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Cytoskeletal Elements of Chick Embryo Fibroblasts Revealed by Detergent Extraction

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Treatment of chick embryo fibroblasts with 0.5% Triton® X-100 extracts most of the cell protein, leaving an organized part of the cell structure attached to the tissue culture dish. This "Triton cytoskeleton" consists largely of intermediate-sized filaments and bundles of microfilaments. SDS polyacrylamide gel electrophoresis reveals that this cytoskeleton is made up of three main proteins. One protein component is 42,000 daltons and co-migrates with muscle actin. The other two components are 52,000 and 230,000 daltons and remain quantitatively associated with the cytoskeleton during the detergent extraction. The possible identity of these three protein components and their organization into a supramolecular structure is discussed.

Key words: chick embryo fibroblasts, cytoskeleton, Triton cytoskeleton, detergent extraction of chick embryo fibroblasts, actin, non-muscle, LETS protein, actin filament bundles, intermediate-sized filaments

INTRODUCTION

Muscle and nonmuscle actins are very similar molecules, but are organized very differently in vivo. The striated muscle myofibril contains individual actin filaments held in an ordered and stable array. In the nonmuscle cell, on the other hand, actin filaments can associate with each other to form bundles of filaments. These bundles are transient structures which form on contact with a substrate (1, 2) or another cell (3), and may participate in a cytoskeleton responsible for the maintenance of the morphologic condition and attachment of the cell.

Spudich and Cooke (4) have demonstrated that bundles can be made in vitro from a partially purified nonmuscle cell actin. Muscle actin or highly purified nonmuscle actin do not form bundles, suggesting that there is a nonmuscle factor(s) involved in the formation of the bundle. Further evidence for such a factor has recently been provided by Cooke et al. (5). Another approach to studying the actin bundles would be to isolate those formed in vivo. In this paper we describe a method which is a first step in that direction. Extraction of cells with Triton X-100 provides a quick and quantitative way of isolating a cytoskeleton that contains the bundles and retains its attachment to the substrate. This cytoskeleton can be used to study both the factors involved in bundle formation and the relation of bundles to attachment.

Other filamentous assemblies, which could be cytoskeletal elements, are microtubules and intermediate-sized filaments (5). Whereas microtubules are not retained during our Triton extraction procedure, intermediate-sized filaments represent a major component of

the Triton cytoskeleton. Another major component in our preparations is a high molecular weight species which appears to be the "LETS" (Large, External, Transformation-Sensitive) protein, a cell-surface component previously implicated in cell adhesion (6, 7). The actin filament bundles, intermediate-sized filaments, and LETS protein may be interacting in some coordinated fashion to effect adhesion, cell-shape changes, and cell movements. Cytoskeleton preparations such as those described here should be very useful in defining these interactions.

MATERIALS AND METHODS

Cells

Primary cell cultures were made from 10-day-old chick embryos (white leghorn, avian leukosis virus-free). Head, limbs, and internal organs were removed, and the rest was minced and incubated at 37°C in 0.25% trypsin in a buffer (Tris-glucose) containing 137 mM NaCl, 5 mM KCl, 5 mM glucose, 25 mM Tris-HCl (pH 7.4), phenol red, penicillin and streptomycin. The resulting single-cell suspension of primary cells was cultured for 3 to 5 days in Medium 199 with 10% tryptose phosphate broth (Difco), 5% calf serum, 1% chicken serum (heated at 56°C for 30 min) and 2% $NaHCO_3$. Secondary cultures of chick embryo fibroblasts (CEF) were plated at a density of 5×10^6 cells/100 mm dish in the above medium omitting the chicken serum, and used experimentally after 2 days, at a density of 1 to 1.5×10^7 cells/100 mm dish.

Detergent Extraction

Monolayers were gently rinsed twice with 5 ml Tris-glucose to which 0.5 mM $MgCl_2$ and 0.025 mM $CaCl_2$ had been added (TGMC buffer). They were then incubated for 10 minutes at room temperature in 1 ml of Triton X-100 (Sigma; polyethylene-glycol [9–10] p-t-octylphenol) in TGMC. The supernatant solution was saved, and the material remaining on the dish was rinsed with 5 ml of TGMC. This material could then be collected for electrophoresis in a volume equivalent to the supernatant solution, i.e., in 1 ml of TGMC, or treated further, e.g., for 10 min at room temperature with sodium lauroyl sarcosine (Schwarz/Mann).

Electron Microscopy (EM)

Gold EM grids (Ladd) were boiled for 10 minutes in concentrated HNO_3, rinsed well with distilled H_2O, sandwiched between a glass coverslip and a layer of formvar, and then coated with carbon. These were x-ray sterilized and included in secondary cultures. After detergent treatment of the cells, the grids including the formvar layer with attached cell structures were lifted off the glass coverslips and stained with 2% aqueous uranyl acetate for 30 sec. They were examined in a Philips 300 electron microscope.

