HEAVY MEROMYOSIN LABELING OF INTERMEDIATE FILAMENTS IN CULTURED CONNECTIVE TISSUE CELLS

IAN K. BUCKLEY, T. R. RAJU, and MURRAY STEWART

From the Department of Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra City, ACT, 2601, Australia, and CSIRO Division of Computing Research, Canberra City, ACT, 2601, Australia

ABSTRACT

Mild treatment with trypsin causes a radical change in the heavy meromyosin (HMM) binding properties of intermediate filaments in glycerinated, myosin-extracted cultured chick embryo connective tissue cells. In non-trypsin-treated cells, HMM labeling of filaments was often indistinct and variable in its distribution. By contrast, in cells treated with trypsin (under conditions which allowed most intermediate filaments to survive), virtually all filaments, including those of intermediate size, decorated with HMM to give distinct arrowhead patterns. We suggest that most intermediate filaments in such cells contain a core of F-actin masked by trypsin-labile accessory proteins.

KEY WORDS intermediate filament · 100-Å filament · F-actin · blocking protein · cell shape · cell motility

Since the pioneering studies of Ishikawa et al. (11, 12), it has been recognized that the filaments present in the cytoplasm of nonmuscle cells are of more than one type. On the basis of their diameter (5-7 nm) and their ability to bind heavy meromyosin (HMM) to give a characteristic arrowhead pattern, all of the cortical filaments and a few subcortical ones appear to be primarily F-actin. These actin-containing filaments are generally referred to as “thin” filaments, by analogy to skeletal muscle (9). In contrast, the predominating filaments of the subcortical cytoplasm have a greater diameter (8-12 nm) (11) and do not label with HMM (12). They are referred to as “intermediate” or “100-Å” filaments (11, 12). These findings, which are consistent with the results of recent antibody- and HMM-fluorescence studies (15, 18, 20, 22), have been confirmed by numerous other investigators (7, 21). Accordingly, there is now widespread acceptance of the view that F-actin is present in the thin filaments of nonmuscle cells but not in any intermediate-sized filaments.

We were prompted to question whether F-actin might lie hidden with some intermediate filaments of cultured connective tissue cells of the “fibroblast” type, for two reasons. First, as seen in the subcortical cytoplasm of critical-point-dried (5), sectioned (2), and negatively stained cells (see below), intermediate and thin filaments are cons-
nected in the form of a three-dimensional network in which the thin filaments arise as low-angle divergent branches from "parent" intermediate-sized filaments. Secondly, tiny structures which may represent cytoplasmic myosin are, in the non-myosin-extracted cell, attached not only to thin filaments but also to intermediate ones (5).

As one approach to the question of whether or not some intermediate filaments contain F-actin, we reinvestigated the binding of HMM within glycerinated cultured connective tissue cells by varying the conditions of treatment before the application of HMM. Believing that nonbinding of HMM to some cytoplasmic filaments could be due to blocking effects of accessory proteins (26), and knowing of the relative resistance of actin to trypsin (27), we have been able to modify the HMM binding patterns by mild trypsin pretreatment. Our results indicate that in cultured chick embryo connective tissue cells which have been glycerinated, myosin-extracted, and mildly trypsinized, virtually all residual filaments, including those of intermediate size, contain F-actin.

**MATERIALS AND METHODS**

**Cell Cultures**

10-day chick embryo cells of connective tissue type were cultured on Formvar/carbon-coated cover glasses in Rose culture chambers as described previously (5).

