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

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ABSTRACT We studied the localization of desmin (skeletin), the major subunit of muscle-type intermediate filaments, by high resolution immunoelectron microscopy in adult chicken skeletal muscle. Immunoferritin labeling of ultrathin frozen sections of intact fixed sartorius muscle showed the presence of desmin between adjacent Z-bands and as strands peripheral to Z-bands, forming apparent connections between the Z-bands with adjacent sarcolemma, mitochondria, and nuclei. We observed no desmin labeling, however, in the vicinity of the T-tubules. In addition, intermediate filaments were morphologically discernible at the level of the Z-bands in plastic sections of glycerol-extracted muscle that had been infused with unlabeled antidesmin antibodies. Our results indicate that the desmin present in adult skeletal muscle, that had previously been detected by immunofluorescence light microscopy, is largely if not entirely in the form of intermediate filaments. The results provide evidence that these filaments serve to interconnect myofibrils at the level of their Z-bands, and to connect Z-bands with other specific structures and organelles in the myotube, but not with the T-tubule system.

The structure and function of the intermediate filaments of striated muscle have been the subjects of increasing interest in recent years, since it was first shown by Ishikawa et al. (10, 11) that a distinct set of 10-nm filaments different from the actin microfilaments was present in developing skeletal muscle. The main protein subunit of muscle type intermediate filaments was isolated from smooth muscle independently by Lazarides and Hubbard (16), who called the 55-kdalton protein desmin, and by Small and Sobieszek (21), who called it skeletin. (We use the former designation here.) In adult skeletal muscle, intermediate filaments are less abundant than in embryonic muscle or than in cardiac or smooth muscle. Two approaches have been used to detect their presence in skeletal muscle. One approach has been morphological, by transmission electron microscopy of the intact tissue. It has been difficult, however, to discern 10-nm intermediate filaments in adult skeletal muscle, although Page (19) did describe a network of filaments encircling the myofibrils at the level of the Z-band in chicken anterior latissimus dorsi muscle. The other approach has been immunofluorescence light microscopy, using antibodies specific for chicken gizzard desmin. With partially extracted and sheared adult skeletal muscle fibers, Lazarides and co-workers (9, 15–17) showed that desmin was present at the periphery of each myofibril at the level of, and surrounding, each Z-band. The resolution of immunofluorescence microscopy is limited, however, and it is impossible to conclude from these results anything about the filamentous state of the desmin, or the detailed ultrastructural relationship of desmin to the Z-band and to other structures in the muscle fiber. These matters are important to understanding the functions of intermediate filaments in skeletal muscle. It has been shown (2, 6) that during myotube development in culture, there is a drastic reorganization of desmin from filamentous strands that are longitudinally distributed in the cell to a highly localized distribution around the Z-bands. Because of this reorganization, and the inability to detect intermediate filaments unequivocally in the mature myotubes, the question has been raised (2) whether in the mature myotube the desmin might be present in a molecular rather than a filamentous form.

To investigate these and related problems further, we carried out electron microscopic immunolabeling of desmin in intact adult chicken skeletal muscle. The much higher resolution of immunoelectron microscopy than of immunofluorescence light microscopy has permitted the distribution of desmin to be determined with greater definition than has hitherto been possible. For this purpose, we used the techniques for the immunolabeling of ultrathin frozen sections of lightly fixed intact tissue developed in this laboratory (23, 26). With this

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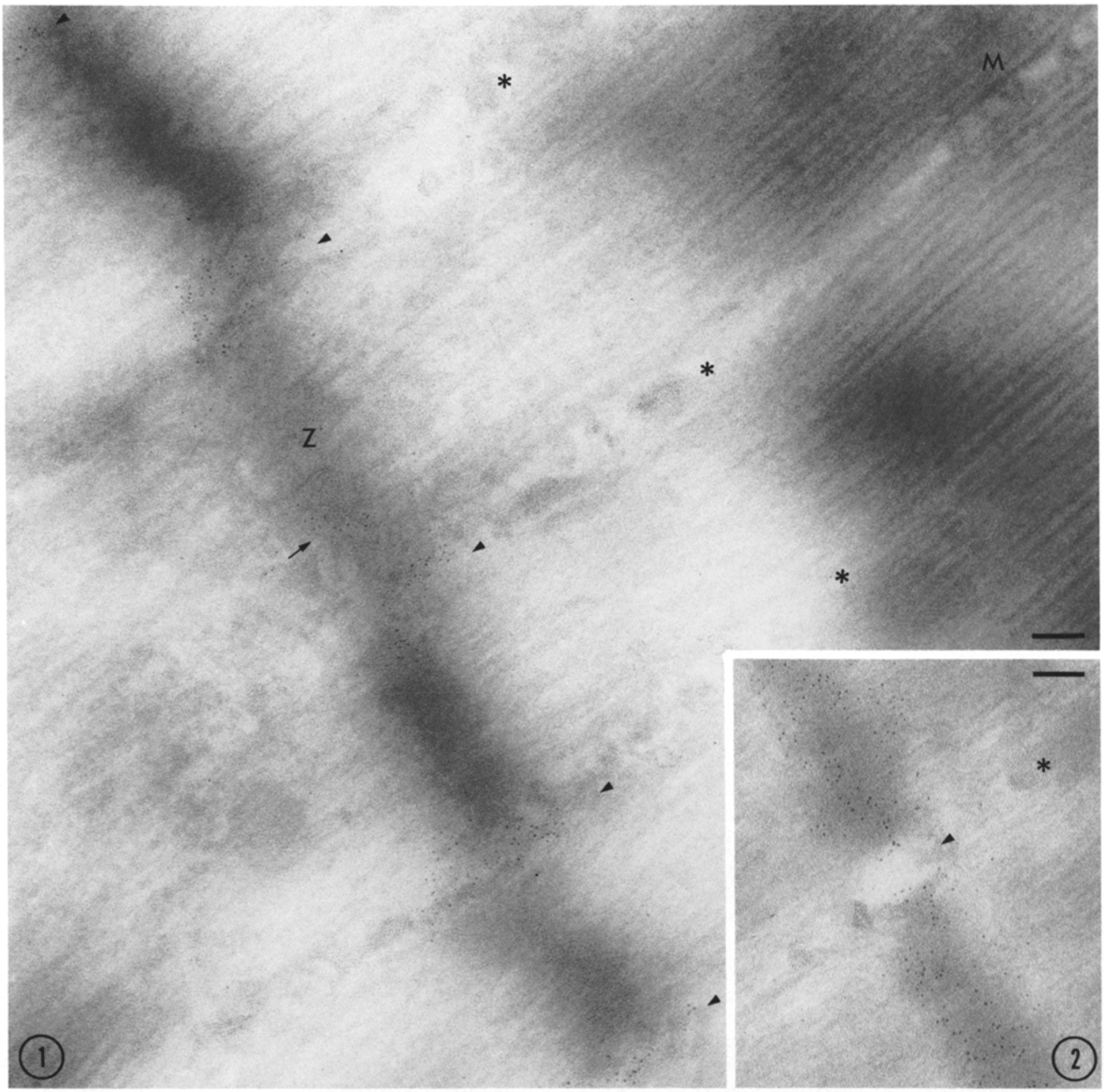