Gel Electrophoresis

Proteins were resolved on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) slab gels in a manner similar to that described by Ames (8), using the discontinuous buffer system of Laemmli (9). To 1 ml of sample, 0.1 ml solubilizing solution (10% SDS, 50% glycerol in 0.05 M Tris, pH 6.8) and 25 μl 2-mercaptoethanol were added. If the sample was material remaining on the dish, then 1 ml TGMC followed by solubilizing solution and 2-mercaptoethanol were added to the dish, and the material was collected by scraping with a rubber policeman. The samples were boiled for 3 min and applied to an acrylamide

gel or stored at −20°C. Twenty μl of sample were applied to a 3% stacking-7.5% separating gel, and electrophoresed at a current of 20 mA/gel. Gels were stained for protein with Coomassie brilliant blue (CB) (8) or for carbohydrate with periodic acid-Schiff's reagent (PAS) (10).

Since whole cells, supernatant solutions, and residues remaining on the dish were all collected in equal volumes (1 ml) and a standard volume (20 μl) applied to the gel, the amount of protein in each fraction could be compared.

Gels were scanned on a Joyce-Loebl microdensitometer. Peaks were cut out and weighed to estimate relative amounts of the same protein in differently treated samples.

Molecular weights (MW) of proteins were estimated using the proteins of rabbit myofibril as standards (11). Since the plot of mobility vs log MW was not linear over the whole range of molecular weights, two lines were drawn, one for low MW (< 75,000 daltons) and one for high MW proteins.

Two-dimensional gels were run by Dr. Peter Rubenstein, Department of Biochemistry and Biophysics, University of California, San Francisco, by the method of O'Farrell (12).

Protein Determination

Samples used for protein determination were solubilized by the same procedure as for electrophoresis above. Protein concentration was determined by the method of Schaffner and Weissman (13).

RESULTS

Triton Extraction

CEF in monolayer were observed by phase contrast light microscopy during extraction with the nonionic detergent Triton X-100 (Fig. 1). Concentrations greater than 0.01% Triton X-100 resulted in a decrease in the opaque appearance of the cells, with the result that the nucleus and nuclear structures became more prominent. Subcellular particles were released into the supernatant solution, but a fibrous cytoskeletal structure which retained the original shape of the cell remained attached to the substrate. In this report, we refer to this structure as the "Triton cytoskeleton." The nuclei and fibrous material remained in their initial disposition during extraction. Thus, Triton appeared to disrupt the plasma membrane and release cell contents without visibly rearranging the Triton cytoskeleton.

When the Triton cytoskeleton was observed at the higher resolution of the electron microscope (Figs. 2, 3), numerous "bundles" of filaments could be seen oriented along the long axis of the cell. These filaments have a diameter of about 7 nm, and the bundles have a diameter of about 0.1 μ and can come together to form larger groupings. Individual larger filaments, 10 nm in diameter, were also seen (Fig. 3). Only small patches of membrane were apparent and the nucleus was opaque.

Electrophoretic gels were used to analyze the component proteins of the Triton cytoskeleton. A range of Triton concentrations revealed that no further material was removed above 0.5% Triton at the cell density used (Fig. 4); thus, 0.5% Triton was selected as the standard concentration for further experiments. Treatment with 0.5% Triton removed about 80% of the total cell protein, leaving 20% with the Triton cytoskeleton. Three major protein components with molecular weights of 42,000, 52,000, and 230,000 were present in the Triton cytoskeleton (Fig. 5). The 42,000 dalton component comigrated

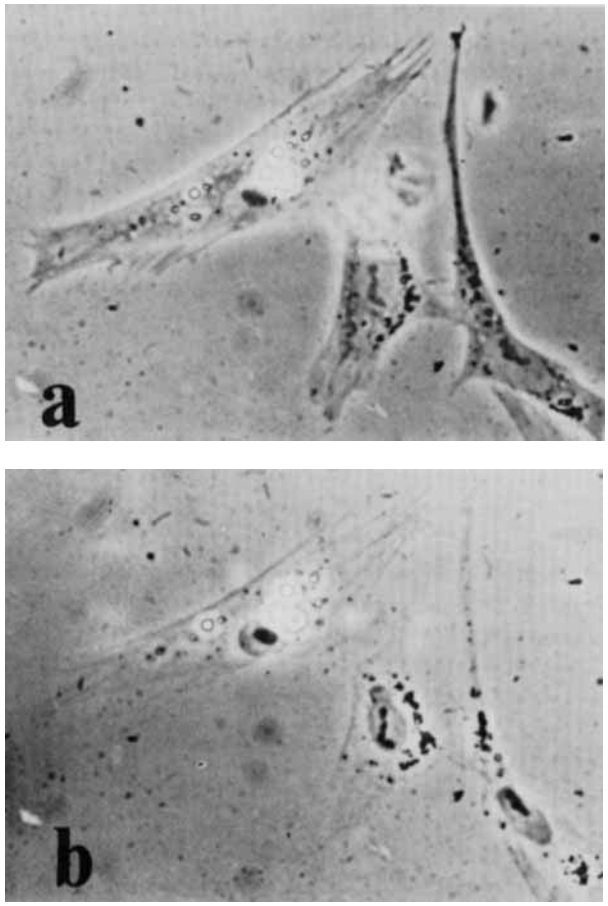

Fig. 1. Triton extraction of chick embryo fibroblasts. Phase contrast micrograph of CEF. 1a shows cells before extraction with 0.5% Triton X-100, and 1b shows the same field after extraction. On extraction, cells become less opaque and lose organelles, but the nucleus and some cytostructure remain in place in the dish. (× 580).

