REDISTRIBUTION OF INTERMEDIATE
FILAMENT SUBUNITS DURING SKELETAL MYOGENESIS
AND MATURATION IN VITRO

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ABSTRACT

The distribution of intermediate filament (IF) subunits during maturation of skeletal myotubes in vitro was examined by immunofluorescence, using antibodies against two different types of chick IF subunits: (a) 58-kdalton subunits of fibroblasts (anti-58K), and (b) 55-kdalton subunits of smooth muscle (anti-55K). Anti-58K bound to a filament network in replicating presumptive myoblasts and fibroblasts, as well as in immature myotubes. The distribution in immature myotubes was in longitudinal filaments throughout the cytoplasm. With maturation, staining of myotubes by anti-58K diminished and eventually disappeared. Anti-55K selectively stained myotubes, and the fluorescence localization underwent a drastic change in distribution with maturation—from dense, longitudinal filaments in immature myotubes to a cross-striated distribution in mature myotubes that was associated with the I-Z region of myofibrils. However, the emergence of a cross-striated anti-55K pattern did not coincide temporally with the emergence of striated myofibrils, but occurred over a period of days thereafter.

KEY WORDS   intermediate filaments   ·
myogenesis   ·   muscle differentiation   ·
immunofluorescence

Reports from this laboratory and from others have demonstrated that the intermediate or 100-Å filament (IF) subunits from different cell types and organisms are not all identical, and can be distinguished immunologically and biochemically (1, 2, 9, 11a, 11b, 21, 29). We demonstrated that antibodies against the 58-kdalton chick fibroblast IF subunit (anti-58K) stained filaments in many different kinds of cultured chick cells, including fibroblasts, chondroblasts, melanocytes, neurons, glia, cardiac and smooth muscle myoblasts, and both replicating presumptive skeletal myoblasts and postmitotic myoblasts and myotubes. Contrastingly, antibodies against the 55-kdalton chick smooth muscle IF subunit (anti-55K) were highly selective and stained only definitive smooth, cardiac, and skeletal muscle cells. Replicating pre-

sumptive skeletal myoblasts were not stained, whereas postmitotic mononucleated cells did stain (8). These results suggested that postmitotic skeletal myoblasts contain a type of IF that is not present in the replicating presumptive myoblasts (2, 8).

Regardless of the type of IF subunit, immunofluorescence studies of many cell types have demonstrated the subunits to be localized to a filamentous network extending throughout the cytoplasm, corresponding to the distribution of IF seen in electron micrographs (for recent reviews see references 1, 2, 5, 6, 15, 17, 26, and 27). We demonstrated that in skeletal myotubes in vitro, both anti-55K and anti-58K bound to abundant longitudinal filaments (2, 8). This pattern of distribution of IF was consistent with the earlier electron microscope description of the 100-Å filaments in myotubes by Ishikawa et al. (19). A very different distribution of antibody to smooth muscle IF subunits (anti-"desmin") was reported in adult skele-

tal muscle by Lazarides and Hubbard (22), however, who found an association of their antibody with Z-bands of myofibrils isolated from mature skeletal muscle.

We have further examined the distribution of 55K and 58K IF subunits during maturation of myotubes in vitro, in which the localization of the antibodies to the two kinds of subunits was compared with the development of striated myofibrils. A unique sequence of events, which consists of a major reorganization of IF subunits with maturation, has now become apparent. During the early period of fusion of postmitotic myoblasts into myotubes—the first 3 or 4 d in culture—there is a rapid *de novo* appearance of 55K subunits in the form of longitudinal filaments throughout the myotubes. Subsequently, as the complement of striated myofibrils increases within the myotubes, the number of IF as discrete organelles diminishes and the distribution of the fluorescent antibody changes dramatically from longitudinally oriented filaments to that of a cross-striated pattern. Rather than coursing longitudinally and parallel to the myofibrils, the 55-kdalton protein now appears associated with the I-Z region of the myofibril. By ~10 d in vitro, most myotubes exhibit only a cross-striated distribution of 55K subunits. In contrast, 58K subunits, which exist in replicating presumptive myoblasts as well as in postmitotic myoblasts and myotubes, assume only a longitudinal filamentous distribution, never redistribute into a cross-striated pattern, and virtually disappear from myotubes by ~1 wk in vitro.

