Electron Microscopic Localization of Cytoplasmic Myosin with Ferritin-labeled Antibodies

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ABSTRACT We localized myosin in vertebrate nonmuscle cells by electron microscopy using purified antibodies coupled with ferritin. Native and formaldehyde-fixed filaments of purified platelet myosin filaments each consisting of ~30 myosin molecules bound an equivalent number of ferritin-antimyosin conjugates. In preparations of crude platelet actomyosin, the ferritin-antimyosin bound exclusively to similar short, 10–15-nm wide filaments. In both cases, binding of the ferritin-antimyosin to the myosin filaments was blocked by preincubation with unlabeled antimyosin. With indirect fluorescent antibody staining at the light microscope level, we found that the ferritin-antimyosin and unlabeled antimyosin stained HeLa cells identically, with the antibodies concentrated in 0.5-μm spots along stress fibers. By electron microscopy, we found that the concentration of ferritin-antimyosin in the dense regions of stress fibers was five to six times that in the intervening less dense regions, 20 times that in the cytoplasmic matrix, and 100 times that in the nucleus. These concentration differences may account for the light microscope antibody staining pattern of spread interphase cells. Some, but certainly not all, of the ferritin-antimyosin was associated with 10–15-nm filaments. In mouse intestinal epithelial cells, ferritin-antimyosin was located almost exclusively in the terminal web. In isolated brush borders exposed to 5 mM MgCl₂, ferritin-antimyosin was also concentrated in the terminal web associated with 10–15-nm filaments.

Although fluorescent antibody studies have established the overall distribution of myosin within some vertebrate nonmuscle cells (1, 3, 8, 11, 19, 20, 22–24), the form and detailed arrangement of myosin has remained a mystery. In part, this is attributable to the low concentration of myosin in the cytoplasm (13, 18); but more importantly, there has been no way to identify myosin positively amidst the plethora of actin and intermediate filaments. Whatever the case, it is clear that a precise molecular localization of nonmuscle myosin is needed to understand actomyosin-based motile mechanisms.

In this study, we report our success using ferritin-conjugated myosin antibodies to locate myosin by electron microscopy. First, we established that our ferritin-antimyosin bound to formaldehyde-fixed and native filaments of purified platelet myosin and that it gave the same light microscope staining pattern of HeLa cells as unlabeled antimyosin. By electron microscopy of the HeLa cells fixed with formaldehyde and rendered permeable to the antibodies with ethanol, we found ferritin-antimyosin concentrated in stress fiber densities. The ferritin-antimyosin also stained the terminal web region of intestinal epithelial cells and isolated brush borders. Blocking and absorption control experiments indicated that the methods had a high signal-to-noise ratio.

MATERIALS AND METHODS

Preparation of Proteins

ANTIBODY PURIFICATION: Immune serum was obtained from rabbit no. 3, immunized, and boosted twice with human blood platelet myosin (3, 17). This serum contained antibodies that reacted only with the tail portion of the myosin molecule (3, 17). Immune IgG was purified from the serum by ammonium sulfate precipitation and DEAE-cellulose chromatography (3). Antimyosin was separated from the remainder of the immune IgG by affinity chromatography over Sepharose-platelet myosin (3). The immune IgG that was depleted of antimyosin by this step is called absorbed IgG and was used in control experiments.

FERRITIN RECRYSTALLIZATION, ACTIVATION, AND COUPLING: Twice recrystallized horse spleen ferritin (Polysciences Inc., Warrington, Pa.) was further purified by recrystallization until only monomeric ferritin was visible in negatively stained preparations, as described by Sternberger (21). 1 ml of 50% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) was pipetted dropwise into 10-mg monomeric ferritin in 2 ml of 0.01 M sodium phosphate, pH 7.3, at 22°C with vigorous stirring to avoid precipitation (7). After 30 min, the reaction mixture was clarified by centrifugation at 4°C for 10 min at 20,000 g. The solution was dialyzed immediately on a 2.5 × 15-cm column of Sephadex G-25 (coarse), equilibrated with 0.01 M sodium phosphate buffer, pH 7.3.
7.3. The void volume containing the glutaraldehyde-activated ferritin was reacted immediately with an equimolar amount of purified antimyosin IgG (or absorbed IgG) for 60-70 h at 4°C while rotating slowly. The reaction mixture was then chromatographed on a 2.5 x 50-cm column of 4% agarose (Biogel A 15m; Bio-Rad Laboratory, Richmond, Calif., 200/400 mesh) equilibrated with 0.1 M Tris-Cl and 0.01 M sodium phosphate, pH 7.3. Ferritin-Ig containing fractions were collected and dialyzed against 0.015 M sodium phosphate, 0.15 M NaCl, pH 7.5.