**Glycerination**

Three variant glycerol solutions were used. These were made by adding 1 vol of glycerol to 1 vol of salt solution. The salt solutions used were (a) Huxley's (9) standard salt solution (SSS): 0.1 M KCl, 1 mM MgCl₂, 6.7 mM K phosphate buffer, pH 7.0, at 4°C; (b) a variant salt solution (VSS) with a phosphate-free buffer: 0.1 M KCl, 5 mM MgCl₂, 10 mM imidazole-HCl, pH 7.0, at 4°C; and (c) a modification of VSS to act as a myosin-extracting solution: 0.1 M KCl, 5 mM MgCl₂, 4 mM ethylene glycol-bis(β-aminoethyl ether)N,N'-tetra-acetic acid (EGTA), 1 mM dithioerythritol, 8 mM Na₃P₂O₈, 10 mM imidazole-HCl, pH 7.0, at 4°C. Before use, the tissue culture medium was aspirated from the culture chamber, and the glycerol solution, precooled to 4°C, was injected into the chamber cavity. Chambers were then stored at 4°C for at least 24 h before subsequent procedures.

**Trypsinization of Glycerinated Cells**

Trypsin (Worthington Biochemical Corp., Freehold, N. J.; TPCK [L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone], 253 U/mg) was used at 5 μg/ml made up in 50% glycerol/50% VSS. The original glycerol solution in the culture chamber was aspirated and replaced with the trypsin-containing solution. This was allowed to stand for 1-3 h at 20°C. Higher concentrations of trypsin or significantly longer times (e.g., > 5 h) resulted in destruction of F-actin and failure to bind HMM.

**Proteins**

HMM was prepared according to Lowey et al. (17) and stored as a stock solution at a concentration of ∼3 mg/ml at −20°C in 50% glycerol, 30 mM KCl, 10 mM Tris-HCl, pH 6.5. Rabbit skeletal muscle actin was prepared according to Kendrick-Jones et al. (13).

**HMM Labeling**

The same batch of HMM was used in all experiments. Before labeling, the culture chamber was disassembled and the cover glass with attached cells was washed in small Petri dishes, using three to four changes of VSS at 4°C. To ensure adequate washing (especially for the removal of unwanted pyrophosphate or trypsin), the cells were left in the final Petri dish for 1 h. For HMM labeling, the above solution was drained off the cover glass and several drops of the stock HMM solution were applied directly to the cells. The treated cells were then stored at 4°C within a covered Petri dish (to prevent drying) for 12 h. At the end of this period, the cells were washed for 10–15 min in several changes of cold VSS to remove glycerol and excess HMM. To establish the specificity of the HMM labeling, the HMM treatment step was also carried out in the presence of 4 mM sodium pyrophosphate (9); this always completely inhibited binding.

**Electron Microscope Procedures**

Treated cells were fixed by placing the cover glass with attached cells in 0.8% glutaraldehyde in VSS for 15 min at room temperature. The cover glass was then transferred to a Petri dish of distilled water and, with fine forceps, small areas of Formvar/carbon were separated and allowed to reach the water surface (cell side down) where they spread out flat. EM grids were applied to these and separated as described previously (4). Negative staining was done with 2% aqueous uranyl acetate. Excess stain was only partially removed with filter paper before rapidly drying in the hot airstream of a hair dryer. Preparations were examined at 80 kV with a Philips EM 301 microscope fitted with a 70-μm objective aperture.

**RESULTS**

**Non-Trypsin-Treated Cells**

**Appearance of Filaments:** The arrangement of filaments in whole glycerinated, myosin-
extracted cultured cells which have been negatively stained (Fig. 1) can be related readily to that found in sectioned and critical-point-dried cells (1–4). Within the veil-like extensions of the cell margin, thin (≈5–7-nm diam) filaments are either diversely oriented or collected into narrow bundles. Deep to the veils, thin filaments are gathered into long bundles or sheet-like arrays (3). Occupying the deeper (i.e., subcortical) cytoplasm there are looser arrays of filaments which are more randomly disposed. In stereoscopic views of critical-point-dried cells, these subcortical filaments form a wide-mesh branching network which cross-links the dorsal and ventral cortical filament arrays (5). In negatively stained preparations, the cross-linking subcortical filaments appear to run between and through the elongate bundles and sheets of thin filaments in a characteristic meandering way (Fig. 1). These subcortical filaments vary considerably in width but the great majority are "intermediate" in size (i.e., 8–12-nm diam) (11). Many subcortical filaments appear to undergo branching, intermediate-sized filaments sometimes giving rise to thin ones (Fig. 1). Unequivocal evidence of this branching comes from viewing stereoscopic pairs of critical-point-dried or sectioned cells (2, 5) and, despite their greater flatness, a similar impression of such branching is gained by examining stereoscopic pairs of negatively stained cells.