FIGURES 1 and 2 All figures are electron micrographs of longitudinal or slightly oblique ultrathin frozen (Figs. 1–7) or plastic (Figs. 8–10) sections of chicken sartorius muscle. Figs. 1 and 2: frozen sections, immunoferritin-stained for desmin (Fig. 1) or α-actinin (Fig. 2). In Fig. 1, ferritin particles representing desmin are localized in narrow areas (arrowheads) between Z-bands (one of them indicated with *Z*), and not at any other levels of interfibrillar spaces (asterisks). By contrast, in Fig. 2, ferritin particles representing α-actinin are found only on Z-bands and nowhere else, neither in the inter-Z-band area (arrowhead) nor at other levels of interfibrillar space (asterisk). In Fig. 1 a group of ferritin particles indicated with an arrow is believed to signify the presence of desmin on the *surface* of the Z-band, rather than background labeling: the fact that this particular Z-band is appreciably less dense than other bands suggests that the section includes the surface of the band and the interfibrillar space. M lines are not well defined in this particular field, but the midlevel of the sarcomere is indicated with an *M*. Bars, 0.1 μm. × 80,000.

methodology, the cellular and tissue ultrastructure in the ultrathin frozen section is highly preserved. Additional experiments were carried out with glycerinated muscle in plastic sections. After the work described in this paper was initiated (25), the studies of Richardson et al. (20) were reported on the immunoperoxidase-labeling of desmin in separated and demembranated myofibers from chicken skeletal and cardiac muscles.

In the accompanying paper (27), we present a detailed immunoelectron microscopic study of desmin localization in chicken cardiac muscle.

MATERIALS AND METHODS

Immunochemical Reagents

Affinity-purified primary rabbit antibodies to chicken gizzard desmin and α-actinin, affinity-purified secondary goat antibodies to rabbit IgG, and ferritin-conjugates of the secondary goat antibodies were all made and characterized as previously described (7, 8, 26).

Specimens and Immunoelectron Microscopy

ULTRATHIN FROZEN SECTIONS: The sartorius muscle of a chicken was brought to a stretched or contracted state by bending or straightening the knee. A thin slice of the muscle was slit, tied to a stick at both ends, excised, and fixed by immersion in a mixture of 3–6% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. The pieces of fixed muscle were trimmed down to cubes of 0.5 mm, infused with 0.6 M sucrose in the phosphate buffer for 30 min or longer, and then placed on a copper mounting block so that the direction of sectioning was parallel to the axis of the muscle fiber. The specimen was then rapidly frozen in liquid Freon 12 and ultrathin-sectioned at −80 to −110°C. We used occasionally 2.3 M instead of 0.6 M sucrose for infusion, in which case liquid N_2 was used for freezing the specimen. The sections were transferred, thawed, indirectly immunolabeled with ferritin-antibody, and finally adsorption stained as described (24).

PLASTIC SECTIONS: In some of the experiments, the muscle strips were first extracted with 50% glycerol in phosphate-buffered saline (PBS) with 1 mM EGTA at −20°C (17) for more than 1 mo. After washing in several changes of PBS at 4°C, the affinity-purified primary antibody to desmin was infused into the tissue pieces for 60 min at room temperature. In other similar experiments, normal rabbit IgG was used in place of the antidesmin antibody. After washing in PBS, the muscle pieces were fixed in a mixture of 3% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature, postfixed in 1% OsO_4 in the same buffer for 1 h, stained en bloc with 2% uranyl acetate for 30 min, dehydrated in a graded series of H_2O-ethanol mixtures to pure ethanol and embedded in Epon 812. Ultrathin sections were cut in a DuPont-Sorvall MT-2B ultramicrotome, and stained with uranyl acetate and lead citrate. All specimens were examined in a Philips EM-300 electron microscope.