**HUMAN PLATELET ACTOMYOSIN AND MYOSIN:** Human platelet actomyosin and platelet myosin were prepared and purified exactly as described by Pollard et al. (17).

**Fluorescence Microscopy**

HeLa cell cultures were grown on glass microscope cover slips (5, 6) and fixed with 3-4% formaldehyde (prepared fresh from paraformaldehyde) in phosphate-buffered saline (PBS: 0.015 M sodium phosphate, 0.15 M NaCl, pH 7.3) for 2-10 min at 37°C. Fixed cells were washed with 130 ml of PBS (in three washes) and dehydrated in 95% ethanol cooled to −20°C for 30-60 s before washing with PBS. After a 60 min incubation with 20-60 μl/ml ferritin-Ig at room temperature, the cover glasses were washed in PBS (as above) before reaction with 80 μg/ml of fluorescein-labeled goat anti-rabbit IgG (Cappel Laboratories, Inc.). Immunofluorescence was observed and photographed with a Leitz Orthoplan microscope (6). For a control, we incubated HeLa cells in 200 μg/ml absorbed Ig-ferritin before reaction with 80 μg/ml fluorescein-labeled goat anti-rabbit IgG (Cappel Laboratories, Inc.).

**Electron microscopy**

**PLATELET MYOSIN FILAMENTS**

We formed platelet myosin filaments by dialysis of myosin in 0.6 M KCl against 0.01 M imidazole-Cl, 50 mM KCl and 2 mM MgCl2, pH 7.0 (filament buffer). 43 μg/ml of these filaments or filaments fixed for 5 min with 4% formaldehyde in filament buffer were reacted with 51 μg/ml of ferritin-antimyosin for 120 min at room temperature. The filaments were washed twice in filament buffer by pelleting at 40,000 g in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, Calif.) and resuspended in filament buffer. 5-10 μl (0.2-0.4 μg) of decorated filaments were applied to carbon-coated Formvar or parlodion EM grids and negatively stained with 1% uranyl acetate for 30 s. Desiccated grids were observed in a Zeiss EM 10A electron microscope.

**PLATELET ACTOMYOSIN**

Platelet actomyosin (200 μg/ml) in 0.05 M KCl, 0.005 M MgCl2, 0.01 M imidazole, pH 7.2 (13, 17) was reacted with 60 μg/ml ferritin-antimyosin for 18 h at 4°C. For a control experiment, platelet actomyosin was mixed with 100 μg/ml purified antimyosin IgG as described above, followed by washing and reaction with ferritin-antimyosin. Similarly, platelet actomyosin was mixed (under identical conditions) with the same concentration of free ferritin. Samples were washed by pelleting and fixed for 30 min in 2.5% glutaraldehyde in 0.05 M KCl, 0.002 M MgCl2, and 0.01 M imidazole, pH 6.8, at 4°C. Pellet surfaces were rinsed with fresh buffer and allowed to warm to room temperature before exposure to 0.1% osmium tetroxide buffered with 0.1 M sodium phosphate, pH 7.0, for 30 min (9). Fixed pellets were rinsed with fresh buffer and then dehydrated embedded, sectioned, and stained.

**HELA CELLS**

HeLa cells were briefly washed with Hank's balanced salt solution (Hank's BSS) before fixation for 3-10 min in 3-4% formaldehyde in Hank's BSS at 37°C. Fixed monolayers were washed with 30 ml of PBS before a 30-60 s dehydration in 95% ethanol cooled to −20°C. Cells were then washed three times with 30 ml of PBS and incubated 1-2 h at room temperature in (a) 60 μg/ml ferritin-antimyosin, (b) 200 μg/ml absorbed IgG-ferritin, or (c) 100 μg/ml unlabeled antimyosin followed by 60 μg/ml ferritin-antimyosin. The samples were then washed with 30 ml of PBS before a second fixation with 2.5% glutaraldehyde buffer in Hank's BSS for 45 min at room temperature. After washing with 30 ml of PBS, the cells were treated with 0.1% osmium tetroxide in 0.1 M sodium phosphate, pH 7.0, for 30 min at room temperature before they were dehydrated, embedded, and sectioned.