MATERIALS AND METHODS

Cultures

Skeletal myogenic cultures were prepared from dissociated 10- to 12-d embryonic chick breast muscle and plated on collagen-coated glass cover slips, as previously described (3). Some cultures were treated with cytosine arabinofuranoside (1 μg/ml) at various times after plating to reduce mononucleated cells. However, unless indicated otherwise, the results presented were obtained without mitotic inhibitors.

Antibodies

The antisera employed were all previously described, and included: Anti-55K, rabbit antiserum against the electrophoretically purified 55-kdalton subunit of reconstituted chick gizzard IF (2, 9). Anti-58K, rabbit antiserum against the 58-kdalton subunit of embryonic chick fibroblasts, electrophoretically purified from fibroblast cytoskeleton preparations (1), kindly provided by

Dr. JoAnn Otto. Anti-light meromyosin (LMM), rabbit antibody against chicken skeletal muscle light meromyosin; the IgG fraction of this antiserum was coupled to fluorescein isothiocyanate (FITC) (10, 23).

Indirect Immunofluorescence

Cultures on cover slips were fixed in −20°C acetone for 10 min, air dried, and then fixed in 2% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min followed by three rinses in PBS for 5 min each. Incubation with anti-55K or anti-58K (either the IgG fraction at 0.5–1.0 mg/ml or whole serum at 1/20 dilution, with identical results) was carried out for 1–3 h at room temperature followed by washing for an equal or greater time. Cultures were then incubated in a 1/20 dilution of FITC- or tetramethylrhodamine isothiocyanate (TRITC)-coupled goat anti-rabbit IgG (Antibodies Inc., Davis, Calif., and N. L. Cappel Laboratories Inc., Cochranville, Pa., respectively), for a similar period. PBS containing 0.5% Triton X-100 and 0.1% SDS was used for all antibody dilutions and for washing. Controls consisted of substitution of preimmune or absorbed immune serum or IgG for immune serum or IgG (2).

Double Imunofluorescence Staining

To visualize myofibrils simultaneously with anti-58K or anti-55K distribution, we doubly stained the cultures according to a procedure developed by Hynes and Destree (18). After first following the above described sequence for indirect staining by anti-55K or anti-58K, using TRITC-coupled second antibody, we then incubated the cultures in normal rabbit serum (1/5 dilution) for 2–3 h, and finally in FITC-coupled rabbit anti-LMM.

Electron Miscroscopy

Cultures were fixed with glutaraldehyde and osmium tetroxide, and embedded in Epon 812. Thin sections were examined in a JEM 100B electron microscope.

RESULTS

Between 48 and 72 h after plating, myogenic cultures contain replicating fibroblasts and presumptive myoblasts, as well as large numbers of postmitotic multinucleated myotubes. As shown in Fig. 1 a, anti-58K stains every cell in the culture dish: the various kinds of mononucleated cells and the immature postmitotic myotubes. In striking contrast, the anti-55K does not stain any of the replicating mononucleated cells (see references 2 and 8), but only stains the immature myotubes (Fig. 1 b). At this stage, the fluorescence is in the form of densely packed filaments oriented primarily longitudinally (Fig. 3 a). This corresponds to their distribution when examined under the elec-
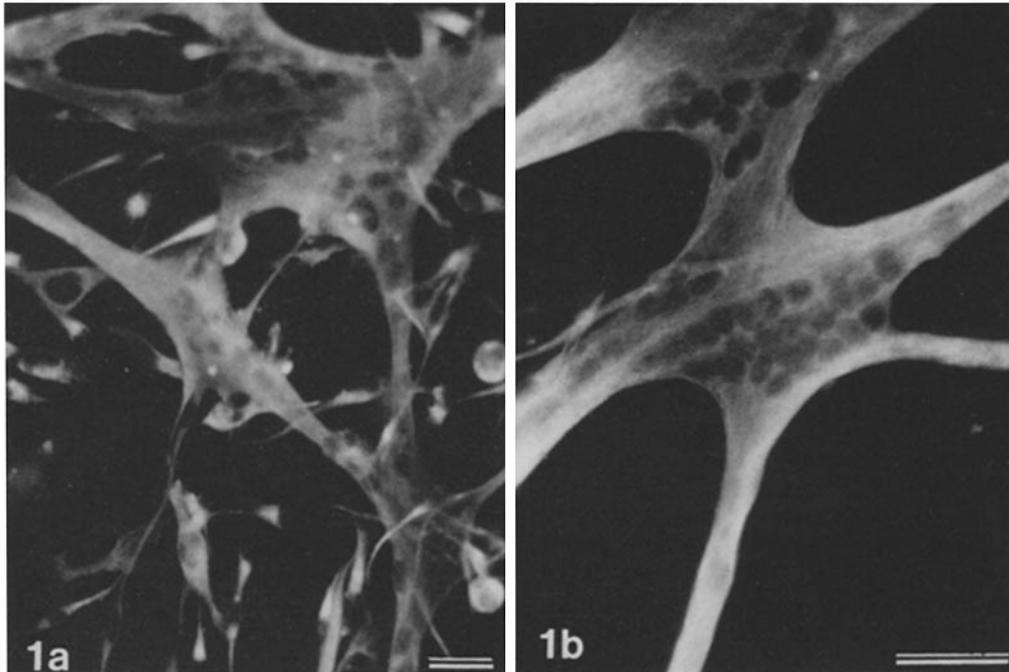
FIGURE 1 Distribution of IF subunit proteins in early skeletal myogenic cultures (3 d in vitro). Two fragments of a single cover slip were stained with anti-58K (a) or with anti-55K (b). Both fields contained similar numbers of mononucleated cells, visible in phase contrast. Anti-58K is bound to both mononucleated cells and immature myotubes (a), while anti-55K stains only myotubes, leaving the replicating mononucleated cells not visible (b). Postmitotic mononucleated myoblasts are not present in this field. Bars, 30 μm; (a) × 300; (b) × 470.

