

Immunoelectron Microscopic Studies of Desmin (Skeletin) Localization and Intermediate Filament Organization in Chicken Cardiac Muscle

K. T. TOKUYASU, ANNE H. DUTTON, and S. J. SINGER

Department of Biology, University of California at San Diego, La Jolla, California 92093

ABSTRACT We studied the localization of desmin (skeletin), the major protein subunit of muscle-type intermediate filaments, in adult chicken cardiac muscle by high resolution immunoelectron microscopic labeling of ultrathin frozen sections of the intact fixed tissues. We carried out single labeling for desmin and double labeling for both desmin and either vinculin or α -actinin. In areas removed from the intercalated disk membranes, we observed desmin labeling between adjacent Z-bands in every interfibrillar space. Where these spaces were wide and contained mitochondria, convoluted strands of desmin labeling bridged between the periphery of neighboring Z-bands and the mitochondria. The intermediate filaments appeared to be organized in a more three-dimensional manner within the interfibrillar spaces of cardiac as compared to skeletal muscle. Near the intercalated disks, desmin labeling was intense within the interfibrillar spaces, but was completely segregated from the microfilament attachment sites (fascia adherens) where vinculin and α -actinin were localized. Desmin therefore appears to play no role in the attachment of microfilaments to the intercalated disk membrane. We discuss the role of intermediate filaments in the organization of cardiac and skeletal striated muscle in the light of these and other results.

Intermediate filaments are thought to contribute to the structural organization and function of striated muscle (for review, see reference 15). In the accompanying paper (23), we examined the distribution of desmin (16) (or skeletin [19]), the 55-kdalton protein which is the major subunit of muscle type intermediate filaments, in adult chicken skeletal muscle by means of high resolution immunoelectron microscopy, employing frozen sections of fixed intact tissue. In this paper, we report a similar study of desmin distribution in chicken cardiac (ventricular papillary) muscle. A previous report of immunoelectron microscopic localization of desmin in chicken cardiac muscle (Fig. 7 in reference 18) was carried out by immunoperoxidase labeling of detergent-treated and fixed tissue. No fields in the vicinity of intercalated disks were shown, perhaps because they were disrupted by the preparative treatment. In our procedures, minimal ultrastructural damage is produced (20).

Although skeletal and cardiac striated muscles show many similarities in sarcomere organization and other features, they also exhibit important structural and ultrastructural differences (4, 5, 17). The cardiac muscle fiber is constructed of many interconnected individual cells, whereas skeletal muscle fibers

are syncytia. Another difference in avian species is that transverse tubules are present in skeletal muscles, as described in our previous paper (23), but are absent in cardiac muscle (4). Furthermore, the arrangements of the myofibrils are more complex in the cardiac muscle fiber, where they merge and branch off one another, than in skeletal muscle, where the myofibrils are very long individual cylindrical units closely stacked together. To the extent that intermediate filaments play a role in the organization of the myofiber, significant differences might be expected in the ultrastructural distribution of desmin in the two muscles. Morphological observations of intermediate filaments in cardiac muscle have been reported previously (1, 6, 13), but because intermediate filaments in adult skeletal muscle have been difficult to discern morphologically, comparative ultrastructural studies of the filaments in the two tissues have been limited. In the present studies we observed similarities and also significant differences in the distributions of desmin labeling in cardiac as compared to skeletal muscle (23). These observations reflect on the function of intermediate filaments in striated muscle organization.

By immunofluorescence light microscopy, Lazarides and Hubbard (16) found labeling for desmin to be present at the

level of the Z-bands in cardiac as in skeletal muscle, and also to be highly concentrated in the regions of the intercalated disks. Largely on the basis of the latter observations, they proposed that desmin may serve to link actin filaments to the intercalated disk membrane (16). The low resolution of light microscopic observations, however, does not allow discrimination of the different domains within the intercalated disk (5), for which immunoelectron microscopic experiments are required. Our evidence is that Lazarides and Hubbard's (16) proposal is incorrect. Desmin labeling is localized to the interfibrillar spaces and is completely segregated from the region of the intercalated disk (the fascia adherens), where actin filaments terminate at the membrane, and where vinculin and α -actinin are concentrated.