with muscle actin, and probably makes up the filaments of the bundles seen in the EM. The actin remaining in the Triton cytoskeleton was slightly less than half of the total cell actin, as estimated by densitometry of SDS gels. The other two major components were nearly quantitatively left on the dish; they were not seen in the Triton supernatant in significant amounts (Fig. 4). None of these three components is extracted by increasing the ionic strength briefly by addition of NaCl to 0.7 M. PAS staining (7) revealed that the 230,000 dalton major component is a glycoprotein (Fig. 6).

A component comigrating with muscle myosin (200,000 daltons on SDS gels) was also found in the Triton cytoskeleton (Fig. 5). A variable amount of this component remained behind after Triton extraction but it was always a minor fraction of the total seen in the whole cell.

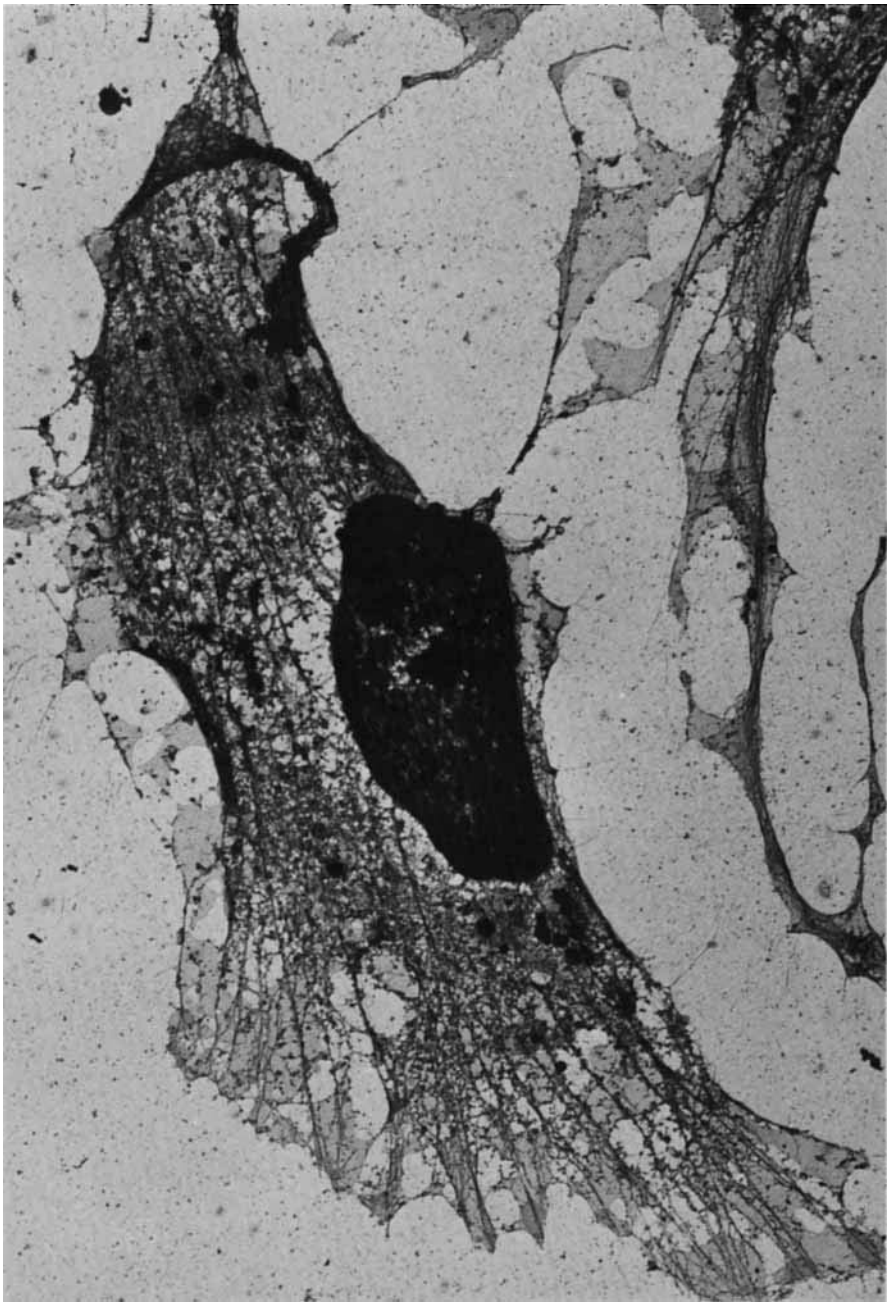

Fig. 2. Electron micrograph of a Triton-extracted CEF (fixed overnight in 4% glutaraldehyde in 0.1 M Hepes buffer, pH 7.4; postfixed 30 min in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, stained 30 min in 2% uranyl acetate in 15% acetone; dehydrated in acetone, and dried from amyl acetate). A portion of a second cell is seen in the upper right corner of the picture. The main features seen at this low magnification (× 3,600) are the darkly staining nucleus, and fibers about 0.1 μ diameter running parallel to the long axis of the cell. Other filamentous material can be seen in between these fibers, and patches of membrane remain.

