Visualization of Longitudinally-oriented Intermediate Filaments in Frozen Sections of Chicken Cardiac Muscle by a New Staining Method

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ABSTRACT When ultrathin frozen sections of chicken cardiac muscle were osmicated, dehydrated in ethanol, embedded in ethyl cellulose, and stained with acidic uranyl acetate, filaments of 10–12 nm width were visualized in wide interfibrillar spaces. Immunostaining of the frozen sections for desmin resulted in exclusive labeling of such filaments. These observations indicated that longitudinally oriented networks of intermediate filaments were present in the interfibrillar spaces, in addition to the transversely oriented networks that surround myofibrils at the level of Z band. As in skeletal muscle (Tokuyasu, K. T., A. H. Dutton, and S. J. Singer, 1983, *J. Cell Biol.* 97:1727–1735), desmin in chicken cardiac muscle is believed to be largely, if not entirely, in the form of intermediate filaments.

The presence of transversely oriented bundles of intermediate filaments at the level of Z band was reported in a number of ultrastructural studies of cardiac muscle (1, 4). In our immunoelectron microscopic study of chicken cardiac muscle (14), however, the major subunit of the filament, desmin, was localized not only at the level of Z band, but additionally along the length of sarcomere in the interfibrillar spaces where large mitochondria were present. The ferritin-antibody indirect labeling for desmin in such spaces often showed longitudinally or obliquely oriented linear arrays, but we could not obtain definitive evidence that they represented intermediate filaments. Furthermore, intense desmin labeling was seen in wide cytoplasmic areas, but it was difficult to judge whether the labeling was distributed diffusely or in overlapping linear arrays (Fig. 3 in reference 14).

These observations raised the possibility that a significant part of desmin in cardiac muscle existed in a nonfilamentous rather than a filamentous form. Here, we report that a new staining method revealed the presence of 10–12 nm wide filaments in wide cytoplasmic areas in ultrathin frozen sections of cardiac muscle and that labeling of desmin occurred exclusively on these filaments. These results indicated that desmin was largely, if not entirely, in filamentous form in this tissue.

MATERIALS AND METHODS

Fixation and Cryoultramicrotomy: The immunoelectron microscopic studies were carried out by immunolabeling of lightly fixed ultrathin frozen sections (11, 15). The left ventricle of adult chicken heart was fixed by perfusing 5 ml of a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 2 mM $CaCl_2$ into the ventricle after severing a peripheral artery. The heart was then opened and the ventricular papillary muscle was excised and cut into small pieces in the fixative. The fixation was continued for 1 h at room temperature in the same fixative. After thorough washing in 0.1 M cacodylate buffer and then with 0.1 M phosphate buffer, the fixed pieces were stored in 0.1 M phosphate buffer with 0.4% paraformaldehyde for up to 1 wk in a refrigerator.

Immunolabeling and Positive Staining: Desmin and myosin were isolated from chicken gizzard according to the method of Lazarides and Hubbard (8) and from the ventricles of chicken heart by a modified method of Kielley and Bradley (6), respectively. Rabbit antibodies to these proteins were affinity-purified and used as the primary antibodies. Ferritin conjugates of affinity-purified goat anti-rabbit IgG (15) were used as the secondary antibodies in indirect immunolabeling.

After the completion of immunostaining, the sections on the grid were briefly fixed with a 2% glutaraldehyde solution and thoroughly washed with 0.1 M phosphate buffer. The grid was then floated on a 1% OsO_4 solution in the phosphate buffer for 15 min, washed twice in distilled water, dehydrated by two washes in ethanol, and finally washed twice in amyl acetate. All washes were carried out for 2 min. The grid was then left in a 1% ethyl cellulose (Fisher Scientific Co., Fair Lawn, NJ) solution in amyl acetate for 10 min. The grid was subsequently picked up with a pair of forceps, immediately touched on its back onto the surface of a piece of hardened filter paper (Whatman filter paper no. 50; Whatman Chemical Separation, Inc., Clifton, NJ), withdrawn along the paper surface to remove the solution on the back side of the grid completely, and left to dry. The formvar supporting film coated with carbon remained intact throughout these procedures. The grid was then floated upside down on a droplet of 2% uranyl acetate in water for 10–15 min, washed on water for 1–2 min, and dried. Since ethyl cellulose is insoluble in water, the sections remained embedded during the staining. The grids were examined in a Philips EM-300 electron microscope.