tron microscope (19). The distribution of anti-58K is similar to that of anti-55K shown in Fig. 3 a. Anti-LMM staining of the same cultures shown in Fig. 1 reveals that modest numbers of striated myofibrils are already present in these myotubes (not shown).

By 5-6 d in culture, all myotubes contain abundant striated myofibrils (Fig. 2 a), as has been demonstrated previously (10). When stained with anti-58K, ~80% of the myotubes now show extensive areas that are completely negative (Fig. 2 b). A few thin myotubes still stain in a longitudinal filamentous pattern. The only subsequent change is a progressive loss of detectable 58K subunits from virtually all myotubes until, by ~10 d in culture, no myotubes bind the anti-58K.

When stained by anti-55K, however, cultures 5–6 d in vitro begin to exhibit faint cross-striations in addition to longitudinal filaments which become increasingly segregated in bundles between myofibrils (Fig. 3 b). By 10–14 d in vitro, virtually all myotubes show prominent cross-striations with anti-55K, and longitudinal staining is for the most

part no longer evident (Fig. 3 c). The intensity of fluorescence of the cross-striations in mature myotubes is considerably less than the intensity of the longitudindally oriented IF in immature myotubes. This is clearly shown in Fig. 4 in which two myotubes showing the two extremes in configuration are present side by side.