**HMM Labeling:** Included here are cells which were glycerinated in the presence of the myosin-extracting solution as well as those glycerinated only with SSS. Because the findings were unaffected by prior myosin extraction, the two are described together. Inasmuch as the negatively stained whole mounts were easy to prepare, it was possible to survey large numbers of cells with comparative ease and rapidity. From the outset, we noted some variability in the HMM binding patterns. Although nearly all cells showed HMM binding, arrowhead formations were sometimes

**Figure 1** Portion of whole cultured cell which was glycerol/myosin-extracted, glutaraldehyde-fixed, and negatively stained. Toward the cell margin, subplasmalemmal cytoplasm (SP) shows only thin filaments. Elsewhere, subcortical filaments, which are mostly intermediate in size, are seen superimposed on bundles of thin cortical filaments. Intermediate filaments often branch, and in some instances, these branches appear as thin filaments (arrowheads). Bar, 0.25 μm. x 52,000.
irregular and indistinct. Rarely (<1%), individual cells showed no binding. In cells which exhibited binding, by far the commonest finding was that the thin filaments in bundles showed arrowhead formations whereas the intervening intermediate filaments did not (12, 21). However, in some cells, while most thin filaments showed HMM binding, some did not; and whereas most intermediate filaments failed to bind HMM, a few did (Fig. 2).

Trypsin-Treated Cells

PRESERVATION OF INTERMEDIATE FILAMENTS: Because of reports that intermediate filaments disappear altogether from some types of cell treated with proteolytic enzymes (24), it was important to establish, as a control, whether the gentle trypsin treatment we employed eliminated these filaments from the glycerinated cells. Depicting the edge of a trypsin-treated cell, Fig. 3 shows characteristic intermediate filaments coursing between bundles of thin filaments, as seen in nontrypsinized cells. Such images suggested that our trypsin treatment had not greatly altered the intermediate filament population. However, to investigate in more detail possible effects of the trypsinization on intermediate filaments, we compared the diameter distribution of subcortical filaments from a group of glycerol/myosin-extracted cells with that from a group of cells that had received the additional trypsin treatment. Three observers measured 20 filaments from each of 10 prints (made at final magnifications of 100,000) from each group. To eliminate subjective bias, the identity of each micrograph was withheld from the observer, and filaments for measurement were selected by dropping a template randomly onto each print. The histograms obtained are shown in Fig. 4. Statistical analysis indicated that these histograms were different (P < 0.01, $\chi^2$ test) and that the trypsin had reduced the mean filament diameter by ~7%. However, the overall distribu-

**FIGURE 2.** Portion of a glycerinated cultured cell, labeled with HMM. Although not always distinct, the thin filaments in bundles show HMM labeling. A few thin filaments outside bundles are not labeled (arrowheads). Nearly all intermediate filaments are unlabeled but occasional ones show an arrowhead pattern (arrows). Bar, 0.25 μm. × 68,000.
Figure 3  Margin of a glycerinated cell which was treated with 5 μg/ml of trypsin for 1 h before fixation and negative staining. In the cytoplasm between bundles of thin filaments there are numerous, diversely oriented filaments of various widths, most of which are intermediate in size (cf. Fig. 1). Bar, 0.25 μm. x 52,000.