RESULTS AND DISCUSSION

Cellular structures in ultrathin frozen sections will be largely destroyed by surface tension, if the sections are simply air-

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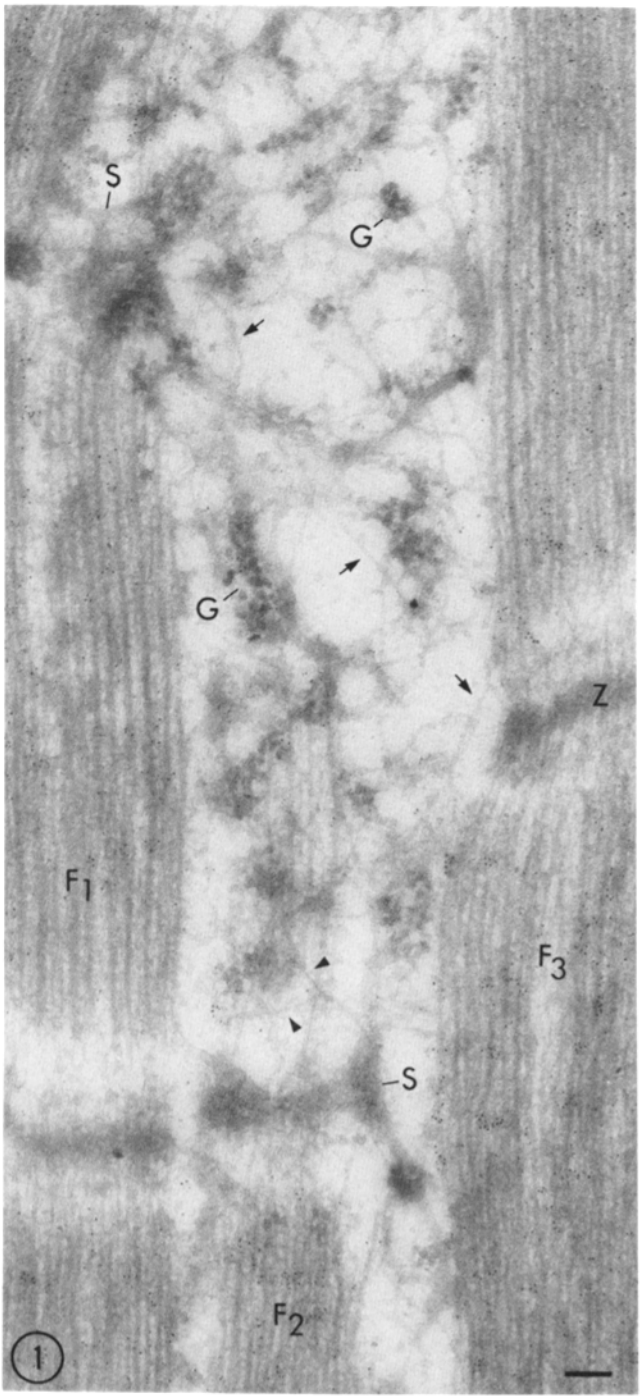

FIGURE 1 The profile of myofibril, F_2, tapers toward the mid-level, whereas the other two myofibrils, F_1 and F_3, show longitudinal profiles in this section that was immunostained for myosin. Ferritin particles are confined in the A bands of myofibrils. Networks of filaments of 10–12 nm width are found in the interfibrillar space (arrows) as well as in superposition with the tapered area of the F_2 myofibril (arrowheads). *G*, glycogen particles. *S*, sarcoplasmic reticulum. Bar, 1 μm. × 60,000.