The development of the cross-striated pattern is not coincident with the assembly of actin and myosin filaments into myofibrils, but occurs a few days later. Double staining of myotubes with anti-55K and anti-LMM reveals that in 5- to 8-d-old cultures all myotubes contain large numbers of prominent striated myofibrils. However, only 50–75% of the myotubes exhibit the cross-striated anti-55K pattern shown in Fig. 3 b. Those myotubes in which the cross-striated anti-55K pattern is not yet present nevertheless contain myofibrils. Furthermore, the reorganization does not occur simultaneously throughout the myotubes. The ends of the myotubes often contain dense longitudinal filaments when the mid-regions already exhibit only a cross-striated pattern. At these in-
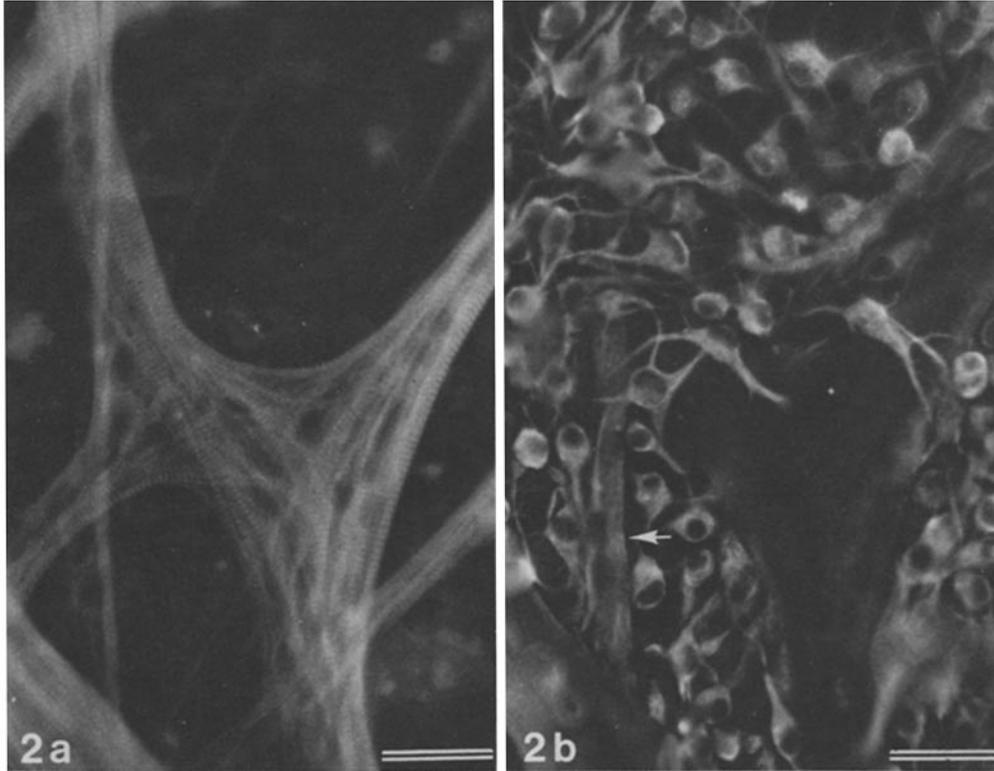
FIGURE 2 Loss of 58K IF subunits from maturing myotubes. A single portion of a myogenic culture, 5 d in vitro, was double stained to reveal both (a) striated myofibrils with FITC anti-LMM and (b) 58K IF subunits with anti-58K and TRITC anti-rabbit IgG. (a) Fluorescein fluorescence; (b) rhodamine fluorescence. In Fig. 2b, the large number of mononucleated cells are all brightly stained by anti-58K, but the large, branching myotube is negative and its presence is revealed only by the striated myofibrils stained by anti-LMM (a). Another myotube (arrow) still exhibits some anti-58K staining. Bar, 30 μm; × 470.

termediate times, there are also scattered regions along myotubes of short stretches of longitudinal staining, especially along the membrane.

Because of the thickness of the intact mature myotubes, many myofibrils are superimposed, and the exact localization of the anti-55K cross-striations with respect to sarcomere bands is difficult to determine. However, some myotubes contain relatively flat lateral regions only one myofibril in depth. When such areas are examined after double staining, it is possible to show that anti-55K staining correlates approximately with the I-Z region (Fig. 5). The uncertainty inherent in determining dimensions of fluorescent structures, and the fact that the myotubes are nearly always contracted after fixation, precludes a reliable distinction between I-band and Z-band association of the bound antibody. We noticed variation in apparent width of the fluorescent cross-striations obtained with anti-55K, but it was not possible to correlate these

variations with the degree of contraction or with the age of the culture.

To determine whether the cross-striated fluorescent anti-55K pattern corresponds to a restriction of IF to the I–Z region, a mature culture yielding a fluorescent anti-55K pattern such as that in Fig. 3c was also prepared for electron microscopy. No consistent distribution of IF appeared that would account for the antibody distribution. IF were seen under the sarcolemma, where they were oriented primarily longitudinally and spanned several sarcomeres. The regions between myofibrils were largely free of discernible IF. In a few instances, however, some suggestions of a concentration of IF in the I–Z region and intertwined among the sarcoplasmic reticulum were noticed. Such a field is shown in Fig. 6.

