radioactivity, it should be noted that four of these coincidences are in the region of the map (lower left-hand corner) where one expects to find free amino acids and very small peptides such as dipeptides (20). Thus, as pointed out by Kaplan and Woodward (20), it is likely that coincidences in this region do not signify major

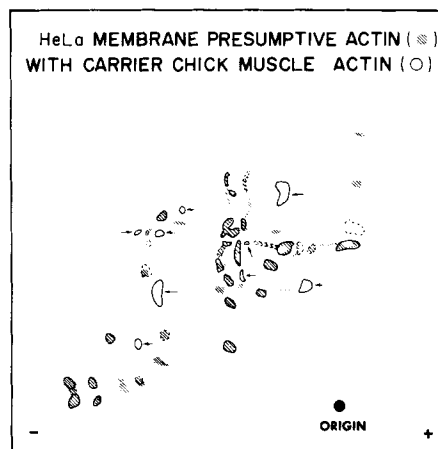


FIGURE 5 Tryptic peptide map of radioactively labeled presumptive membrane actin of HeLa cells co-mapped with carrier chick muscle actin. Cross-hatched areas, solid and dashed outlines as in Fig. 4.

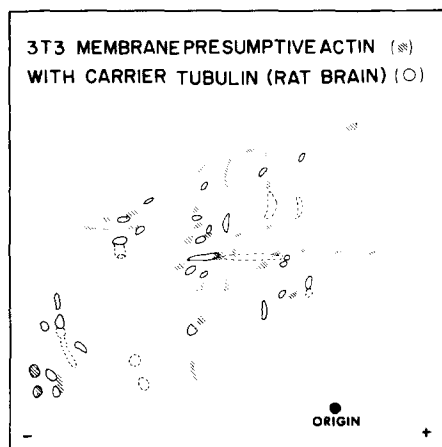


FIGURE 6 Tryptic peptide map of radioactively labeled presumptive membrane actin of 3T3 cells co-mapped with carrier rat brain tubulin. Cross-hatched areas, solid and dashed outlines as in Fig. 4.

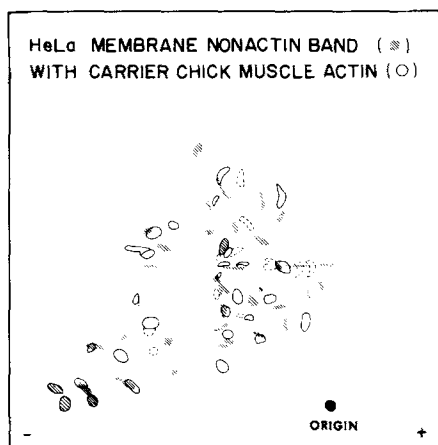


FIGURE 7 Tryptic peptide map of radioactively labeled nonactin membrane protein of HeLa cells co-mapped with carrier chick muscle actin. Cross-hatched areas, solid and dashed outlines as in Fig. 4.

similarities in primary structure. We conclude that both controls show the expected result—that peptide maps of nonidentical proteins do not coincide.

Removal of Actin from Membranes

As a further test of the identity of the presumptive actin, we extracted the membranes with a solution (28, 21) in which actin filaments should depolymerize to globular monomers. This treatment (Fig. 8) removed approximately 80% of the

actin from the 3T3 membranes and about 60% of the actin from the HeLa membranes. These results suggest that at least some of the actin is associated with the membranes in its filamentous form, and that it is removed from the membranes because it has been depolymerized. Electron micrographs of extracted 3T3 membranes (not shown) appeared to contain a smaller number of thin filaments; however, this observation is difficult to compare with the data of Fig. 8 because it could not be quantitated, and because the extraction also removed nonactin proteins from the membranes. We do not know why the HeLa membrane actin appears more resistant to extraction than the 3T3 actin.