RESULTS

In longitudinal sections cut through the interior of the myofibrils, immunoferritin labeling for desmin was confined to the small gaps between Z-bands of adjacent myofibrils (Fig. 1). A control for the desmin labeling was provided by immunolabeling a similar section for α-actinin (Fig. 2). The α-actinin labeling was confined to its known localization, the Z-band, and was absent from the inter-Z-band spaces between myofibrils where desmin labeling was found. Other sections grazing the surfaces of the myofibrils could be recognized by the

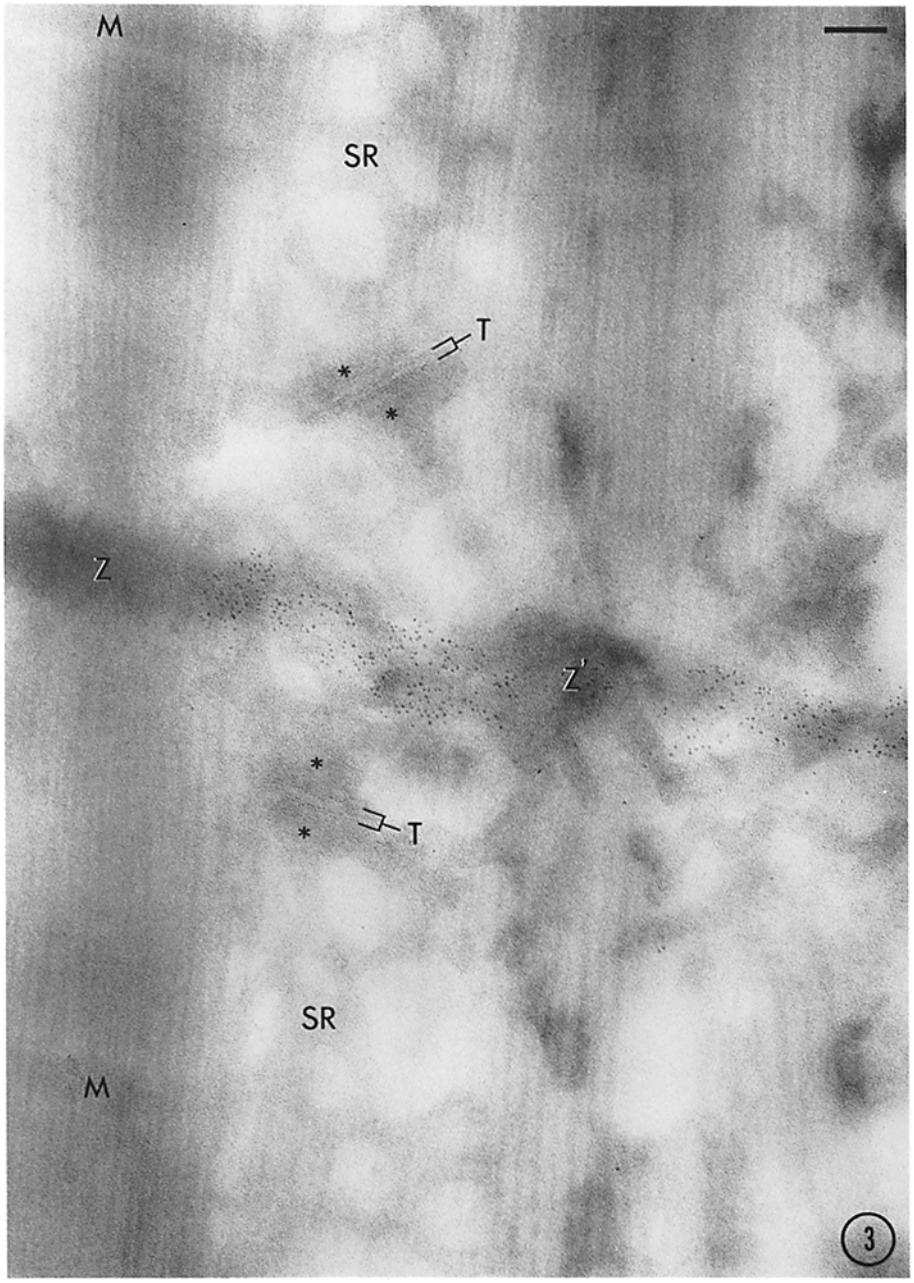


FIGURE 3 Longitudinal section of contracted leg muscle, tangentially passing through the surface of a myofibril. A Z-band of the myofibril is skimmed (*Z'*) and networks of sarcoplasmic reticulum (*SR*) surrounding the myofibril are visible. Narrow bands of ferritin particles representing desmin form a continuous belt with Z-bands of adjacent myofibrils (*Z* and *Z'*). These ferritin bands and transverse tubules (*T*) are completely separated from each other. Lateral cisternae of the triads (asterisks) are obliquely sectioned and do not show clear profiles, but they are seen to be continuous with *SR*. *M*, M-line. Bar, 0.1 μm. × 80,000.