DISCUSSION

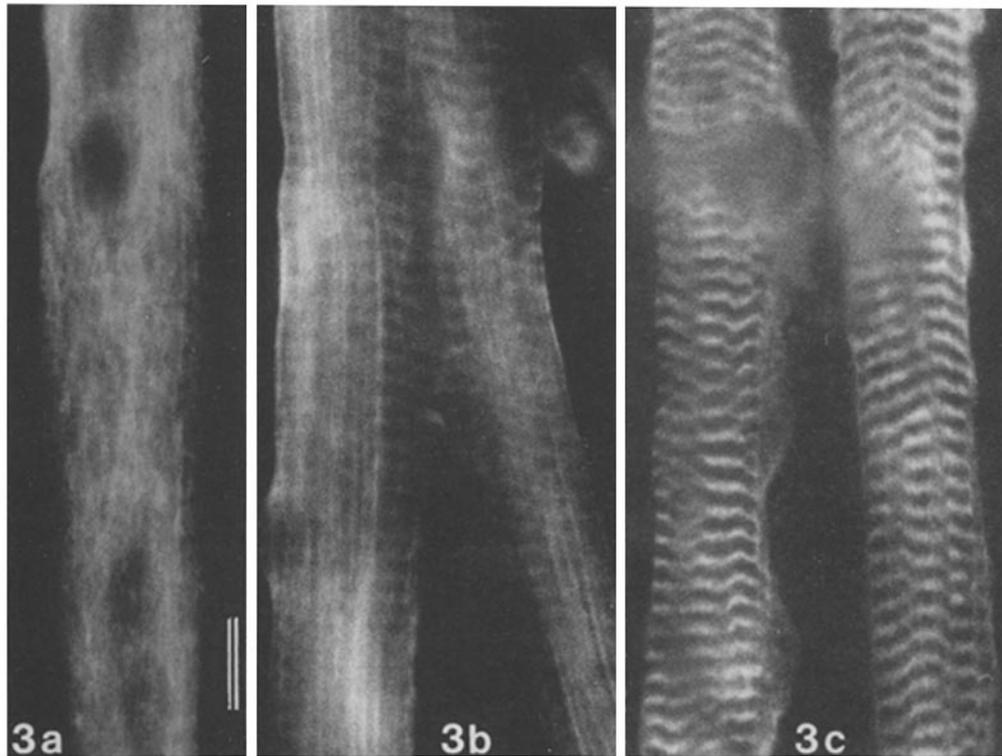The kinds of molecules synthesized by replicating

FIGURE 3   Localization of 55K IF subunits in myotubes at three stages of maturation. All cultures were stained with anti-55K. (a) Immature myotubes, 2 d in vitro; fluorescence is in fine, longitudinal filaments throughout the cytoplasm. (b) Intermediate stage, 6 d in vitro. fluorescent cross-striations have appeared, and longitudinal filaments are diminished and segregated into bundles. (c) Mature myotube, 22 d in vitro. Fluorescent cross-striations are prominent and longitudinal staining has largely disappeared. Bar, 10 μm; × 1,200.

presumptive myoblasts differ qualitatively from those synthesized by their postmitotic daughters, the mononucleated myoblasts or myotubes (for a recent review, see Holtzer [14]). This has been demonstrated for myosin heavy and light chains (4, 10, 25), actins (12, 24, 28), creatine kinase (30), and acetylcholine receptors (7). To this list can now be added the protein subunits that comprise the IF. Clearly, the replicating presumptive myo- blasts synthesize the 58-kdalton protein, whereas their daughter postmitotic myoblasts and myotubes initiate the synthesis of what is likely to be the product of another structural gene, the 55-k-dalton protein (8). In contrast to the above instances in which muscle-specific molecules progressively increase with maturation, the findings described here imply a unique developmental sequence for the 55K IF subunits. The changes in amount and distribution of antibody binding suggest that after the initial appearance of this major muscle-specific molecule, there is subsequently (a)

a diminution in its amount and (b) a change in its morphological arrangement.

Present evidence supports the interpretation that the molecules that bind anti-55K in the pattern of longitudinal IF in immature myotubes are identical to those that bind this antibody in a cross-striated pattern in mature myotubes. The antibodies were raised against the purified 55K subunit of chicken smooth muscle IF, which consists of at least two charge variants (9, 20). The data indicate that both of the major 55K components are identical in smooth and skeletal muscle and are present in the same proportions (21). Moreover, absorption of anti-55K with smooth muscle IF subunits abolishes the immunofluorescence staining of both patterns in skeletal myotubes. Therefore, it is likely that anti-55K is binding to identical determinants in the mature and immature myotubes.