HMM Labeling: Trypsin treatment before HMM labeling had a dramatic effect: virtually all filaments in all cells exhibited distinct labeling. Besides the subplasmalemmal filaments and the parallel filaments lying in bundles and sheets, the more diversely oriented filaments of the subcortical cytoplasm showed clear arrowhead patterns. As seen in Fig. 5, these meandering HMM-labeled filaments vary considerably in diameter, some appearing twice the width of those in the bundles. The distinctness of the binding in thin negatively stained preparations is illustrated in Fig. 6. Where wide-caliber HMM-decorated filaments split to form two branches, both are clearly labeled with HMM (Fig. 6).

To obtain a more quantitative appreciation of the increase in HMM labeling brought about by the trypsin treatment, we used a protocol similar to that employed to investigate changes in filament diameter. Using a sampling template, 20 filaments were selected from each of 10 micrographs taken from trypsin-treated and non-trypsin-treated groups of cells. Filaments were categorized according to whether or not they bound...
FIGURE 4 Histograms of filament diameters. (a) Cytoplasmic filaments without trypsin treatment. (b) Cytoplasmic filaments after trypsin treatment. (c) Rabbit skeletal muscle F-actin.

DISCUSSION

Our major finding is that in glycerinated, myosin-extracted cultured chick embryo connective tissue cells of the fibroblast type, mild trypsin treatment (which causes relatively minor changes in the intermediate filament population) allows virtually all residual filaments to label with HMM. This finding indicates that not only thin filaments, but also most intermediate filaments of these cells contain F-actin. This conclusion differs from that of Ishikawa et al. (12) and many others (21), but it should be stressed that our results were obtained under significantly different conditions. When we carried out the HMM labeling procedure on cells which had been glycerinated in the presence of either a standard salt solution (9) or a myosin-extracting solution (5), our results were essentially similar to those of others. It was only when the HMM labeling procedure was preceded by mild trypsinization that the labeling pattern was strikingly different. A likely interpretation of this finding is that trypsin degrades various accessory proteins which normally block the binding of HMM to underlying actin filaments. Once these accessory proteins are removed, the underlying F-actin can bind HMM and form characteristic arrowhead complexes. Implicit in this interpretation is that many intermediate filaments of cultured fibroblasts contain an actin core, perhaps in the form of multiple (~two to five) strands of F-actin. Multistrandedness is suggested by the variable widths of intermediate filaments (11) and by the branching of thin filaments from intermediate filaments, especially when both show characteristic arrowhead labeling patterns with HMM (cf. Fig. 5).

We have been encouraged in the above interpretation of how trypsin might promote the HMM labeling of underlying actin (through the removal of trypsin-labile blocking proteins) by Tilney's (26) studies on the acrosomal process of Limulus sperm, as these studies have shown that a specific accessory protein associated with F-actin can have such an HMM blocking effect. These studies are of additional possible relevance because the molecular weight of the HMM blocking protein in Limulus (55,000) is identical or close to that of certain proteins characteristic of the intermediate-sized filaments of some types of vertebrate cell (25). It may be significant that, in many attempts to isolate these vertebrate cell intermediate filament proteins in pure form, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic analyses have shown the co-purification of actin (e.g., references 16, 25). If we think of the specific intermediate filament proteins as accessory proteins which are normally associated with underlying strands of F-actin, then such findings are readily reconciled with our own. However, in some studies, polyacrylamide gel analyses of proteins from intermediate filament extracts of neurons (14, 23) and smooth muscle cells (24) show
Figure 5 Portion of a glycerinated cultured cell treated with 5 µg/ml of trypsin for 1 h followed by HMM. All filaments show HMM labeling. Parallel arrays of thinner HMM-labeled filaments lie in bundles. Lying between the bundles and meandering across them are diversely oriented HMM-labeled filaments some of which are twice the width of those in the bundles (arrowheads). Bar, 0.25 µm. × 68,000.

only one band, that of the intermediate filament protein, and no trace of actin. Such results indicate that, in these cells, intermediate filaments represent a specially differentiated filament which does not contain F-actin. Accordingly, it will be necessary to investigate the intermediate filaments of each type of cell to determine which contain F-actin and which do not.