Treatment with Cytochalasin B

In certain cells, cytochalasin B disrupts microfilaments which bind heavy meromyosin (34) and therefore are probably actin. We therefore tested its effects on the association of actin with the membranes. 3T3 cells were removed from the culture dishes by the same brief trypsinization used in the initial stage of membrane preparation and were incubated at 37°C for 2 h in suspension with 10 $\mu\text{g}/\text{ml}$ of cytochalasin B and 1% dimethyl sulfoxide (DMSO). This concentration of cytochalasin B is sufficient to cause 3T3 cells attached to the surface of a culture dish to withdraw their processes, round up, and become highly refractile under phase-contrast microscopy within 30 min. HeLa cells in growth medium with sera were treated with 1 $\mu\text{g}/\text{ml}$ of cytochalasin B and 0.1% DMSO for 2 h at 37°C, at which time many cells had blebs on their surfaces (30). The lower concentration of cytochalasin B was used for HeLa cells because membranes isolated from HeLa cells treated with 10 $\mu\text{g}/\text{ml}$ of cytochalasin B were found to be grossly contaminated with aggregates of cytoplasmic particles. Membranes were then prepared as usual, except that the same concentration of cytochalasin B and DMSO used to treat the cells was also present in the media used to wash and homogenize the cells. When compared to control membranes prepared in the same way from cells incubated for 2 h in DMSO without cytochalasin B, these membranes showed no significant changes in the concentration of actin (Fig. 9).

DISCUSSION

Three criteria indicate that actin is associated with membranes isolated from 3T3 and HeLa cells: (a) the membranes contain a component with the

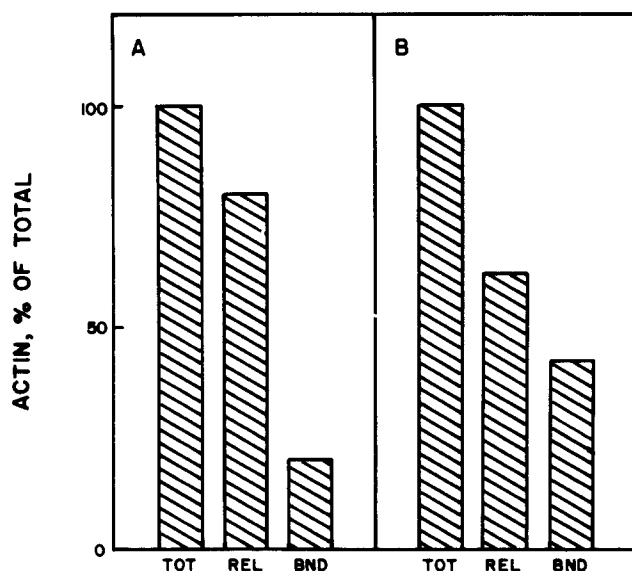


FIGURE 8 Removal of actin from membranes under actin depolymerizing conditions. Membranes were incubated for 18 h at 4°C in the presence of 0.2 mM ATP, 1 mM imidazole, pH 7.4. An aliquot of the total mixture was set aside, and the remainder was centrifuged for 15 min at 1,400 rpm on the 269 rotor of an International centrifuge. The resulting supernate was saved and the membrane pellet resuspended in water. The amount of actin in each fraction was then calculated from the protein content and the percentage of the total protein which had the electrophoretic mobility of actin (see Materials and Methods). The amount of actin in each fraction was then expressed relative to the amount of actin in the unextracted membranes normalized to 100%. *TOT* refers to total membrane mixture, *REL* to material released into the supernate, and *BND* to the material remaining bound to the membrane. Fig. 8 A shows results with 3T3 membrane, Fig. 8 B with HeLa membranes.

electrophoretic mobility of authentic actin; (b) peptide maps of the presumptive actins eluted from polyacrylamide gels are generally coincident with peptide maps of authentic muscle actin; (c) treatment of the membranes with low ionic strength solutions, which should depolymerize actin filaments to globular monomers, removes actin from the membranes.

The variability in actin peptide patterns on different maps (Figs. 4-7) is a natural consequence of the relatively large number of manipulations required between extraction of the protein from the gel and final thin-layer electrophoresis. This may be further aggravated by the presence of a trypsin-insensitive actin core. By comparing Ninhydrin to autoradiographic peptides on the same thin-layer plate, the inherent imprecision in comparing side-by-side maps even under the best of circumstances is avoided. Thus, in our experiments, only precise coincidence of Ninhydrin and autoradiographic peptides qualifies as identity of the peptides and not analogous position on separate plates.

Other experiments not reported here strengthen our identification of actin. Peptide maps of chymotryptic digests of 3T3 membrane-associated actin are predominantly coincident with those of chymotryptic peptides of chick muscle actin. Tryptic peptides of actin associated with membrane vesicles prepared from chick embryo fibroblasts by the method of Perdue et al. (26) also show a high degree of coincidence with tryptic peptides of chick muscle actin. Homogenates of HeLa cells contain a component which interacts with added myosin to give the following responses which are characteristic of actomyosin: (a) addition of ATP lowers the viscosity, presumably owing to dissociation of the hybrid actomyosin formed by HeLa actin and added myosin; (b) the component of HeLa cells which coelectrophoreses with authentic actin can be precipitated as a hybrid actomyosin when purified myosin is added to the homogenate and the ionic strength is lowered.