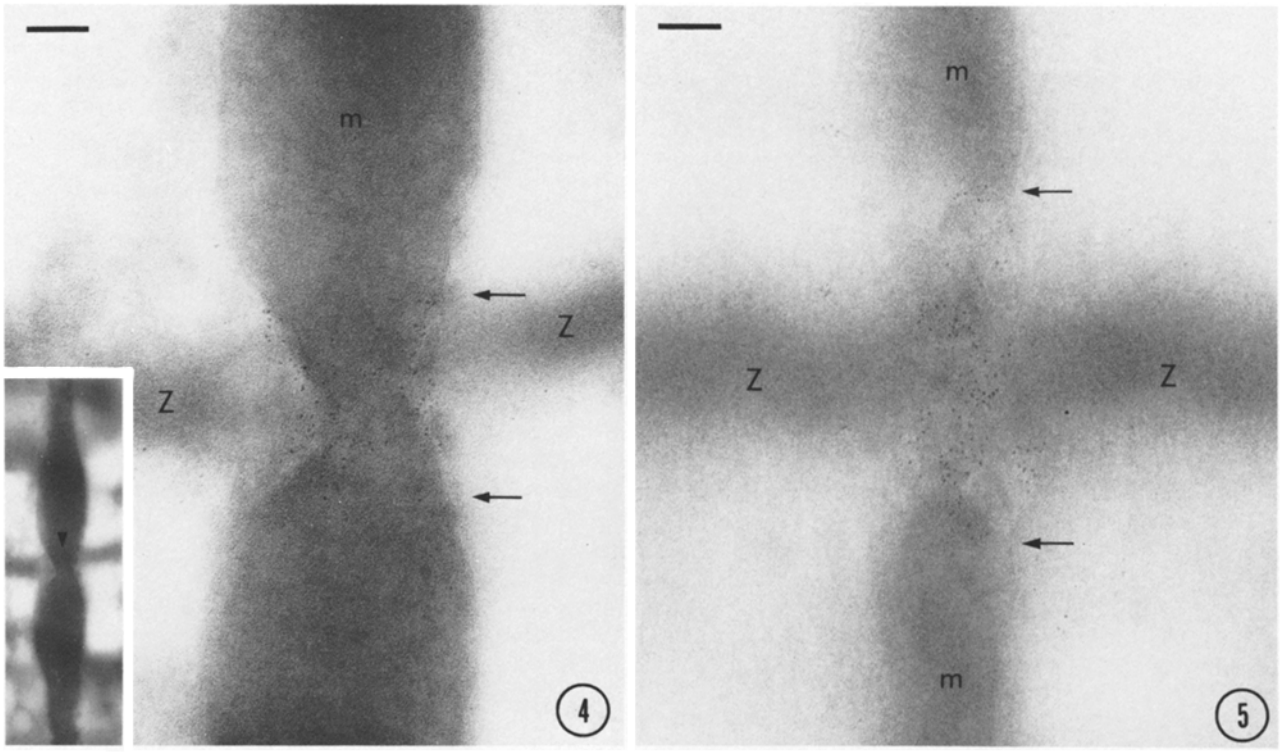
presence of the elements of the sarcoplasmic reticulum (Fig. 3). On such sections, we found desmin labeling in bands that appeared to circumscribe the myofibril at the level of the Z-band. In all such specimens, as is clear in Figs. 1 and 3, the widths of the bands of desmin labeling in the longitudinal direction were closely similar to the widths of the associated Z-bands. Furthermore, there was no indication that desmin-labeled strands crossed over the interfibrillar space between two Z-bands that were one or more steps out of register (i.e., only immediate adjacent Z-bands appeared to be linked by desmin labeling). Another feature of the surface sections was the presence of T-tubules, a pair of which we generally found symmetrically disposed and at some considerable distance from the Z-band, whether the muscle was contracted (Fig. 3) or relaxed (not shown). We never found any significant labeling for desmin within ~0.2 μm of the T-tubules. Also, the sarcoplasmic reticulum itself was generally not labeled for desmin except at the Z-band level (Fig. 3; see also Fig. 6). These results suggest that desmin is not associated with the T-tubules or the sarcoplasmic reticulum in these muscles.

It is well known that mitochondria in skeletal muscle are generally small and elongated, and are confined to the interfibrillar spaces with their long axes parallel to the axis of the muscle fiber. Mitochondria are usually symmetrically disposed across a Z-band plane, and those spanning across the plane are often constricted at the level of the plane (*inset*, Fig. 4). In a section which appears to have grazed the constricted portion of such a mitochondrion (Fig. 4), immunolabeling for desmin was found to cross the constriction between the Z-bands of adjacent myofibrils. On the other hand, where a Z-band plane separated two mitochondria (Fig. 5), we observed desmin labeling in the interfibrillar space to extend from the periphery of a Z-band to the edges of both mitochondria, well beyond the confines of the longitudinal widths of the Z-bands themselves. Longitudinally directed arrays of desmin labeling are occasionally seen to extend continuously over the distance of a few sarcomeres in the peripheral areas of the myofiber where mitochondria are densely packed (not shown).