MATERIALS AND METHODS

Fixation and Cryoultramicrotomy: We carried out the immunoelectron microscopic studies by single and double immunolabeling of lightly fixed ultrathin frozen sections (20). The three-stage fixation procedure described previously (22) was slightly modified in the present study. The initial fixation was carried out by perfusing 5 ml of 8% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, with 2 mM CaCl_2 into the ventricle after severing a peripheral artery of chickens. The heart was then opened and the ventricular papillary muscle was excised and cut to small pieces in the same fixative. After remaining in the fixative for 0.5 h, the pieces were treated with a mixture of 8% paraformaldehyde and 20 mM ethylacetimidate (EAI) in the same buffer for 5 min. This step was necessary to preserve the antigenicity of vinculin (12). The muscle pieces were then finally placed in a mixture of 4% paraformaldehyde and 4% glutaraldehyde in the same buffer for 1 h. For the purpose of confirming that the EAI treatment did not alter the distribution of desmin, some pieces were processed from the first step directly to the third. The fixed pieces were stored in 0.1 M phosphate buffer with 0.4% formaldehyde for up to 1 wk in a refrigerator.

Immunolabeling and Positive Staining: Desmin (16) and vinculin and α -actinin (10, 11) were isolated from chicken gizzards. Affinity-purified rabbit antibodies to desmin and guinea pig antibodies to vinculin and α -actinin were used as the primary antibodies in the indirect immunolabeling procedure. Affinity-purified and cross-absorbed goat antibodies to rabbit IgG and to guinea pig IgG were conjugated to ferritin and Imposil (iron-dextran), respectively, and used as the secondary antibodies. These preparations and procedures have been described in detail (11, 12).

Prior to immunolabeling, the thawed ultrathin frozen sections were conditioned with a 2% gelatin solution for single labeling of desmin and with a mixture of 2% gelatin and 2% dextran for double-labeling of desmin and either vinculin or α -actinin. After the completion of immunolabeling, the sections were fixed briefly with a 2% glutaraldehyde solution, thoroughly washed on water and adsorption-stained as described previously (21).

RESULTS

A single large field of a desmin-labeled ultrathin frozen section of the ventricular papillary muscle, in a region removed from the intercalated disk membranes, is shown in Fig. 1*a* and *b*. Several features of the desmin distribution are revealed in this one figure.

(*a*) Where the interfibrillar space was narrow (between Z_1 and Z'_1 , and between Z_3 and Z'_3) ferritin labeling for desmin was generally restricted to the interspace at the level of the adjacent Z-bands in a manner similar to that observed with skeletal muscle (23).

(*b*) A myofibril running diagonally from the right upper corner to the left lower corner in Fig. 1*a* was single at the level of the Z_2 -band (white arrow) but branched into two subfibrils at the level of the Z_3 -band (black arrow). The subfibrils were separated from each other even more widely at the level of the Z_4 -band, allowing for the intercalation of a mitochondrion in the interspace. Extensive desmin labeling was found in the inter-subfibrillar space at the Z_4 -level. A significant but lesser degree of labeling was also recognized at the branching point

at the Z_3 -level, but no labeling was detectable at the corresponding area where the myofibrils were merged at the Z_2 -level.

(*c*) Within wide interfibrillar spaces, there was generally a larger amount of desmin labeling than in the narrower interfibrillar spaces of cardiac muscle or skeletal muscle (23). Where mitochondria were located, the labeling at the level of the Z-bands was more broadly dispersed in the longitudinal direction than the thickness of the Z-band. Where the mitochondria were slender, the degree of label dispersion was similar to that in skeletal muscle (interfibrillar space between Z_2 and Z'_2 ; compare with Fig. 5 of reference 23). However, in wider interfibrillar spaces where large mitochondria were present, long ribbons of desmin labeling extended from the level of the Z-band to the middle region of a sarcomere (white arrowheads, in the left upper corner of Fig. 1*a* and right lower corner of Fig. 1*b* where an interfibrillar space was tangentially sectioned) and formed weblike arrays presumably representing an intertwined complex of intermediate filaments. Such ribbons often reached from the periphery of the Z-bands to the surface of the mitochondria (black arrowheads). In some instances, it appeared possible to trace a continuous ribbon of label from the level of one Z-band through the wide interfibrillar space to the Z-band one sarcomere distance removed (white arrowheads from 0 to Z''_2).