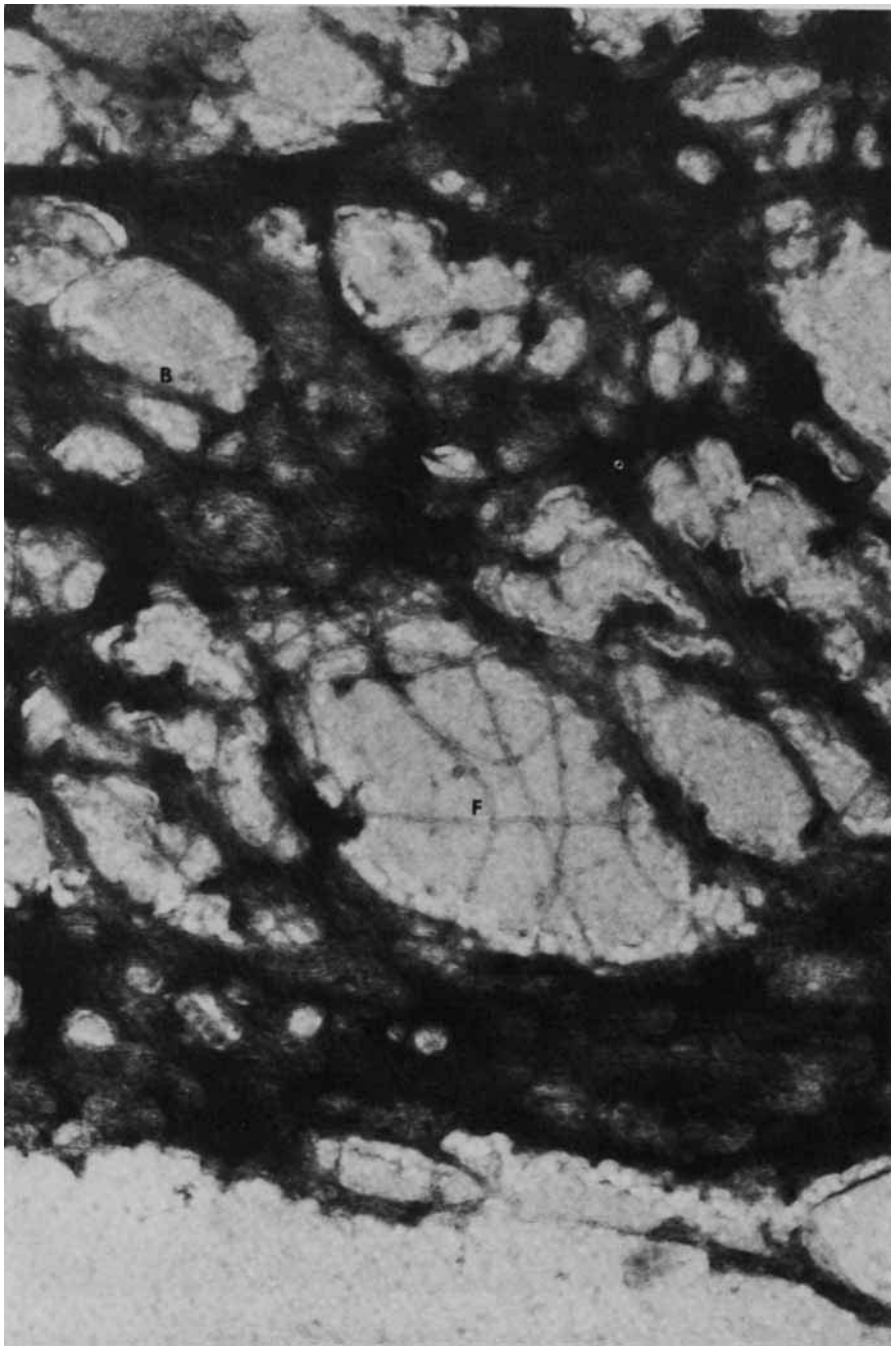

Fig. 3. Electron micrograph of a portion of a Triton-extracted CEF, negatively stained for 30 sec with 2% aqueous uranyl acetate and air dried. This higher magnification (× 50,000) reveals that the fibers seen in Fig. 2 are bundles of actin filaments (B) about 7 nm in diameter. The individual filaments seen are all larger, about 10 nm (F); no individual actin filaments are seen.