The Z-bands of neighboring myofibrils are normally in register across the muscle fiber, as is seen in Figs. 1–5. This lateral alignment of the bands, however, can be readily disturbed during specimen preparation. In such areas, when adjacent Z-bands were longitudinally displaced by as much as 1 μm (Fig. 6), rows of desmin labeling bridged between the edges of each of the displaced Z-bands and remained confined to the interfibrillar spaces. The displaced Z-bands were still parallel to one another, as were the myofibrils themselves. In Fig. 6, the Z-band on the left side (labeled *1*) was apparently displaced from its site of association with the plasma membrane (arrowhead) which was still in register with the Z-band (labeled *5*) five myofibrils further away. A longitudinal row of desmin labeling extended between the site at the plasma membrane and the edge of the displaced Z-band 1.

The nuclei in skeletal muscle fibers are elongated structures extending across several sarcomeres. At sites where Z-bands were apposed to the nuclear envelope (Fig. 7) linear arrays of desmin labeling were seen connecting the Z-band to the envelope.

To obtain further information about the nature of the desmin-labeled structures located between Z-bands of adjacent myofibrils, glycerol-extracted muscle that was infused with unlabeled antidesmin antibodies, fixed, and then plastic-



FIGURES 4 and 5 Frozen sections, tangentially skimming the constricted portion of a mitochondrion in Fig. 4 (arrowhead in the inset of Fig. 4, a low magnification picture of the same field) and longitudinally sectioning through two individual mitochondria in Fig. 5. Ferritin particles representing desmin cover the constricted portion that is located at the level of Z-bands (*Z*) in Fig. 4, and the interspace between the two mitochondria (*m*) that are symmetrically arranged in relation to the Z-bands (*Z*) in Fig. 5. In both figures, the dispersion of ferritin particles in the longitudinal direction is appreciably greater than the width of the Z-bands (arrows). Bars, 0.1 μm. × 80,000.