The remaining figures (Figs. 2–5) are all of areas surrounding the intercalated disk regions of the plasma membrane of the cardiac muscle cells. Figs. 2 and 3 are of sections doubly immunolabeled with ferritin-antibody conjugates for desmin and Imposil-antibody conjugates for vinculin. A complete segregation of the two labels is evident, with the desmin labeling confined to the interfibrillar spaces, and vinculin labeling confined to the fascia adherens domain of the intercalated disk where the microfilaments are attached. No vinculin labeling was found at the Z-bands (22). In Fig. 2, the Z-band and the fascia adherens appear to form a structural continuum within a single myofibril, in the same manner as was previously described in a morphological study of cat myocardium (17). It is of interest that there was no desmin labeling at the junction site between the Z-band and the fascia.

In Fig. 4, double immunolabeling for desmin and α -actinin showed again a complete segregation of the two labels, with desmin labeling confined to the interfibrillar spaces, and α -actinin labeling associated with both the fascia adherens region of the intercalated disk as well as at the Z-band.

Where two myofibrils were widely separated along the longitudinal segment of the intercalated disk, desmin labeling was observed to be longitudinally dispersed throughout the interfibrillar space, but to be particularly concentrated near the desmosomes (Fig. 5). However, the densities immediately adjacent to the desmosomes (<30 nm from the desmosome membrane) were not labeled for desmin.

DISCUSSION

Although cardiac and skeletal muscles are both striated and share a similar sarcomeric organization, the multicellular character of cardiac muscle results in structural features that are distinctly different from skeletal muscle (4, 5, 17). Where cardiac muscle cells make contact with one another, the cell membrane (the intercalated disk) has characteristic transverse and longitudinal segments. The transverse segments, where strong associations are made longitudinally to neighboring cells in the fiber, are differentiated into distinct domains, one of