If membranes contain discrete actin-binding sites, as suggested by several ultrastructural obser-

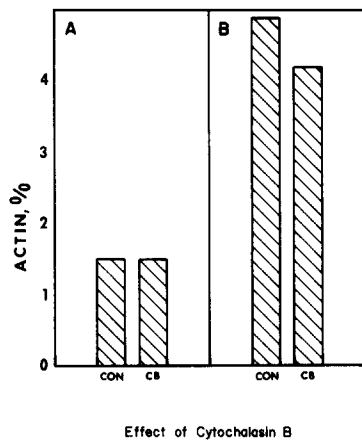


FIGURE 9 Effect of cytochalasin B on the concentration of actin in membranes from 3T3 (Fig. 9 A) and HeLa (Fig. 9 B) cells. *CON* = control cells incubated with 1% DMSO (3T3) or 0.1% DMSO (HeLa). *CB* = cytochalasin B-treated cells incubated with 10 $\mu\text{g}/\text{ml}$ cytochalasin B and 1% DMSO (3T3) or 1 $\mu\text{g}/\text{ml}$ cytochalasin B and 0.1% DMSO (HeLa) for 2 h. The actin concentration (expressed as percent of total protein with the electrophoretic mobility of actin) was determined as described in Materials and Methods.

variations of apparent attachment or very close association of actin filaments with membranes (24, 28, 36, 42, 44, 45), then one might expect that most cellular actin would fractionate as an intrinsic component of the membranes, and therefore that the concentration of actin in the membrane fraction would be higher than in the homogenate. However, inspection of the gel electrophoretic patterns of the membrane and homogenate (Fig. 3) and quantitative densitometry of many membrane preparations indicate that the concentration of actin in the membrane is about the same as in the homogenate.

We would not expect this result if actin were tightly and exclusively associated with the plasma membrane *in vivo*. However, the fraction of cellular actin which has formed filaments is not known for any nonmuscle cell. Therefore, it is possible that only a small percentage of the total cellular actin is polymerized and attached to the membranes. On the other hand, even if all the actin of the cell were normally in the filamentous form and if all filaments were associated with the membranes either by direct attachment or indirectly via myosin cross bridges, it is possible that many of these connections would be disrupted during the isolation procedure. Clearly, we need additional

data to understand why the actin concentrations in the cell and the membrane fraction are similar.

Actin or thin filaments with the morphology of actin have been found to be associated with certain cellular processes occurring at the cell surface. Examples include formation of the cleavage furrow in cell division (27, 32), phagocytosis (31), pseudopod withdrawal (13, 41, 43), amoeboid movement (10), filopodia formation (44, 9), cell contact leading to contact inhibition (18), and ruffling in lymphocytes and fibroblasts (7). Although the molecular mechanism of these movements is unknown, a theoretical argument suggests that cytoplasmic actins could exert tension via attachment to cell membranes. To transmit force, actin must be anchored at one or more points, and actin filaments are regularly seen near the plasma membrane of many cells where they would be in a position to facilitate the processes listed above. Specific models have already been proposed in which membrane-associated actin mediates exocytosis (3), nerve growth cone formation (5), and endocytosis (1). Two of these models suggest that actin lies in the plane of the membrane, but electron microscopy of membranes purified from *Acanthamoeba* (28) and intestinal brush border (36) has shown only actin filaments perpendicular to the membrane. However, it is still possible that other, functionally important membrane-associated actin lies parallel to or in the plane of the membrane because actin filaments, or even filaments decorated with heavy meromyosin, would probably be very difficult to identify by electron microscopy if the filaments were parallel with and very close to the membrane.

Treatment of the cells with cytochalasin B did not significantly change the actin content of the membranes. Superficially, this result seems inconsistent with earlier reports that cytochalasin B causes the disappearance of morphologically identifiable microfilaments. We did not attempt to verify these ultrastructural studies ourselves. However, these results could be reconciled if cytochalasin B disrupts microfilaments without changing the intracellular localization of the actin (which we presume forms the microfilaments). Investigation of this question and others such as the significance of the fraction of actin which appears to resist extraction from the membrane is now underway.

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