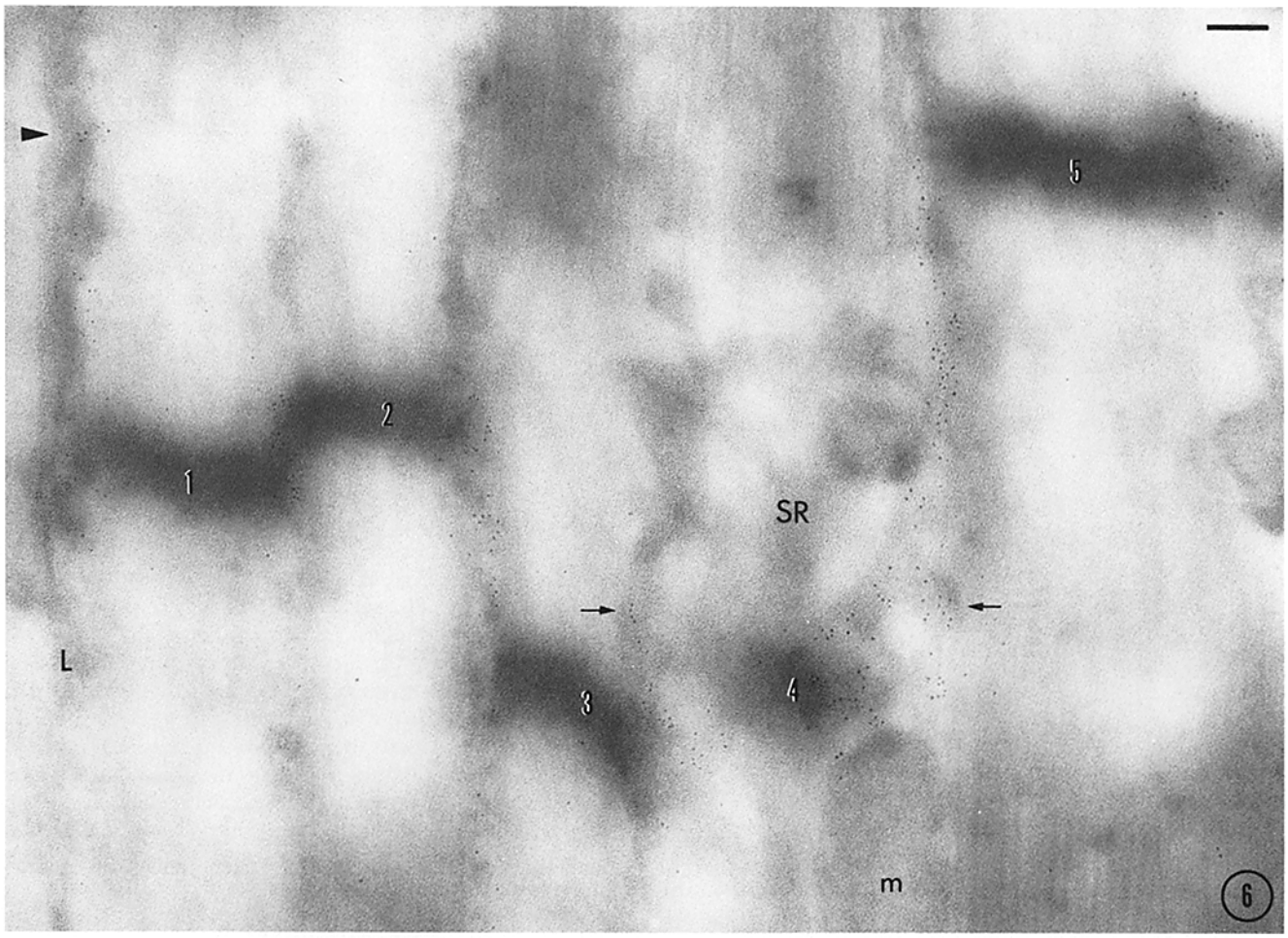


FIGURE 6 In this frozen section, displacements of Z-bands (numerals *1–5*) from the in-register position are seen. Ferritin particles representing desmin are still confined within interfibrillar spaces and form continuous strands between the bands, even when the stepwise displacement reaches nearly 1 μm (between the fourth and fifth bands). A row of ferritin particles is also recognized between a site (arrowhead) on the sarcolemma (*L*) and the first band (*1*). A network of sarcoplasmic reticulum (*SR*) is seen in the area between two arrows, which indicates that an interfibrillar space is included within the section in this area. The strand of ferritin particles from the third to the fourth and further to the fifth band is superimposed over parts of the SR network. *m*, mitochondrion. Bar, 0.1 μm. × 80,000.

embedded, was examined in thin sections. In sections skimming the surface of several neighboring myofibrils (Fig. 8), arrays of apparently continuous bundles of filaments traversed the surface (arrow at Z_1 and region between arrows at Z_2) at the level of Z-bands, the width of a bundle corresponding to the width of the Z-bands. In such sections, the filaments were apparently rendered more highly visible by the attachment of the unlabeled antidesmin antibodies, since in the absence of the specific antibodies, corresponding filaments were not unequivocally discernible (arrowheads in Fig. 9). Removal of cytosol during the glycerol extraction may also have aided the visualization of intermediate filaments. In longitudinal sections through the interior of myofibrils, cross-sections (arrowheads in Fig. 10) or oblique profiles (not shown) of 10–15-nm diameter filaments could be discerned in every interspace between adjacent Z-bands. Up to as many as 10 cross-sectioned filaments were observed in a single inter-Z-band space (Fig. 10).

DISCUSSION

From the time that intermediate filaments were recognized as a distinct class of cytoplasmic filaments, it has become increasingly clear that they serve to link together various structures and organelles inside cells (for review, see reference 15). In striated muscle, it has been suggested that the intermediate filaments play a role in the highly organized stacking of the myofibrils. However, while intermediate filaments were readily visualized by transmission electron microscopy in cardiac muscle (1, 4, 12, 22) and developing skeletal muscle (10, 13), they have generally not been clearly recognized in adult skeletal muscle (see Fig. 9, *inset*), although isolated reports of their presence have appeared (2, 19). By immunofluorescent light microscopic observations, desmin, the major protein subunit of muscle-type intermediate filaments (16, 20) was shown to be associated with the periphery of adult skeletal muscle fibers at the level of their Z-bands (9, 16). The low resolution of the method, however, could not reveal whether the desmin associated with the Z-bands was in a filamentous or nonfilamentous form. A nonfilamentous form for desmin has been raised as a distinct possibility (2). It would have some analogy to the case of another filamentous protein, actin, which under particular circumstances can be constrained to exist in nonfilamentous forms, by complexation either with other cytoplasmic proteins (for review, see reference 14) or with other peripheral proteins of the erythrocyte skeleton (18).

The immunoelectron microscopic labeling experiments de-

FIGURE 7 Ferritin particles representing desmin form a continuous row between a Z-band (*Z*) and a site (arrowhead) on the envelope of the nucleus (*N*). Bar, 0.1 μm. × 80,000.

scribed in this paper provide more definitive information on these and related matters. First of all, they establish that the desmin in adult skeletal muscle is largely, if not completely, in a filamentous state (see also reference 20). This is strongly suggested by the linear arrangements of the ferritin-antibody labels for desmin, particularly on frozen ultrathin sections grazing the surfaces of the myofibrils (Fig. 3), and on sections where neighboring Z-bands have been displaced (Fig. 6). These linear arrays are to be expected for filamentous structures. More directly, the use of unlabeled antidesmin antibodies in glycerinated muscle has clearly delineated filament structures of a diameter consistent with that of intermediate filaments partially covered with antibodies. Such filaments could be recognized on sections grazing the surfaces of myofibrils (Fig. 8) and in cross-section (Fig. 10) or in extended form in each of the interspaces between adjacent Z-bands. It is to be noted that the distribution of the ferritin-antibody labels in frozen sections is co-extensive with that of morphologically discernible intermediate filaments in plastic sections (compare Fig. 1 with Fig. 10 and Fig. 3 with Fig. 8). All of the present observations are consistent with the notions that intermediate filaments are circumferentially arranged around Z-bands, and that a continuous mesh of the filaments surrounds the bands, the mitochondria, and the nucleus at each level of Z-bands. We have seen no indications of linear connections between Z-bands formed by intermediate filaments.