The staining of the longitudinal filaments in early myotubes by anti-58K as well as by anti-55K suggests the presence of 58K subunits as well as
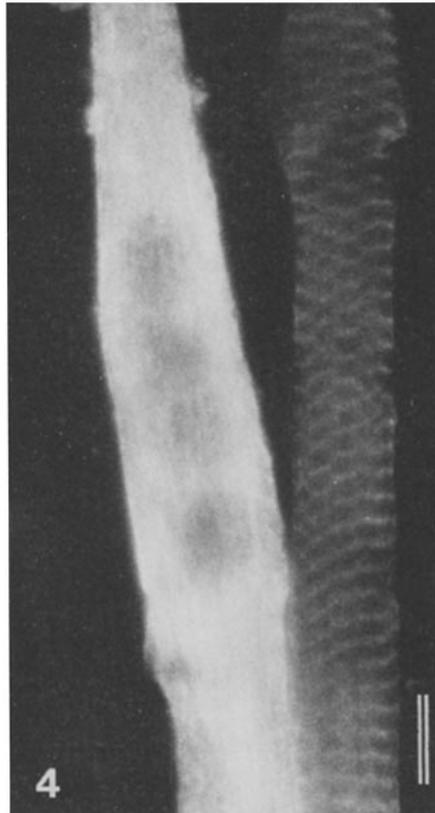
FIGURE 4 Myotubes, 13 d in vitro (treated with cytosine arabinofuranoside from day 2 to day 5), stained with anti-55K. These myotubes exhibit the two extremes in fluorescent localization of 55K IF subunits and demonstrate the difference in overall fluorescence intensity. The simultaneous presence of myotubes of such different degrees of maturation was rare in cultures not treated with mitotic inhibitors, although variation in degree of maturation of individual myotubes in a given culture is always evident. Bar, 10 μm; $\times$ 1,200.

55K subunits; therefore there either are two different sets of IF or hybrid IF composed of both types of subunits. Because the replicating presumptive myoblasts contain 58K subunits, their presence in the postmitotic myoblasts and myotubes might indicate the time required for their degradation. The observation that anti-58K staining of myotubes eventually disappears completely and never assumes a cross-striated distribution indicates that protracted synthesis of 58K subunits does not occur normally in myotubes. Immunological analysis of anti-58K indicated a weak recognition of 55K subunits by anti-58K, as evidenced by a faint precipitin band between anti-58K and purified gizzard 55K subunits (9), and by the ability of the purified 55K subunits to absorb out the staining of 58K in fibroblasts by anti-58K (1). The fact that anti-58K does not stain cross-striations in mature myofibrils suggests that cross-reaction under the immunofluorescence staining conditions used is unlikely. Similar immunodiffusion and absorption experiments demonstrate that anti-55K does not recognize 58K subunits.