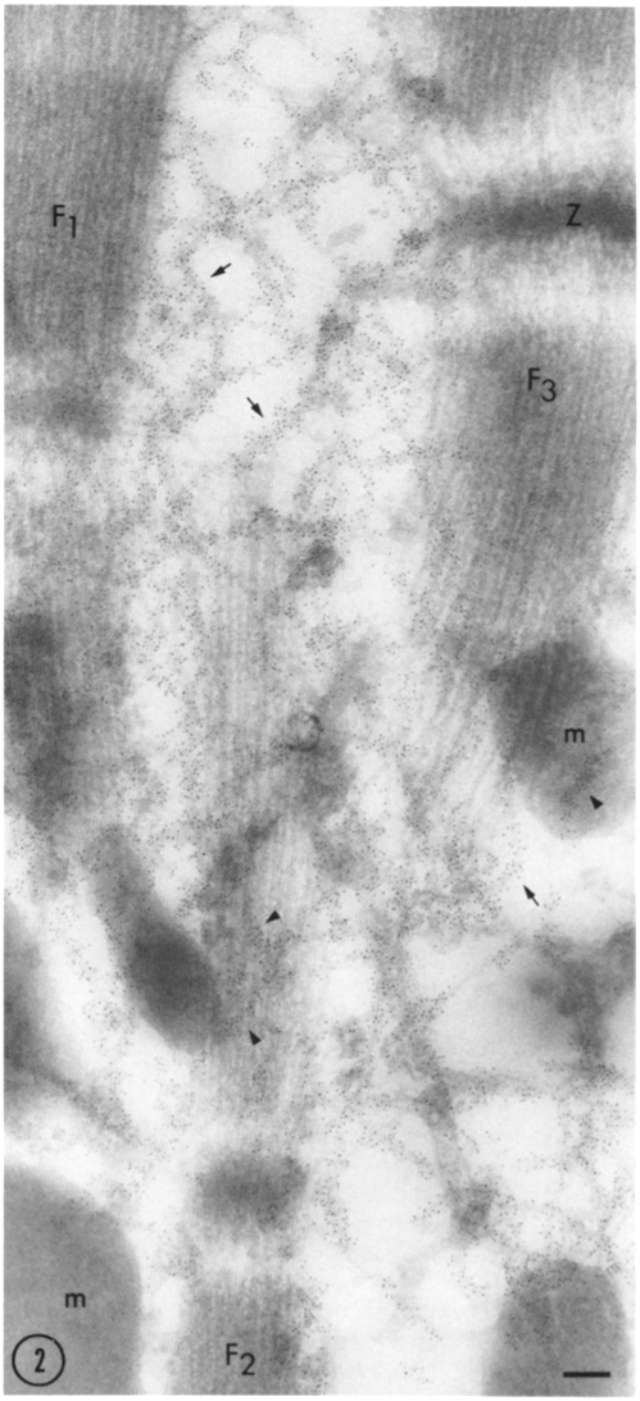

FIGURE 2 The profile of myofibril, F_2, tapers toward the top, while those of the other two myofibrils, F_1 and F_3, taper toward the bottom in this section that was immunostained for desmin. Networks of extensively labeled coarse filaments of 30–40 nm width are observed in the interfibrillar spaces (arrows) as well as in superposition with tangentially skimmed areas of mitochondria (*m*) and myofibrils (arrowheads). They resemble the networks of 10–12-nm filaments in Fig. 1 in overall configuration. *Z*, Z band. Bar, 1 μm. × 60,000.

dried. Therefore, to visualize the effects of positive staining upon the structures, it is essential to take certain measures to protect the sections against the surface tension at the time of drying. We reported earlier that such protection could be provided by embedding the section in hydrophilic polymers such as methyl cellulose and polyethylene glycol after (9) or at the time of (10) staining. These polymer embedding methods preserved membrane structures well (5, 12, 13, 14) but were not very effective for the visualization of filamentous structures.

To observe cellular structures with a definition similar to that seen in conventional plastic-embedded sections, fixation

of frozen sections with OsO_4 and dehydration with chemical solvents were considered to be necessary. The sections would then be embedded in a hydrophobic polymer and stained with aqueous staining solutions through a thin layer of the material. In our previous study (9), osmicated frozen sections were successfully embedded in nitrocellulose (parlodion) but the penetration of aqueous staining solutions was inadequate. We have now found that staining solutions do permeate into sections embedded in ethyl cellulose, which is a water-insoluble polymer but is more hydrophilic than nitrocellulose.