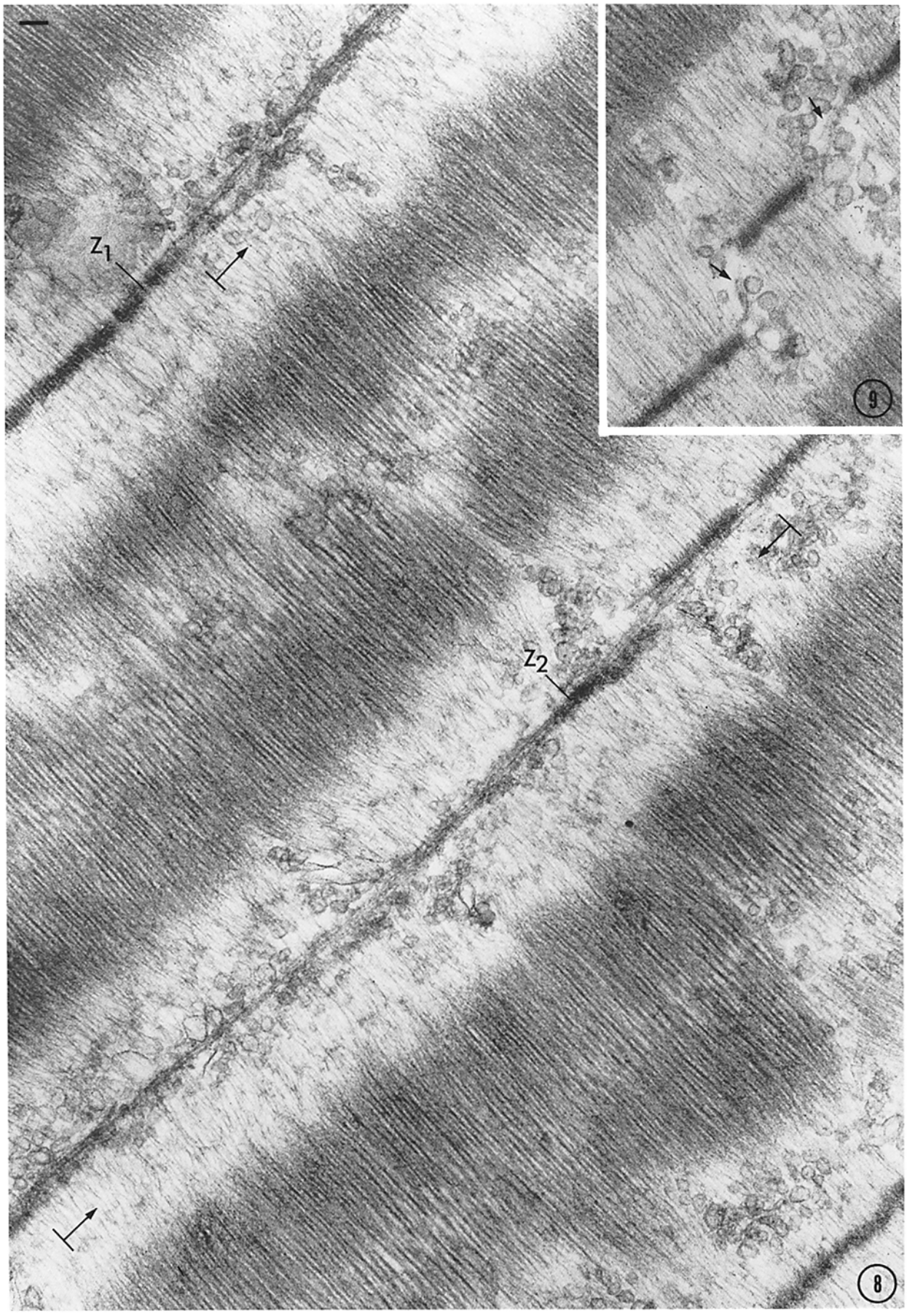
Seen in cross-section (Fig. 10), as many as 10 intermediate filaments can course through the space between adjacent Z-bands, but there is no indication that they exhibit a highly regular arrangement with respect to one another. These results and the morphological findings in Fig. 8 indicate that a band of several desmin filaments is associated with each Z-band. The longitudinal width of the filament band generally corresponds closely to the width of the associated Z-band (see, however, Fig. 5). To account for this correspondence, it seems reasonable to propose that some type of direct or indirect lateral linkage of at least some of the filaments to components at the periphery of the Z-band may be formed. Perhaps other linkages are also formed between the filaments themselves.

Such filament-Z-band or filament-filament linkages may be relatively weak and readily broken. This is suggested by the observations that adjacent Z-bands that are considerably displaced from one another remain connected by continuous strands of desmin (Fig. 6), as if a partial unravelling of the intermediate filaments from around the periphery of the Z-band occurred under the shearing stress.

Viewed in the context of our results, the finding (2, 6) that in cultures of developing myotubes the immunofluorescent labeling for desmin becomes associated with the Z-bands only some time after adjacent myofibrils have become aligned in register, raises many interesting questions. The process whereby intermediate filaments that are originally longitudinally oriented in the cytoplasm of the myotube achieve their final lateral disposition surrounding the Z-bands is obscure, but may be usefully analyzed by further immunoelectron microscopic studies of developing myotubes.

Our results also bear upon the interactions between desmin-containing intermediate filaments and other cellular structures. On the basis of desmin immunofluorescent labeling and other light microscopic experiments, Lazarides and his co-workers (9, 17) discussed the possible participation of desmin in linking Z-bands not only to one another, but also to the T-tubule system in skeletal muscle, and to the plasma membrane of the myofiber. At the resolution of the light microscope, however, such discussion was perforce speculative. At the electron microscopic level of resolution, on the other hand, it was feasible to address these and related questions directly. We found no evidence that desmin is implicated in the attachment of the T tubules to the myofibril. In chicken sartorius muscle, a pair of T-tubules is associated with each sarcomere of a myofibril, symmetrically located at some considerable distance from the