1 Herman, J. M., N. Crisona, and T. D. Pollard, 1980, relations between cell activity and the distribution of cytoplasmic actin and myosin. Manuscript submitted for publication.

**Brush Border Isolation**

Murine brush borders were isolated exactly according to the procedure of Moorecker and Tilney (12) in isotonic buffers and were washed three times with 0.06 M KCl, 0.005 M MgCl2, 0.001 M EGTA, 0.001 M dithiothreitol, 0.01 M imidazole, pH 7.3, before incubation with either 60 μg/ml ferritin-antimyosin or absorbed ferritin-IgG for 60-120 min at 25°C. Brush borders were washed, fixed, and prepared for EM thin sectioning.

**Ferritin Counting Methods**

**myosin filaments:** We counted the number of ferritin grains associated with formaldehyde-fixed or native filaments of platelet myosin in negatively stained preparations. The sample consisted of 54 filaments of native myosin and 43 filaments of fixed myosin from several EM grids. The number of ferritin grains bound to filaments were calculated along with the mean values and the standard deviation from the mean.

**HeLa cells:** The sample consisted of (a) 20 electron micrographs obtained from uranyl acetate and lead citrate stained thin sections of HeLa cells incubated in 60 μg/ml ferritin-antimyosin, (b) 20 electron micrographs of unstained thin sections from HeLa cells that were incubated with 200 μg/ml ferritin-absorbed IgG, or, (c) 20 electron micrographs of unstained thin sections from HeLa cells incubated with 100 μg/ml antimyosin followed by 60 μg/ml ferritin-antimyosin. We counted the ferritins in 20-30 representative 0.5 μm2 areas of each micrograph at a magnification of ×78,000, which included stress fiber densities, those regions of stress fibers adjacent to the densities, regions of general cytoplasm, and nucleoplasm.

**RESULTS**

**Preparation of Ferritin-Antimyosin**

After reaction of a 1:1 molar (5:1 wt) ratio of glutaraldehyde-activated ferritin with purified antimyosin, virtually all protein was recovered as a single peak (Fig. 1) which eluted at Kd = 0.45 ahead of the position of free ferritin (Kd = 0.56). The 5% of the IgG that was un conjugated eluted from the agarose column at Kd = 0.67. The entire ferritin-IgG peak contained antigen-combining activity based upon indirect fluorescent staining of HeLa cells. This demonstrated that the bulk of the ferritin and Ig were conjugated in a 1:1 molar ratio. Immuno-electrophoresis of platelet myosin vs. ferritin-antimyosin produced a single precipitin line (not shown).

**Purified Platelet Myosin Filaments**

Ferritin-antimyosin bound to synthetic platelet myosin and was visualized in negatively stained specimens as a luent...
annulus with a 6-nm electron dense center caused by the iron-containing core of ferritin. In positively stained specimens, only the central iron core was seen. Up to 30 ferritin-antimyosin conjugates were associated with each filament (29.9 ± 0.6) (Fig. 2a), or about one ferritin-antibody complex per myosin molecule in these filaments (13). Neither longer incubation times (>120 min) nor higher concentrations of ferritin-antimyosin yielded increased decoration of synthetic platelet myosin filaments. Similar results were obtained with formaldehyde-fixed platelet myosin filaments (27.1 ± 0.5). In contrast, ferritin-labeled absorbed IgG that was depleted of antimyosin by affinity chromatography bound sparingly to these myosin filaments. Only three to five ferritins bound to each filament (Fig. 2b) reacted with a concentration of absorbed ferritin-IgG that was three times the ferritin-antimyosin concentration used in Fig. 2a.

Platelet Actomyosin

Platelet actomyosin is a heterogeneous mixture of contractile proteins and membranes (13) including all of the antigens that might have contaminated the myosin used for immunization. In these specimens, short 10–15-nm wide filaments similar to purified myosin filaments were decorated with ferritin-antimyosin (Fig. 3a). Neither thin filaments nor membranous material bound the ferritin-labeled antibodies (Fig. 3b). In these and other thin sections, only the 6-nm iron core of ferritin is seen. An average of 8–12 ferritin-antimyosin conjugates were associated with each filament cross section (Fig. 3b). Pretreatment of platelet actomyosin with unlabeled antimyosin blocked the binding of ferritin-antimyosin to the 10–15-nm wide filaments (Fig. 3c).