These findings raise a number of questions: (a) Can the cross-striated anti-55K distribution in mature cultured myotubes be accounted for solely by the presence of IF in the I-Z region, or does a portion of the 55K protein exist in nonfilamentous form? A distinct set of filaments in this region has not been unequivocally demonstrated in sections of mature in vivo muscle. Our electron microscope examination of the cultured myotubes has also yielded equivocal evidence. However, if IF exist in a circumferential or radial orientation around the I or Z region (13), they might easily escape detection in longitudinal sections. Comparing Figs. 3 c and 6 raises the issue of the sensitivity of the labeled-antibody technique. It has been cal-
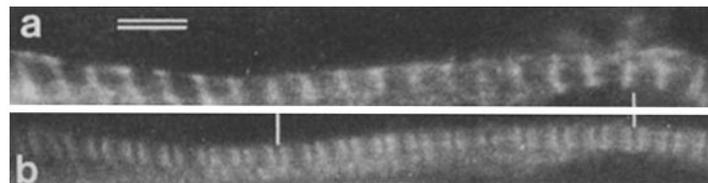
FIGURE 5 Peripheral portion of a myotube double stained with anti-55K and anti-LMM to show the position of 55K subunits relative to myofibril bands in mature myotubes. (a) TRITC fluorescence of anti-55K; (b) FITC fluorescence of anti-LMM. Vertical lines indicate the I-Z region of the myofibril in Fig. 5 b as delineated by anti-LMM binding to the lateral edges of the A band (23). The anti-55K fluorescence in Fig. 5 a is approximately aligned with the I-Z region. Small particles of debris (not included) served to align Fig. 5 a and b. Bar, 5 μm; $\times$ 1,800.
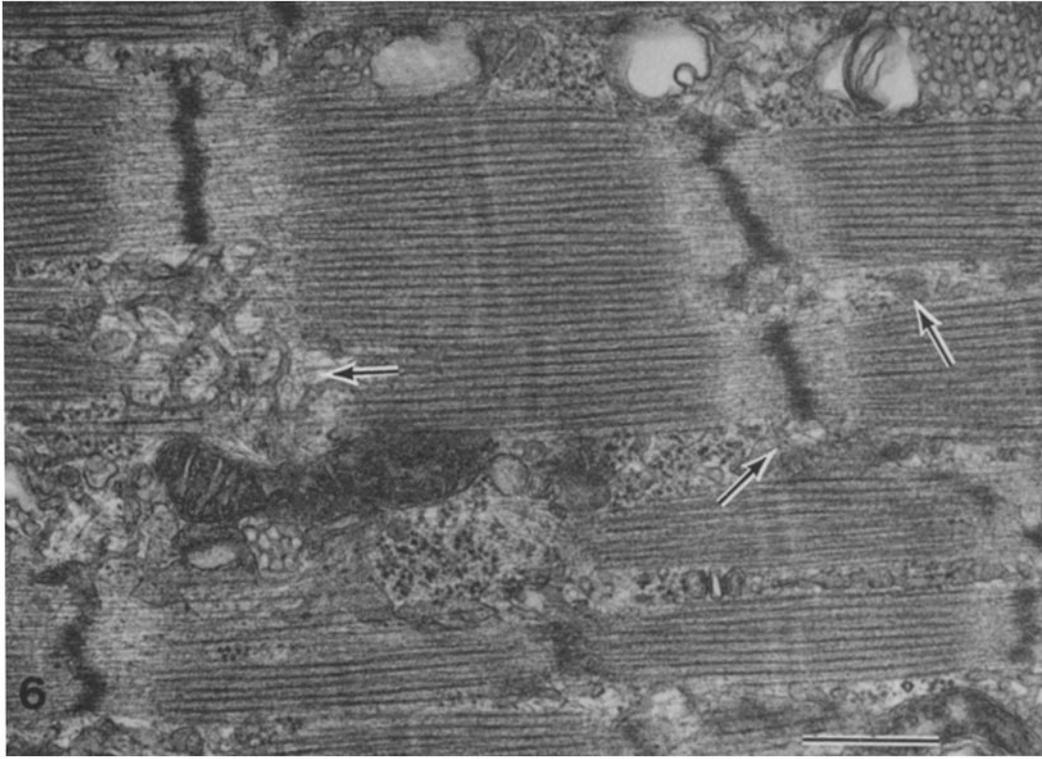
FIGURE 6   Mature myotube, 20 d in vitro, showing suggestions of IF (arrows) between myofibrils near the I-Z region and among the elements of the sarcoplasmic reticulum (left center). Such fields were seen infrequently. See also reference 20a. Bar, 0.5 μm; × 39,000.

culated (16) that 10^3 molecules of a protein could be detected by ths technique, providing the antigen was present in a locus 0.1 μm in diameter. At this time, however, we cannot with confidence state that what is observed in Fig. 6 are 100-Å filaments, and that what is observed in Fig. 3c is wholly confined to 100-Å filaments. The resolution of this question must await EM-level immunocytochemical analysis. (b) What is the mechanism by which the 55K subunits become redistributed? One possibility is that most or all of the IF present in immature myotubes become degraded, and that the 55K subunits newly synthesized at later stages are arranged differently. This redistribution would require the intervention of other molecules that either inhibit the assembly of 55K subunits into longitudinal IF, restrict their distribution, promote their assembly into an alternative form, or catalyze their degradation in a spatially selective way. Finally, although the immunofluorescence results suggest a decrease in the amount of 55-kdalton protein with maturation, it is possible that the antigenic site has become less accessible to the antibody.

The drastic reorganization of the protein subunits of which the muscle-specific IF are composed is, to our knowledge, without precedent as a major event in cytodifferentiation. Further experiments are needed to determine what the underlying mechanism may be. It is conceivable that the redistribution of IF subunits may be analogous to the reorganization of microtubules during mitosis, or to the insertion, removal, and rearrangement of molecules of the plasmalemma in different physiological states.

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