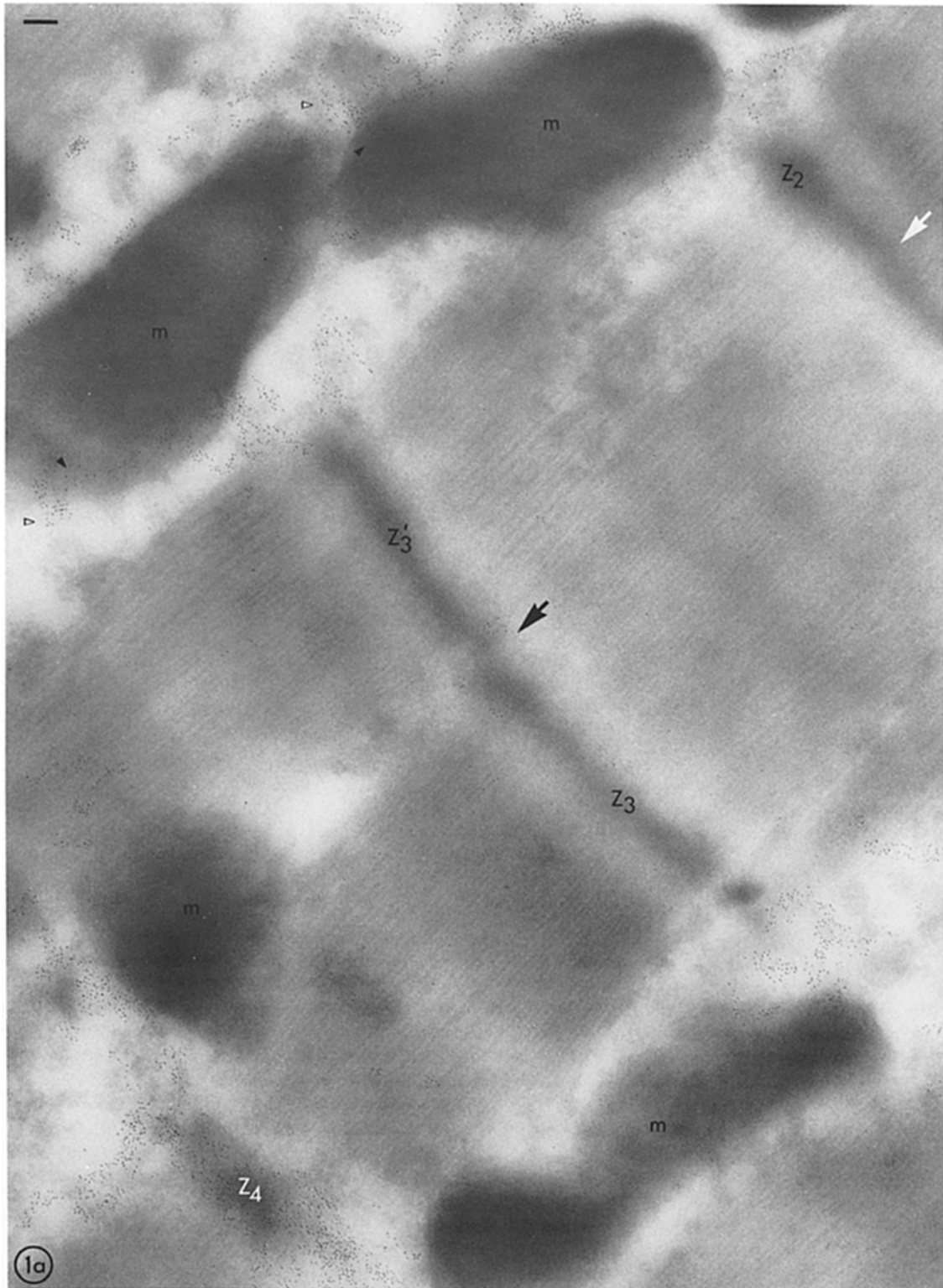


FIGURE 1 All figures are electron micrographs of longitudinal ultrathin frozen sections of chicken ventricular papillary muscle, immunolabeled singly for desmin (with ferritin conjugates in Figs. 1 and 5) or doubly for desmin and vinculin (with ferritin and Imposil conjugates, respectively, in Figs. 2 and 3) or for desmin and α -actinin (with ferritin and Imposil conjugates, respectively, in Fig. 4). Fig. 1: A single field divided in two parts, a and b. The field is segmented into five sarcomeric zones by four levels of Z-bands (Z_1 to Z_4). White Z markings (Z'_2 and Z_4) indicate those Z-bands that were tangentially skimmed at the surface, and an 0 marking at the Z_3 level indicates the expected location of a Z-band where the section included the interfibrillar space but missed the Z-band per se. In the interfibrillar space where thin mitochondria, m_1 and m_2 , are located, the labeling is more or less confined to the Z level (areas between Z_1 and Z'_1). In the interfibrillar spaces where mitochondria are wide, the labeling is much more spread longitudinally (left upper corner of a and right lower corner of b) often in the form of continuous arrays of weblike strands. They often reach the midsarcomeric areas (white arrowheads) and appear to terminate at the surfaces of mitochondria (dark arrowheads). Significant labeling is found where the branching of a myofibril occurs at the Z_3 level (large dark arrow) but not at the corresponding, nonbranching area at the Z_2 level (large white arrow). Asterisks, sarcoplasmic reticulum. *m*, mitochondria. Bar, $0.1 \mu\text{m}$. $\times 63,000$.