HeLa Cells

LIGHT MICROSCOPY: To establish that our ferritin-antimyosin bound to HeLa cells like antimyosin without ferritin attached (references 3 and 4 and footnote 1), we examined HeLa cells by fluorescence microscopy. Direct staining with rhodamine-antimyosin (Fig. 4a and b) and indirect staining with ferritin-antimyosin (Fig. 4c and d) gave identical punctate staining along stress fibers. In cells without stress fibers, both reagents gave diffuse staining (not shown). In control experiments, absorbed IgG-ferritin did not stain.

ELECTRON MICROSCOPY: The formaldehyde/ethanol fixation procedure that we used for light microscopy gave both remarkably good ultrastructural preservation (Fig. 5) and allowed ferritin-antibodies access to the cytoplasmic matrix, presumably through perforations in the cell membrane (Fig. 6). Prominent microtubules, organelles, thin filaments, and intermediate filaments are routinely well preserved by this fixation procedure (Figs. 5 and 6).

In cells incubated with ferritin-antimyosin, the ferritin was concentrated within the dense regions of stress fibers (Fig. 7). The concentration of ferritin in these dense regions (80–110/0.5 μm²) was five to six times that in those regions of stress fibers adjacent to the stress fiber electron densities (Fig. 7), 20 times that elsewhere in the cytoplasmic matrix, and 100 times that within the nucleus (<1 ferritin/0.5 μm²). The stress fiber densities were 0.3–0.7-μm long and consisted of amorphous and filamentous material, including a few 10–15-nm wide filaments decorated with ferritin-antimyosin (Fig. 7). Most of the ferritin was not associated with clear filaments. The inter-dense regions of stress fibers (Fig. 7) were composed primarily of 6-nm-wide filaments but infrequently there were 10–15-nm filaments labeled with ferritin, in addition to more numerous ferritins not associated with any filamentous material. The specificity of the ferritin-antimyosin staining of fixed HeLa cells was tested by treating with either absorbed IgG-ferritin (Fig. 8a) or by pretreating specimens with unlabeled antimyosin before treating with ferritin-antimyosin (Fig. 8b). In both cases, no ferritin was detected in either stained (Fig. 8) or unstained thin sections. Ferritin counts throughout the cyto-
Figure 4. Fluorescent antibody staining of HeLa cells. Fluorescence (a and c) and phase contrast (b and d) images of interphase HeLa cells stained either (a) directly with 20 µg/ml rhodamine goat anti-human platelet myosin or (c) indirectly with 20 µg/ml ferritin-antimyosin followed with 80 µg/ml fluorescein goat anti-rabbit IgG. Bar, 10 µm.

Plasm of unstained control sections were 1–2/0.5 µm², so that the signal-to-noise ratio was ~70:1 in the stress fibers.

Brush Borders

Ferritin-antimyosin is also concentrated in the terminal web region of intestinal brush borders isolated in an isotonic buffer (12) with 5 mM MgCl₂ (Fig. 9a and b), and confirming previous light microscope studies (1, 11). As noted previously (10), 10–15-nm wide filaments are present within the terminal web region under these conditions and many of the ferrittins are clustered around them (Fig. 10). In favorable sections (Fig. 10), these myosin filaments can be seen bridging two adjacent microvillar actin bundles as they insert into the terminal web supporting the model proposed by Mooseker and Tilney (12). Neither the actin within the microvilli nor the intermediate filaments were decorated (Fig. 9b).

Discussion

Technique

Our goal was to localize myosin in nonmuscle cells by electron microscopy, and we found that treatment of lightly-fixed, ethanol-extracted cells with ferritin-labeled and affinity-purified antibodies was satisfactory for this purpose. It is conceivable that we might have obtained denser staining with an indirect method, but the direct approach is sufficiently sensitive, with about one ferritin per myosin molecule. Further, blocking controls are possible only with direct staining; and, direct staining involves only a single antigen-antibody reaction, which may contribute to the high signal-to-noise ratio we obtained. Another approach for localizing brush border myosin has been to use indirect methods and peroxidase labeled antibodies (2). As in our experiments, the label was concentrated in the terminal web. An advantage of the ferritin method is more discrete labeling.