Our observation that, in glycerinated cells which have not been trypsin-treated, HMM labeling gives less distinct arrowhead patterns which are inconstant in their distribution and that, in rare instances, a cell shows no labeling, requires some comment. As we used the same batch of HMM and followed the same labeling procedure throughout all our experiments, we cannot ascribe the indistinctness and variability in the distribution of labeling to any lack in actin-binding activity of our HMM. This conclusion is supported by the observation that every time we carried out HMM binding on cells which had received prior trypsin treatment, the arrowhead configurations were always distinct and quite constant in their distribution. The finding that the HMM-labeled filaments of cultured fibroblasts which have only been glycerinated are often indistinct and "fuzzy" in appearance is not new (8). Previously, as such cells have been fixed, embedded, and sectioned, this fuzziness has been attributed to a combination of the processing involved and the tilting of the filaments within the section (21). However, because distinct arrowhead patterns have been depicted in sectioned material (12), we think it more likely that this indistinctness of the arrowhead pattern reflects an interference with HMM binding by trypsin-labile blocking proteins which, to a variable extent, affect all the filaments in these glycerinated cells. Although this interference is usually minimal in the case of the thin filaments, it may be sufficient to produce fuzzy or otherwise
FIGURE 6 More detailed view of HMM binding pattern after trypsin treatment. In several places (arrowheads), HMM-labeled intermediate filaments are seen to branch to form two filaments, both of which exhibit HMM labeling. Bar, 0.25 μm. × 96,000.

atypical decorated filaments (8, 21). It has been our experience with negatively stained whole cell mounts (which can be rapidly surveyed in large numbers) that, although nearly all intermediate filaments remain unlabeled, occasional ones show recognizable HMM binding. Had such material been embedded and sectioned, it seems likely either that such filaments would not be recognized as HMM-labeled or that, if recognized as labeled, they could be interpreted as labeled thin filaments.

If the mechanical force for organelle movements is based on an actomyosin system, then a core of F-actin within certain intermediate filaments could be functionally meaningful (2, 9, 10, 21). This is because organelles reside and move within the subcortical cytoplasm, the filaments of which are nearly all intermediate in size (11). Indeed, the constant association between organelles and intermediate filaments has led to the view (25) that intermediate filaments must in some way be responsible for organelle movements. If many intermediate filaments contain F-actin, this could provide one component of a shear-force-generating system for organelle movement. Another way in which actin within intermediate filaments might be functionally meaningful is in the maintenance of cell shape. Although it has long been recognized that much filamentous actin occurs in the cell cortex (7, 12, 21), it has been assumed that relatively little exist in the deeper cytoplasm. Our results, indicating a core of F-actin within many subcortical intermediate filaments, support the view (19, 20) that there may exist, throughout the cytoplasm of some cells, a supporting actin-based network which is important for cell shape determination.

It is a pleasure to acknowledge the capable and willing assistance of David Irving and Marj Lee.

Received for publication 1 February 1978, and in revised form 29 March 1978.
REFERENCES

1. Buckley, I. K. 1974. Subcellular motility: a correlated light and electron microscopic study using cultured cells. Tissue Cell. 6:1-20.

2. Buckley, I. K. 1975. Three-dimensional fine structure of cultured cells: possible implications for subcellular motility. Tissue Cell. 7:51-72.

3. Buckley, I. K., and K. R. Porter. 1967. Cytoskeletal fibrils in living cultured cells. A light and electron microscopic study. Protoplasma. 64:349-380.

4. Buckley, I. K., and K. R. Porter. 1975. Electron microscopy of critical point dried whole cultured cells. J. Microsc. (Oxf.). 104:107-120.