The Triton cytoskeleton was further analyzed on two-dimensional gels (Fig. 7). A large spot running in the same position as muscle actin was seen. There were two spots at the 52,000 dalton position in the electrophoretic dimension. These were close in pK_i, and both were more acidic than actin; the spot which contained most of the protein was the less acidic of the two (to the left on the gel). The 230,000 major component did not focus well and appeared as a streak on the gel.

A variety of other cell types were also treated with Triton. Some (Rous sarcoma virus-transformed CEF, HeLa, rat hepatic tumor cells, D. discoideum) responded by coming off the dish. The cells which responded in this way either lacked or had reduced amounts of the 230,000 dalton component in SDS gels of whole cells. NRK (normal rat kidney) cells, on the other hand, did not come off the dish upon Triton extraction, and the SDS gel pattern of proteins remaining on the dish was very similar to that of CEF. There were again three major bands, the middle one migrating more slowly (54,000 daltons) than in CEF (52,000 daltons).

Rabbit myofibrils extracted with Triton (11) gave a very different electrophoretic pattern than did CEF (Fig. 5). When adjusted to the same actin concentration, extracted rabbit myofibrils have far more myosin than CEF cytoskeletons. Furthermore, at this concentration no components are detected in CEF cytoskeleton samples running in the positions of other major myofibril proteins, such as α-actinin or troponin. There is a component that appears to comigrate with tropomyosin. Conversely, the myofibril does not have proteins running in the positions of the 52,000 or 230,000 dalton CEF proteins.

Sarkosyl Extraction

Further extraction of the Triton cytoskeleton with 0.1% sarkosyl, an anionic detergent, releases about two-thirds of the remaining protein; increasing the concentration released no further material (Fig. 8). Most of the 52,000 dalton component and some of the actin are released into the supernatant solution during this treatment (Fig. 8). The material is released in low molecular weight form, since it cannot be sedimented by centrifugation at 200,000 × g for 2 hr. None of the 230,000 dalton protein is released by sarkosyl. The most obvious detectable change by light microscopy is the disappearance of the nucleus. The EM reveals that the bundles and 10 nm filaments are gone, with only 7 nm filaments remaining (Fig. 9); a ghost of the nucleus remains.

DISCUSSION

We have extracted substrate-attached tissue culture cells with Triton X-100, an approach similar to that used by Yu et al. (14) with red blood cell ghosts, to expose a cytoskeleton which retains some of the original cellular structure. We have carried out a correlated gel electrophoretic and electron microscopic analysis on this cytoskeleton.

The composition of the isolated cytoskeleton seems rather simple. Eighty per cent of the total cell protein has been removed, and there are only three major protein components on SDS-PAGE gels. Although a number of minor components are apparent, they are present in at least an order of magnitude of lower concentration. Some of these may prove important as the nature and functions of the three major bands are investigated.

We have already seen (Fig. 5) that the composition of the cytoskeleton differs markedly from that of a myofibril. Bray and Thomas (17) have shown that the actin-to-myosin ratio in CEF is different from that in skeletal muscle but similar to smooth muscle. Smooth muscle also contains many intermediate-sized filaments and is probably more analogous to CEF than is skeletal muscle.

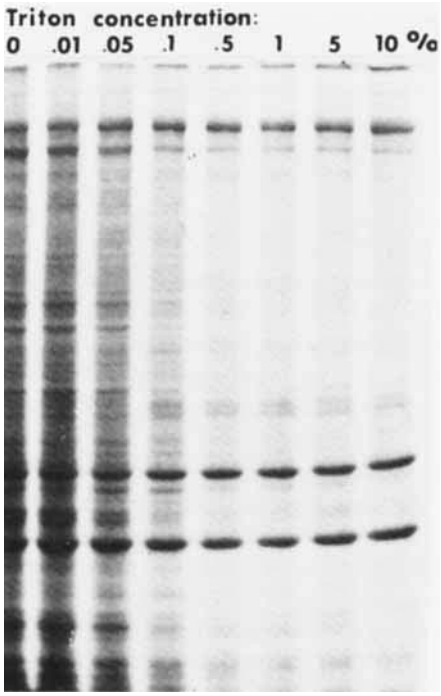

Fig. 4. SDS polyacrylamide gel of the material remaining attached to the tissue culture dish after extraction with increasing concentrations of Triton X-100. The standard volume of samples were applied to each channel; see section on Materials and Methods. It can be seen that no further material is removed above 0.5% Triton under the experimental conditions used.