We found that ethanol treatment of formaldehyde-fixed cells was an excellent way of preparing cells for antibody staining at both the light and electron microscope levels. The advantages are retention of the major cytoplasmic constituents (Fig. 5), including >90% of the actin and myosin, and good preservation of ultrastructural details (Fig. 5), including microtubules. In comparison, detergent extraction removes considerably more protein and frozen sections have little ultrastructural detail. In addition, we understand that J. Sanger and J. Sanger (personal communication) have found independently that...
Figure 7: Electron micrograph of HeLa cell stress fibers with densities. This specimen was sectioned and prepared as in Figs. 5 and 6. The inset is magnified to show the association of the ferritin-labeled antimyosin with the stress fiber densities. Some individual 10-15-nm filaments are seen within the decorated densities. Regions between densities are composed primarily of 6-nm wide filaments. Bars, 0.25 μm.

Ethanol extraction can be used to introduce myosin fragments into fixed cells.

Cytoplasmic Myosin

Although earlier studies established the overall distribution of myosin in HeLa cells (references 3 and 4 and footnote 1) and intestinal epithelial cells (1, 11), little was known about the form of the myosin or its detailed distribution before our study (14-16). It was suggested that the punctate pattern of stress fiber fluorescence was the result of staining individual myosin filaments (3, 4). However, our main findings are that most of the antimyosin labeling in both HeLa and intestinal epithelial (I. Herman, unpublished observations) cells are not associated with obvious filaments and that the labeling patterns probably represent local differences in the concentration of cytoplasmic myosin.

The current study does not provide any final answers about the form of cytoplasmic myosin, but it strongly suggests that at least part of the myosin is not assembled into filaments. Although the ferritin-antimyosin labeled 10-15-nm filaments in HeLa cells, some of it was not associated with such filaments. The formaldehyde fixation is capable of preserving platelet myosin filaments (Figs. 2 and 3) so that the apparent absence cannot be blamed entirely on the fixation procedure. Consequently, other factors will have to be sought to explain why myosins that readily form filaments after purification do not do so in the cell.

Certainly modifications in the cellular environment can determine whether the myosin forms filaments, as in the case of the brush border region of the intestinal epithelial cells. In both fixed, intact cells, and isolated brush borders, ferritin-antimyosin was concentrated in the terminal web, but 10-15-nm myosin filaments were seen only in the isolated brush borders and not in labeled intact cells (I. Herman, unpublished observations). It has been shown (10) that Mg<sup>2+</sup> is important for the observation of these filaments, but additional work will be required to understand the mechanism of its action.

We found the correlation between the fluorescent- and the ferritin-antimyosin staining patterns within the cytoplasm of well-spread HeLa cells to be particularly interesting. From the fluorescent antibody staining experiments reported here (Fig. 4) and from earlier studies (3, 4), one gets the impression that all antimyosin is confined to the bright spots along stress fibers. These bright, 0.3-0.7-μm spots coincide with the decorated stress fiber densities. However, ferritin-antimyosin is also present throughout the cytoplasm in concentrations above the background level. The punctuated fluorescence image of HeLa cell stress fibers obtained from antimyosin staining must simply be caused by differences in the cytoplasmic myosin concentration, with stress fiber densities possessing the highest myosin concentration. This fivefold variation of ferritin-antimyosin along the stress fiber (refer to Fig. 7) provides sufficient contrast in the light microscope to give one the false impression that there is no antimyosin between the brightly stained spots.
The authors express their sincere thanks to Pamela Maupin for her technical assistance and Dr. D. Murphy for his comments on this work.

Supported by National Institutes of Health Research grant GM 26338 and a Muscular Dystrophy Association of America Postdoctoral Fellowship award to I. M. Herman.

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FIGURES 9 and 10. Electron micrographs of thin sections of intestinal brush borders reacted with 60 μg/ml ferritin-antimyosin. Fig. 9a, stained section; 9b, unstained section. Ferritin-antimyosin is localized exclusively within the terminal web. Intermediate filaments and microvilli are devoid of ferritin. Bar, 0.2 μm. Fig. 10 is a higher magnification to show ferritin associated with 10-15-nm filaments. The ends of a 10-15-nm filament are demarcated by arrowheads. Bar, 0.1 μm.