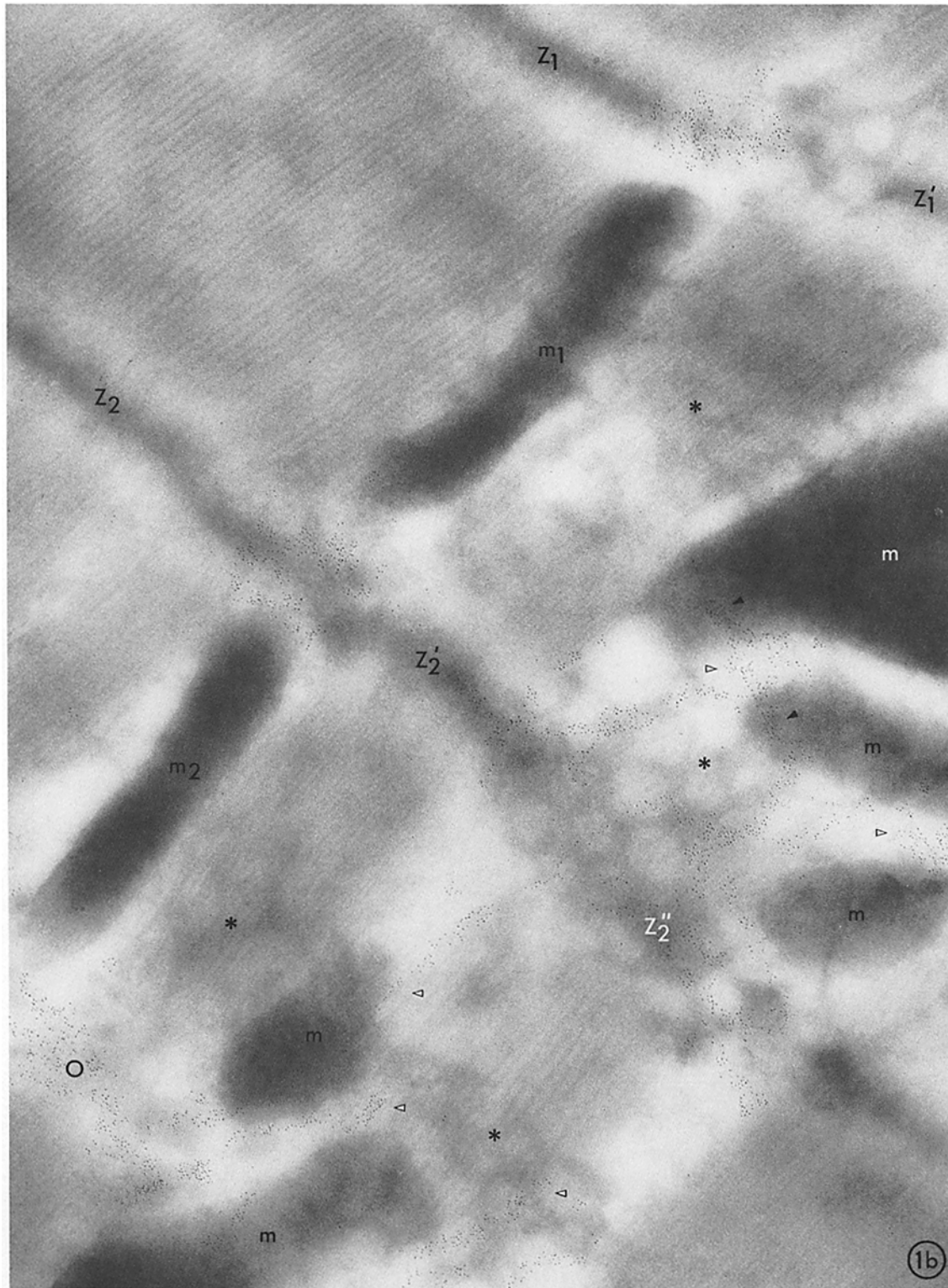


FIGURE 1 *continued*

which is the fascia adherens. Actin filaments are not only terminally attached at Z-bands, as in skeletal muscle, but also at the fascia adherens. Earlier we showed (22) by double immunoelectron microscopic labeling for vinculin and α -actinin that both proteins are present at the fascia adherens, with vinculin situated closer to the membrane at that site than α -actinin. (This steric relationship of the two proteins at the fascia adherens is also evident from a comparison of the

individual labeling experiments of Figs. 3 and 4.) Here we have demonstrated that in the region of the intercalated disk membrane, desmin is completely segregated from the fascia adherens and is localized exclusively to the interfibrillar spaces (Figs. 2-4). The double-labeling experiments show that the absence of desmin labeling near the fascia adherens cannot be ascribed to an inaccessibility of the antibody to the antigen, since either vinculin or α -actinin is labeled there in the same specimens.



FIGURE 2 The intercalated disk forming the boundary between two cardiac muscle cells changes its course from the transverse segment (white arrowhead) to the longitudinal segment (dark arrowhead). In a single myofibril demarcated by pairs of short parallel lines, a Z-band (Z, at left) is continuous with the fascia adherens (F, center) of the intercalated disk. No desmin labeling is seen at this Z-fascia junction. Extensive labeling of desmin (ferritin) is localized in the interfibrillar spaces where mitochondria (*m*) are located. Labeling of vinculin (Imposil), on the other hand, occurs only along the fascia adherens, but not on the Z-band nor in the interfibrillar spaces. Bar, 0.1 μm . $\times 72,000$.

Thus, our evidence does not support the speculation of Lazarides and Hubbard (16) that desmin mediates the attachment of actin filaments to the intercalated disk.

In the interfibrillar spaces near intercalated disks, desmin is associated with desmosomes, but at some distance (~ 30 nm) removed from the desmosomal membrane. This is consistent with immunocytochemical experiments (8) that indicate that a specific set of high molecular weight intracellular proteins is associated with the densities immediately peripheral to the membrane at the desmosomes. These proteins may mediate the lateral attachment of the desmin intermediate filaments to the desmosomal membranes.