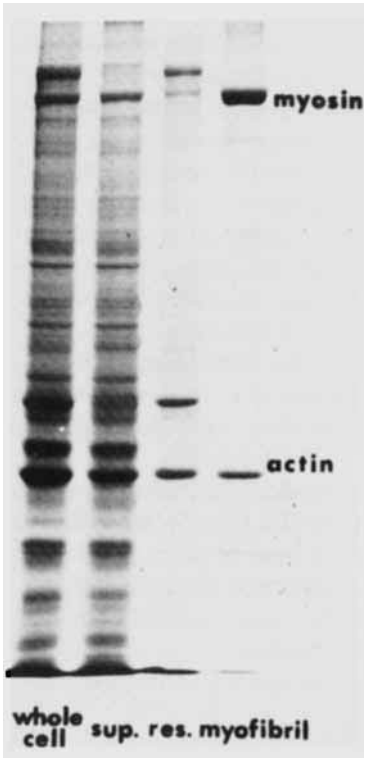

Fig. 5. SDS polyacrylamide gel of fractions obtained by extraction with 0.5% Triton X-100. The first channel is total cell protein, the second, protein released into the supernatant, and the third, protein remaining associated with the tissue culture dish (Triton residue). The fourth is a rabbit skeletal muscle myofibril, also extracted with 0.5% Triton X-100. The amount of protein applied to the gel in channels 3 and 4 is adjusted to contain equal amounts of actin (standard volume applied to channel 3; 3.5 μg protein applied to channel 4). The Triton residue contains two major bands in addition to actin; comparison with the supernatant shows that both of these bands are almost quantitatively left behind in the residue.

Fig. 6. PAS vs CB-stained gels of the Triton cytoskeleton. Samples of the Triton cytoskeleton are applied to both channels. The first, to which 10 times the standard volume of sample was applied, was stained with PAS to demonstrate sugar groups. The second channel was stained with CB to demonstrate proteins. A comparison shows that the high molecular weight component is a glycoprotein.

Fig. 7. Two-dimensional gel of Triton cytoskeleton. The first dimension was isoelectric focusing, run in the horizontal direction from basic (on the left side) to acidic (on the right). The second dimension was SDS-PAGE, run in the vertical direction; the buffer conditions were like those of the one-dimensional gels previously shown. The lowest major spot corresponds in position to rabbit muscle actin. The 52,000 dalton band is resolved here into two spots, both more acidic (to the right) than the actin spot. The 230,000 dalton component is seen as a streak at the top of the gel.

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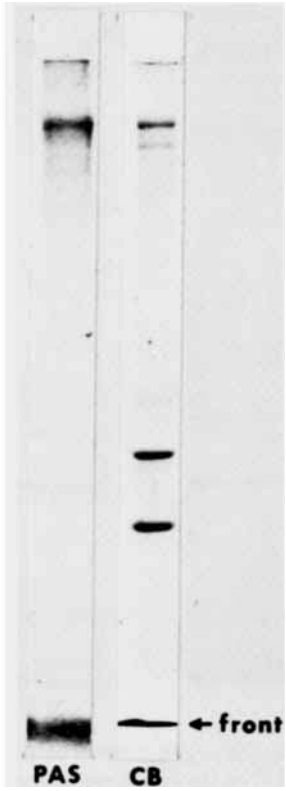

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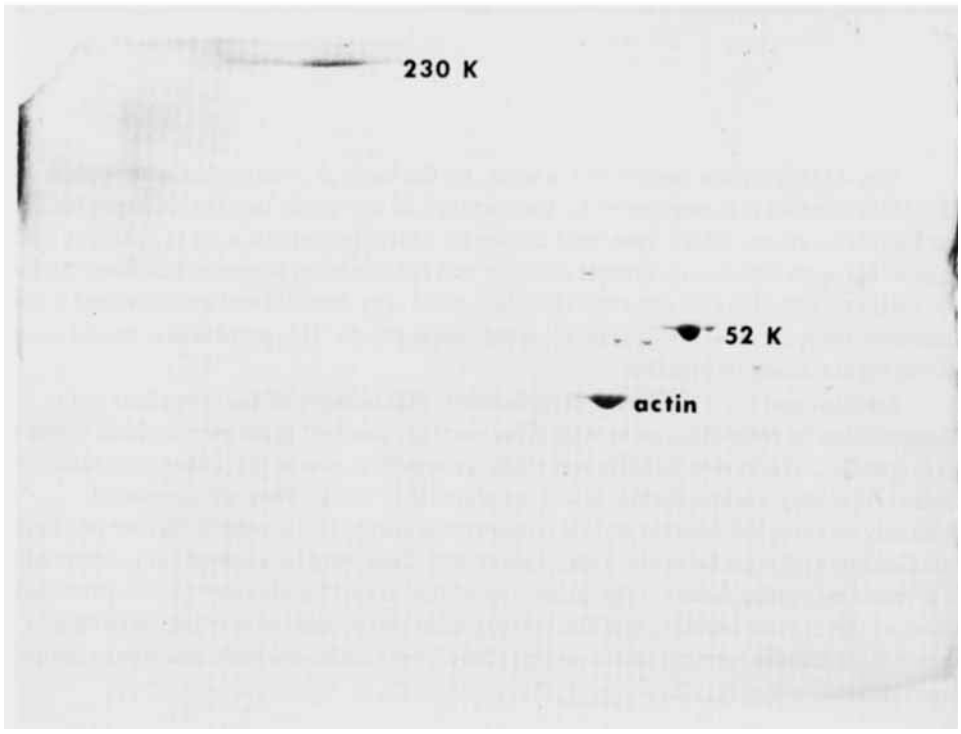