5. Buckley, I. K., and T. R. Raju. 1976. Form and distribution of actin and myosin in non-muscle cells: a study using cultured chick embryo fibroblasts. J. Microsc. (Oxf.). 107:129-149.

6. Burnside, B., and A. M. Latches. 1976. Actin filaments in apical projections of the primate pigmented epithelial cell. Invest. Ophthalmol. 15:570-575.

7. Clarke, M., and J. A. Spudich. 1977. Non-muscle contractile proteins: the role of actin and myosin in cell motility and shape determination. Annu. Rev. Biochem. 46:797-822.

8. Goldman, R. D. 1975. The use of heavy meromyosin binding as an ultrastructural cytochemical method for localizing and determining the possible functions of actin-like microfilaments in nonmuscle cells. J. Histochem. Cytochem. 23:529-542.

9. Huxley, H. E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J. Mol. Biol. 7:281-308.

10. Huxley, H. E. 1973. Muscular contraction and cell motility. Nature (Lond.). 243:445-449.

11. Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol. 36:538-555.

12. Ishikawa, H., R. Bischoff, and H. Holtzer. 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. J. Cell Biol. 43:312-328.

13. Kendrick-Jones, J., W. Lehman, and A. G. Szent-Györgyi. 1970. Regulation in molluscan muscles. J. Mol. Biol. 54:313-326.

14. Lasek, R. J., and P. N. Hoffmann. 1976. The neuronal cytoskeleton, axonal transport and axonal growth. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 347-360.

15. Lazarides, E. 1976. Aspects of the structural organization of actin filaments in tissue culture cells. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 374-394.

16. Lazarides, E., and B. D. Hubbard. 1976. Immunological characterization of the subunit of the 100 Å filaments from muscle cells. Proc. Natl. Acad. Sci. U. S. A. 73:4344-4348.

17. Lowey, S., H. S. Slatter, A. G. Weeds, and H. Baker. 1969. Substructure of the myosin molecule. I. Subfragments of myosin by enzyme degradation. J. Mol. Biol. 42:21-29.

18. Osborn, M., W. W. Franke, and K. Weber. 1975. Visualization of a system of filaments 7-10 nm thick in cultured cells of an epithelialoid line (Pt K2) by immunofluorescence microscopy. Proc. Natl. Acad. Sci. U. S. A. 74:2490-2494.

19. Pollard, T. D. 1976. Cytoskeletal functions of cytoplasmic contractile proteins. J. Supramol. Struct. 5:317-334.

20. Pollard, T. D., K. Fujikawa, R. Niederman, and P. Maupin-Szamier. 1976. Evidence for the role of cytoplasmic actin and myosin in cellular structure and motility. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 689-724.

21. Pollard, T. D., and R. R. Weihing. 1974. Actin and myosin in cell movements. Crit. Rev. Biochem. 2:1-65.

22. Sanger, J. W., and J. M. Sanger. 1976. Actin localization during cell division. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1295-1316.

23. Shelanski, M. L., S.-H. Yen, and V. M. Lee. 1976. Neurofilaments and glial filaments. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1007-1020.

24. Small, J. V., and A. Soriesz. 1977. Studies on the function and composition of the 10-nm (100 Å) filaments of vertebrate smooth muscle cells. J. Cell Biol. 23:243-268.

25. Starger, J. M., and R. D. Goldman. 1977. Isolation and preliminary characterization of 10-nm filaments from baby hamster kidney (BHK-21) cells. Proc. Natl. Acad. Sci. U. S. A. 74:2422-2426.

26. Tilney, L. G. 1975. Actin filaments in the acrosomal reaction of Limulus sperm. J. Cell Biol. 64:289-310.

27. Tilney, L. G. 1977. Actin: its association with membranes and the regulation of its polymerization. In International Cell Biology 1976-1977. B. R. Brinkley and K. R. Porter, editors. The Rockefeller University Press, New York. 388-402.