In the interfibrillar spaces that are well removed from the intercalated disks, desmin labeling is also found, mostly in the form of linear strands associated with the periphery of each Z-band. Such linear arrays of desmin labeling are most likely due to intermediate filaments (1, 6, 13) as has been directly established for skeletal muscle in the accompanying paper (23). (See also reference 18.) However, whereas in skeletal muscle the width of a bundle of desmin strands in the longitudinal direction generally corresponds closely to the width of the Z-band, suggesting a close and specific association of the intermediate filaments with the Z-band periphery, in cardiac muscle, convoluted strands of desmin labeling splay out longitudinally into the interfibrillar spaces well beyond the width of the Z-bands (Figs. 1*a* and *b*). In the wider interfibrillar spaces, the strands of desmin labeling often appear to make contact with the mitochondria located there.

These differences in the organization of the desmin inter-

mediate filaments between cardiac and skeletal muscle very likely reflect differences in the arrangements and stacking of the myofibrils in the two striated muscle fibers. In the skeletal muscle syncytium, the individual myofibrils are relatively uniform in diameter and are closely stacked together with their long axes parallel and with their Z-bands in register. The interfibrillar spaces are usually narrow, accommodating relatively small and slender mitochondria. In cardiac muscle, however, the myofibrils are not individual structures over long distances, but instead merge with and branch from one another along the length of the fiber. The interfibrillar spaces are irregular in size, narrow where Z-bands of adjacent myofibrils are in close register, and wide where relatively large mitochondria are frequently intercalated (14). If during development of cardiac muscle this irregular organization of the myofibrils is produced before desmin becomes extensively associated with the periphery of the Z-bands, as in the case of cultured skeletal muscle myotubes (2, 9), then it is understandable that the desmin filament organization might be correspondingly irregular. Longer filaments that were more loosely arranged would come to interconnect the peripheries of adjacent Z-bands that were separated by the larger interfibrillar spaces. Such filaments were also shown above to splay out in the longitudinal direction and reach the surfaces of mitochondria at the mid-sarcomeric area. It is even distinctly possible that some Z-bands at successive levels in the longitudinal direction are interconnected by the intermediate filaments in cardiac muscle, whereas we have no firm evidence that such connections occur in skeletal muscle (23).