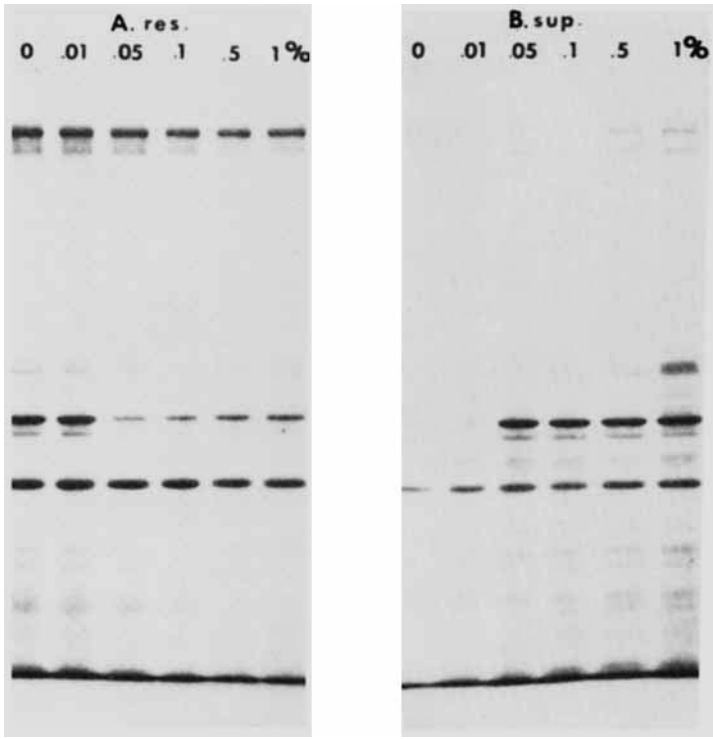

Fig. 8. SDS polyacrylamide gel of the Triton cytoskeleton extracted with increasing concentrations of sarkosyl. (A) Protein remaining behind on the dish at the indicated sarkosyl concentrations. The sample in the first channel, which was treated with buffer only, is equivalent to a Triton cytoskeleton. (B) The corresponding protein extracted into the sarkosyl supernatant at the indicated sarkosyl concentrations.

The 42,000 dalton component is actin, on the basis of comigration with muscle actin. This identification is strengthened by the presence of numerous bundles of filaments in the EM preparations, which have been shown by others to contain actin (15, 16). A comparison between Triton supernatant solution and cytoskeleton suggests that about half of the total cell actin may be present in bundles, since only bundles and no individual 7 nm filaments are seen in the Triton cytoskeleton preparations. This preparation should thus be useful for the study of bundles.

Spudich and Cooke (4) have demonstrated that bundles of Dictyostelium actin filaments can be reconstituted in vitro from partially purified actin preparations. These in vitro bundles, like in vivo bundles and those we report in situ in the Triton cytoskeleton, consist of closely packed filaments and are about 0.1μ thick. They are composed primarily of actin but contain several minor components. If the actin is further purified, bundles can no longer be made. This suggests that there may be a minor component which is a "bundle-forming factor." The properties of our in situ bundles can be compared with those of the in vitro bundles, and the cytoskeleton can be used as starting material in a search for a bundle-forming factor and to study how bundle assembly and disassembly are regulated.

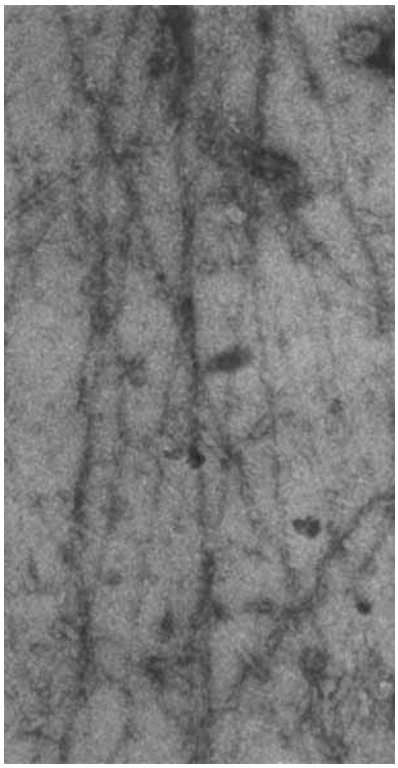

Fig. 9. Electron micrograph of the material remaining on the dish after sarkosyl extraction of the Triton cytoskeleton, negatively stained with 2% aqueous uranyl acetate. Ten nm filaments are no longer seen, and most of the actin bundles are dispersed into individual 7 nm filaments. (Compare with Fig. 3) (× 68,000).