When frozen sections of cardiac muscle were osmicated, dehydrated in ethanol, embedded in ethyl cellulose and positively stained with acidic uranyl acetate in the embedded state, networks of filaments were recognized in wide cytoplasmic areas. In frozen sections that were immunostained for myosin, these filaments remained unlabeled (Fig. 1)[1], which indicated that they were not disarranged myosin filaments. On the other hand, the diameters of such filaments, 10–12 nm (Fig. 1), suggested that they were intermediate filaments. When the frozen sections were indirectly immunostained for desmin, the filaments were exclusively labeled and became recognizable as coarse filaments of 30–40-nm diam due to the added thickness of the antibodies and ferritin particles (Fig. 2). In sections that tangentially skimmed the surface of myofibril, longitudinally oriented filaments were seen to cover the entire length of the sarcomere (dark arrowheads in Fig. 3), whereas those filaments near the Z bands were found to be transversely oriented (white arrowheads). Abundant networks of such filaments were also observed in the cytoplasmic cones that capped the termini of the elongated nucleus of the muscle cell (not shown).

These observations indicated that there were longitudinally dispersed networks of mostly individual intermediate filaments in interfibrillar spaces, in addition to the transversely oriented networks that encircled myofibrils at the level of Z band. The greater the width of the interfibrillar space, the more abundant the longitudinal networks appeared to be. At the present time, the exact relation of these two sets of networks is not known. The courses of some of the filaments of the longitudinal networks appeared to be unaltered at the level of Z band (a pair of o-markings in Fig. 3).

Myofibrils in skeletal muscle were laterally in register across the muscle fiber but those in cardiac muscle were often out of register, sometimes as much as one half length of sarcomere (e.g., see Fig. 1 in reference 3). This seemed to be related to a basic feature of cardiac muscle: transverse segments of the intercalated disk that occurred at the locations corresponding to Z bands were considerably greater than the Z bands in overall thickness. Consequently, two neighboring myofibrils in register will become out of phase as one of them passes a transverse segment of an intercalated disk at one level until the other passes another transverse segment at a different level. This, together with the facts that myofibrils are branched in cardiac muscle and the cell volume occupied by mitochondria is far greater in cardiac muscle (7) than in skeletal muscle (2), indicate that the organization of cardiac muscle is much more complex than that of skeletal muscle. The considerable difference in the distribution of intermediate filaments between the two types of striated muscles reported here and in