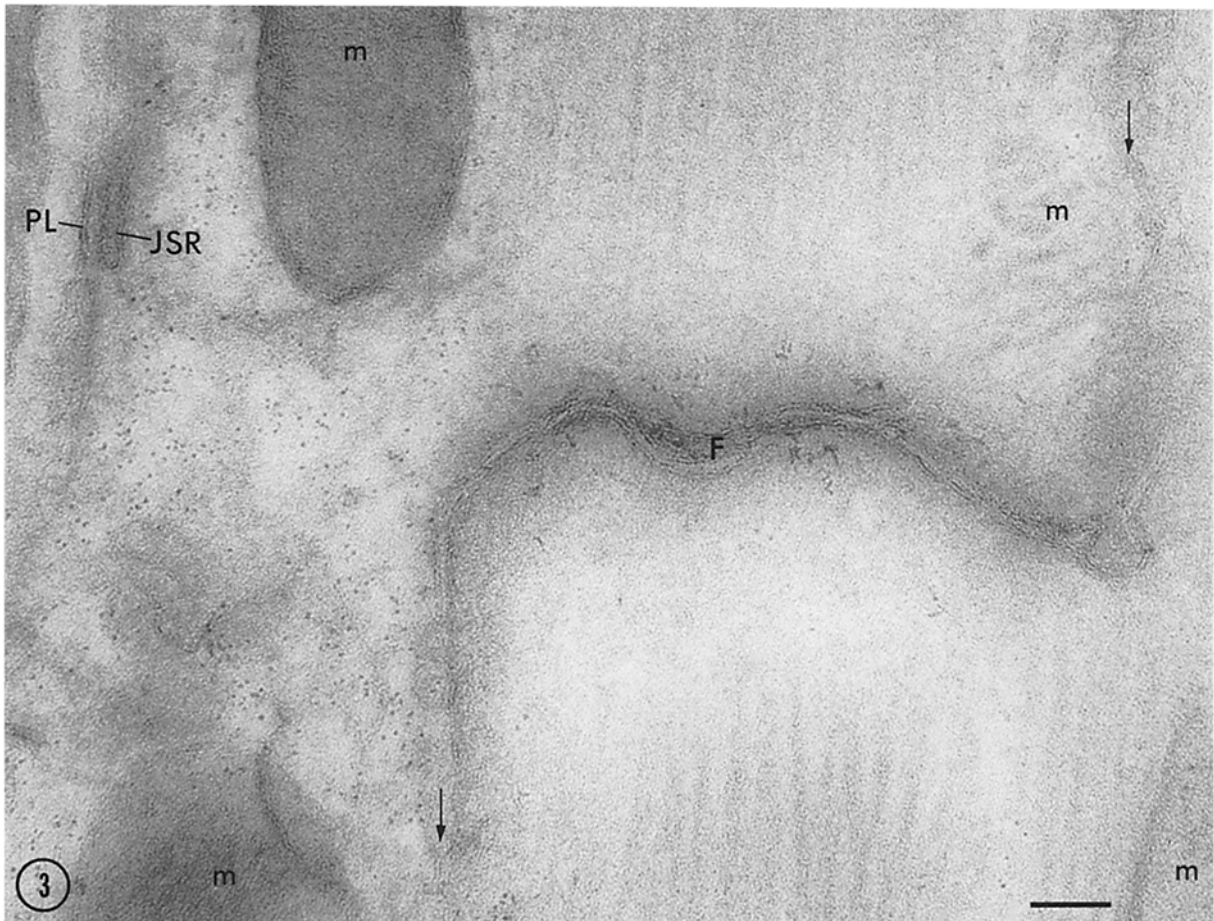


FIGURE 3 Vinculin labeling (Imposil) is localized at the fascia adherens (F), while desmin labeling (ferritin) is found exclusively outside the myofibril. Longitudinal segments of the intercalated disk are indicated with arrows. *m*, mitochondria. *PL*, plasmalemma. *JSR*, junctional sarcoplasmic reticulum. Bar, 0.1 μm . $\times 100,000$.

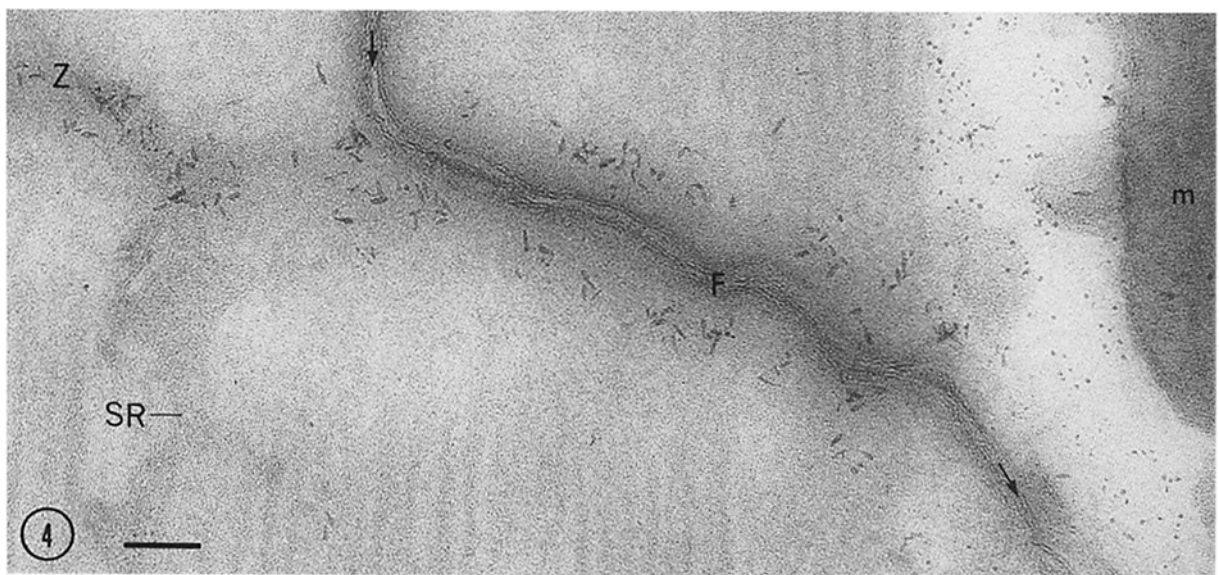


FIGURE 4 α -Actinin labeling (Imposil) is found on the fascia adherens (F) as well as on the Z-band (Z), whereas desmin labeling (ferritin) is seen exclusively outside the myofibril. Note that the Imposil particles in this figure are further removed from the plasma membrane of the fascia adherens than those in Fig. 3. Longitudinal segment of the intercalated disk is indicated by arrows. *m*, mitochondria. *SR*, sarcoplasmic reticulum. Bar, 0.1 μm . $\times 100,000$.