FIGURES 8 and 9 Fig. 8: plastic section of glycerinated sartorius muscle, immunostained en bloc with unlabeled antidesmin antibodies before fixation and plastic embedding. Linear profiles of intermediate filaments are clearly recognizable at the levels of Z-bands at the places where the surfaces of myofibrils are tangentially sectioned (arrow at the Z_1 level and between two arrows at the Z_2 level): vesicular structures such as are also found in interfibrillar spaces in Fig. 9 are seen along the intermediate filaments here. Note that the width of the intermediate filament bundles in the longitudinal direction is similar to that of the Z-band. Because of a slightly oblique direction of the section, boundaries of the myofibrils are not clearly discernible but from the average width of myofibrils in the vicinity (e.g., Fig. 9), it can be estimated that this figure covers more than six myofibrils at the Z_2 level. Careful inspection reveals that an apparently continuous array of intermediate filaments at the Z_2 level is discontinuous at a few points, e.g., at the point of the Z_2 marking. Fig. 9: glycerinated muscle, from which this section is derived, was infused with normal IgG instead of antidesmin antibodies. Otherwise, it was processed in the same manner as the preparation shown in Fig. 8. Careful inspection suggests the presence of filamentous structures in the inter-Z-band spaces (arrows) but they are generally not as clearly defined as those in Fig. 8. Bars, 0.1 μm. × 48,000.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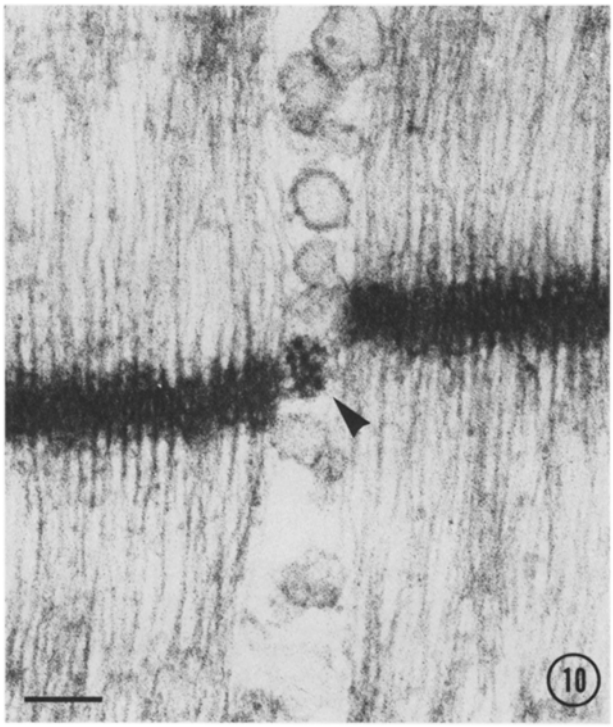

FIGURE 10 This section and the one in Fig. 8 originated from the same specimen block. Here, cross-profiles of about 10 intermediate filaments (arrowhead) are recognized in the space between Z-bands. Bar, 0.1 μm. × 100,000.

Z-band even in the contracted state (*T* in Fig. 3). Desmin labeling was uniformly absent from the vicinity of the T-tubules (Fig. 3). Similar conclusions were obtained by Richardson et al. (20). On the other hand, we have obtained evidence for some type of linkage involving desmin intermediate filaments, between the sarcolemma (arrowhead at left in Fig. 6) and the adjacent Z-band (Z_1 in Fig. 6) in a section in which the lateral register of adjacent myofibrils was disturbed. An intermediate filament connection is the likely significance of the continuous strand of desmin labeling found between these two sites.

The mitochondria of skeletal muscle, which supply the energy for its contraction, are advantageously located in the narrow spaces between adjacent myofibrils. They appear also to be regularly disposed with respect to the individual sarcomeres. We have obtained evidence that desmin intermediate filaments may determine these mitochondrial dispositions (Figs. 4 and 5). In the *inset* to Fig. 4, a mitochondrion is shown which is constricted around its middle, the constriction occurring at the level of the Z-bands of adjacent myofibrils. Around the constriction, desmin labeling was observed (Fig. 4), suggesting that the intermediate filaments may have surrounded the mitochondrion and may even have induced the constriction. Other mitochondria contained between two planes of Z-bands appear also to be in contact with desmin intermediate filaments at their edges (Fig. 5) extending from the Z-bands. That some type(s) of binding exists between the desmin filaments and specific sites appropriately disposed on the mitochondrial surface is suggested by these results.

Intermediate filaments connecting Z-bands to nuclear envelopes have been observed morphologically, particularly in cardiac muscle (1, 4). Our results (Fig. 7) indicate that the filaments that can form such connections in skeletal muscle contain desmin. These connections may be functionally important, since it is known (3, 5) that the nuclei in striated muscle cells undergo dramatic changes in conformation during the contraction-relaxation cycle correlated with changes in the sarcomere length. The nuclei shorten during contraction by infoldings in their envelopes. Desmin intermediate filament linkages between Z-bands and the nuclear envelope may play a role in these nuclear shape changes.

Our evidence suggests that there may be specific linkages formed by desmin intermediate filaments at a wide variety of different sites, including the periphery of the Z-band, the sarcolemma, the surface of mitochondria, and the nuclear envelope. If such linkages exist, their nature is entirely unknown, but it is likely that more than one type of molecular association would have to be invoked to account for the apparent diversity of linkage sites. There may well be other proteins than desmin itself that are involved in linking the intermediate filaments to these sites.

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