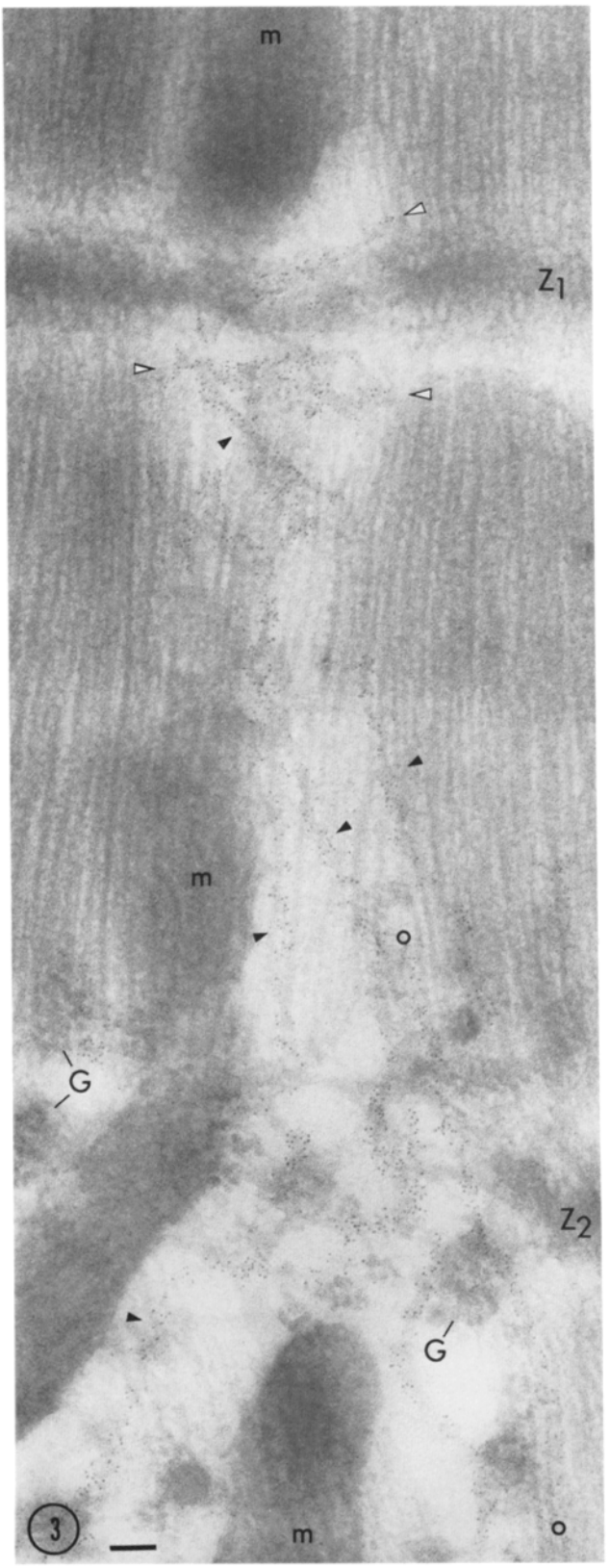

FIGURE 3 Ferritin particles indicating the presence of desmin cover coarse filaments of 30–40 nm width (arrowheads) in the area where the surfaces of myofibril and mitochondria (*m*) are tangentially skimmed. Longitudinally directed filaments cover the sarcomere (dark arrowheads), whereas transversely oriented ones are found near the level of Z_1 band (white arrowheads). One of the longitudinally directed filaments (indicated with a pair of *o*-markings) maintains its linear course through the level of Z_2 band. G, glycogen particles. Bar, 1 μm. × 60,000.

[1] All figures are electron micrographs of longitudinal ultrathin frozen sections of chicken ventricular papillary muscle, immunoferritin-stained for ventricular myosin (Fig. 1) or for desmin (Figs. 2 and 3).

our previous papers (13, 14), is believed to be closely related to the organizational difference between them.

In previous morphological studies of mammalian hearts (1, 4) intermediate filaments were reported to penetrate into myofibrils. This apparent penetration, however, could be artificially created by superposition of the filaments with myofibrils within the sections, as observed in the present figures. Another complication is that adjacent myofibrils are often very closely apposed to each other without any appreciable space between them (3). Intermediate filaments passing through such boundaries could be conceived as penetrating into myofibrils. In our past (13, 14) and present immunoelectron microscopic studies, we were not able to obtain unequivocal evidence that the intermediate filaments penetrated into myofibrils.

Recently, Wang and Ramirez-Mitchell (16) extensively extracted rabbit skeletal muscle myofibrils with KI and observed that a complex cage-like structure remained surrounding the sarcomere. We showed in our previous study of chicken skeletal muscle (13) that transversely oriented intermediate filaments surround myofibrils at the level of Z band. Longitudinally directed arrays of desmin labeling were occasionally seen to span a distance of a few sarcomeres, but it occurred only in the interfibrillar spaces where a large number of mitochondria were packed (unpublished observation). In chicken cardiac muscle, longitudinally oriented intermediate filaments were much more abundant than in skeletal muscle, as described above, but the distribution of the filaments was variable in different interfibrillar spaces as well as at different levels of Z bands. It is possible that significant differences existed between avian and mammalian species in the organization of intermediate filaments in striated muscles. It is, however, much more likely that the reported cage-like structure was composed of not only desmin, but several other protein species that have not been hitherto identified.

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