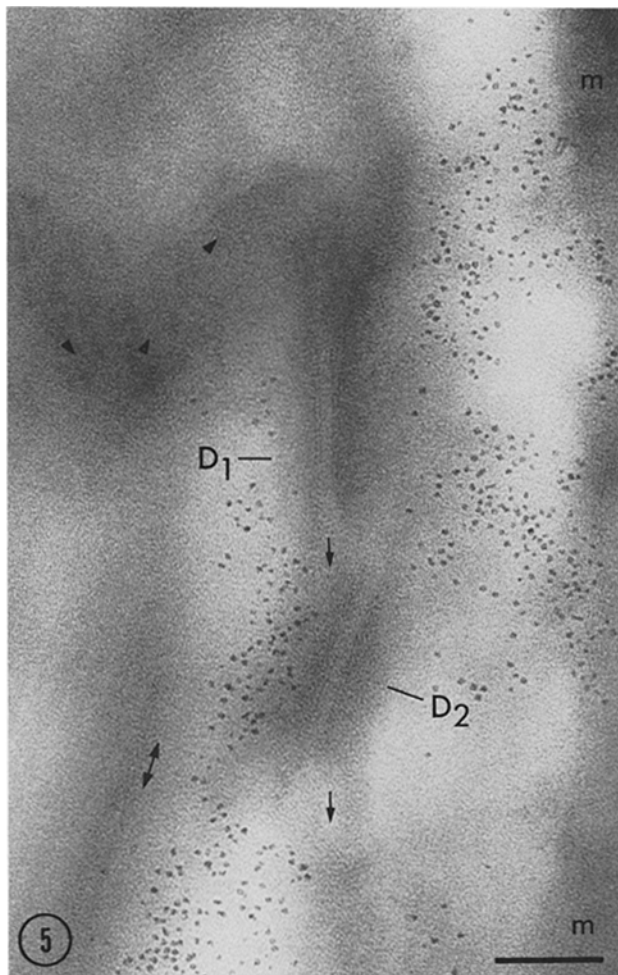


FIGURE 5 A desmosome (D_1) is found at the end of the transverse segment of an intercalated disk (arrowheads) which is not clearly defined due to the oblique sectioning. Another desmosome (D_2) is located on the longitudinal segment (single arrows). Desmin labeling (ferritin) is localized near but not on the dense plaques of the desmosomes, separated by ~ 30 nm from the membrane. A double-headed arrow indicates the direction and the lateral boundary of the myofibril on the left. *m*, mitochondria. Bar, $0.1 \mu\text{m}$. $\times 140,000$.

The convoluted appearance of desmin filaments in the wide interfibrillar spaces suggests that these filaments do not exert strong constraints on the relative positions and separations of myofibrils in cardiac muscle fibers. A similar conclusion might be derived for the case of skeletal muscle from results in the accompanying paper (Fig. 5 of reference 23). It is also noteworthy that in cardiac muscle where two myofibrils appeared to merge into one (at Z_2 level in Fig. 1*a*), and where a Z-band formed a junction with an intercalated disc within a single myofibril (Fig. 2), there was no desmin labeling. These pieces of evidence, together with the *post-hoc* association of desmin with the Z-bands in cultured skeletal myotubes (2, 9), raise questions about the precise role of desmin intermediate filaments in myofibrillar organization and function. Perhaps their role is not that of mediating myofibrillar organization during development but that of maintaining that organization against

the stresses that arise during the repeated cycles of contraction and relaxation that occur once the muscle has become functional. Other roles for the desmin intermediate filaments are suggested from the apparent association of the filaments with mitochondria mentioned above and with mitochondria and nuclei in the case of skeletal muscle (3, 6, 23). These associations may serve to maintain the positions of the mitochondria and nuclei with respect to adjacent sarcomeric units during the contraction-relaxation cycle (3, 7).

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