The second major component in the Triton cytoskeleton, the 52,000 dalton protein, can be accounted for by the numerous 10 nm filaments seen in this structure in the EM. Their identity is suggested by the fact that sarkosyl extracts the 52,000 dalton component from the Triton cytoskeleton and the 10 nm filaments are no longer seen in the EM. This protein has about the same subunit molecular weight as filaments of similar diameter from nerve and smooth muscle (18, 19).

The third component is a 230,000 dalton glycoprotein which is reduced in amount in transformed cells. These are properties described for LETS protein (20–25). Hynes (26) has treated hamster fibroblast cells with NP-40, a detergent very similar to Triton. He has demonstrated that LETS is not removed by this procedure using fluorescent anti-LETS or by iodinating surface proteins including LETS before extraction. His work lends support to the identification of our 230,000 dalton component as LETS. The LETS protein has been postulated to function in adhesion (6, 7), and its quantitative retention in a Triton cytoskeleton which remains attached to the dish is consistent with this idea. Perhaps the release of some cell types (e.g. Rous transformed CEF) from the dish upon detergent extraction is due to the reduction or absence of LETS in these cell types.

How these three major components are associated in the cytoskeleton is not resolved. There is evidence that both the 230,000 dalton component and actin are involved in attachment and that bundles may associate with the plasma membrane at attachment

plaques (27). The 230,000 dalton component might be a transmembrane protein (28) and interact with actin, or be indirectly linked to actin via another protein. The actin bundles and the 10 nm filaments may interact directly, or only be physically entangled in the Triton cytoskeleton. Our isolated cytoskeleton preparations should prove very useful in establishing whether or not any of these various interactions occur and, if so, the functional significance of those interactions.

ACKNOWLEDGMENTS

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REFERENCES

1. Goldman, R. D., Bushnell, A., Schloss, J., and Wang, E. : J. Cell Biol. 63:113a (1974).
2. Clarke, M., Schatten, G., Mazia, D., and Spudich, J. A.: Proc. Natl. Acad. Sci. USA 72:1758 (1975).
3. Heaysman, J. E. M., and Pegrum, S. M.: Exp. Cell Res. 78:71 (1973).
4. Spudich, J. A., and Cooke, R.: J. Biol. Chem. 250:7485 (1975).
5. Goldman, R., Pollard, T., and Rosenbaum, J. (Eds.): "Cell Motility," Vol. III, in press, New York: Cold Spring Harbor Laboratory (1976).
6. Yamada, K. M., Yamada, S. S., and Pastan, I.: Proc. Natl. Acad. Sci. USA 72:3158 (1975).
7. Yamada, K. M., Yamada, S. S., and Pastan, I.: Proc. Nat. Acad. Sci. USA 73:1217 (1976).
8. Ames, G. F.-L.: J. Biol. Chem. 249:634 (1974).
9. Laemmli, U. K.: Nature 227:680 (1970).
10. Zacharius, R. M., and Zell, T. E.: Anal. Biochem. 30:148 (1969).
11. Etlinger, J. D., Zak, R., and Fischman, D. A.: J. Cell Biol. 68:123 (1976).
12. O'Farrell, P. H.: J. Biol. Chem. 250:4007 (1975).
13. Schaffner, W., and Weissman, C.: Anal. Biochem. 56:502 (1973).
14. Yu, J., Fischman, D. A., and Steck, T. L.: J. Supramol. Struct. 1:233 (1973).
15. Lazarides, E., and Weber, K.: Proc. Natl. Acad. Sci. USA 71:2268 (1974).
16. Ishikawa, H., Bischoff, R., and Holtzer, H.: J. Cell Biol. 43:312 (1969).
17. Bray, D., and Thomas, C.: Biochem. J. 147:221 (1975).
18. Shelanski, M. L., Albert, S., DeVries, G. H., and Norton, W. T.: Science 174:1242 (1971).
19. Cooke, P.: J. Cell Biol. 68:539 (1976).
20. Hynes, R. O.: Proc. Natl. Acad. Sci. USA 70:3170 (1973).
21. Hynes, R. O.: Cell 1:147 (1974).
22. Hynes, R. O., and Humphreys, K. C.: J. Cell Biol. 62:438 (1974).
23. Yamada, K. M., and Weston, J. A.: Proc. Natl. Acad. Sci. USA 71:3492 (1974).
24. Stone, K. R., Smith, R. E., and Joklik, W. K.: Virology 58:86 (1974).
25. Wickus, G. G., Branton, P. E., and Robbins, P. W.: In "Control of Proliferation in Animal Cells," Clarkson, B., and Baserga, R. (Eds.). Cold Spring Harbor Laboratory, p. 541 (1974).
26. Hynes, R. O., Destree, A. T., and Mautner, V.: In "Membranes and Neoplasia: New Approaches and Strategies" (V. T. Marchesi, Ed.), New York: Alan R. Liss, Inc., pp. 189–201 (1976).
27. Abercrombie, M., Heaysman, J. E. M., and Pegrum, S. M.: Exp. Cell Res. 67:359 (1971).
28. Hunt, R. C., and Brown, J. C.: J. Mol. Biol. 97:413 (1975).