Remodeling of cytoskeleton and triads following activation of v-Src tyrosine kinase in quail myotubes

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SUMMARY

To study the cellular signals underlying the regulatory mechanisms involved in maintenance of sarcomeric integrity, we have used quail skeletal muscle cells that reach a high degree of structural maturation in vitro, and also express a temperature-sensitive mutant of the v-Src tyrosine kinase that allows the control of differentiation in a reversible manner. By immunofluorescence and electron microscopy we show that v-Src activity in myotubes leads to an extensive cellular remodeling which affects components of the sarcomeres, the cytoskeleton network and the triad junctions. We have previously shown that activation of v-Src causes a selective dismantling of the I-Z-I segments coupled to the formation of aggregates of sarcomeric actin, α-actinin and vinculin, called actin bodies. We now show that intermediate filaments do not participate in the formation of actin bodies, while talin, a component of costameres, does. The I-Z-I segments are completely dismantled within 24 hours of v-Src activity, but the A-bands persist for a longer time, implying distinct pathways for the turnover of sarcomeric subdomains. Immunofluorescence labeling of markers of the triad junctions demonstrates that the localization of the α1 subunit of the dihydropyridine receptor is disrupted earlier than that of the ryanodine receptor after tyrosine kinase activation. Furthermore, the location of junctional sarcolemmal reticulum and transverse tubule membranes is maintained in myotubes in which the I-Z-I have been removed and the regular disposition of the intermediate filaments is disrupted, supporting a role for sarcolemmal reticulum in the proper positioning of triad junctions. Altogether these results point to a tyrosine kinase signaling cascade as a mechanism for selectively destabilizing sarcomere subdomains and their tethering to the cytoskeleton and the sarcolemma.

Key words: Tyrosine kinase, Sarcomere, Triad, v-Src, Myogenesis, Transverse-tubule

INTRODUCTION

Skeletal muscle cells are characterized by highly ordered arrays of interdigitating thick and thin filaments organized in sarcomeres. The regular arrangement of the contractile apparatus is matched by that of the sarcoplasmic reticulum (SR) membrane system which serves as calcium store, and of the transverse (T) tubules, tubular membranes continuous with the sarcolemma. The SR and the T-tubule membranes are closely apposed and interact at triad junctions, sites of excitation-contraction (E-C) coupling. Molecular components of the junctions are the ryanodine receptor protein (RyR), the calcium-release channel of the SR, and the dihydropyridine receptor (DHPR), a voltage-dependent calcium-release channel associated with the T-tubules. The sarcomeres are integrated by a complex array of intermediate filaments that link the Z-discs of myofibrils to the peripheral cytoskeleton and the sarcolemma (Small et al., 1992). A specialized component of the peripheral cytoskeleton are the costameres, rib-like structures at the plasma membrane level made up of vinculin (Pardo et al., 1983) and a number of other proteins, such as talin, spectrin and dystrophin. This elaborate membrane skeleton forms connections both with the intracellular microfilament network and with the extracellular matrix (Small et al., 1992).

The expression and assembly of the contractile proteins, the integration of the contractile apparatus with the cytoskeleton and specialized membrane compartments during myofibrillogenesis, as well as the maintenance of the differentiated cellular architecture, are of key importance for the function of muscle. One approach to investigate myofibrillogenesis has been to study myogenic cells in culture since they recapitulate much of the differentiation and maturation process occurring in vivo. An additional advantage to using in vitro systems is that the process of differentiation can be modulated, allowing the study of both myofibrillar assembly and disassembly. Growth factors, phorbol esters or taxol, for example, have been used to interfere with the assembly process of myofibrils and triads (Toyama et al., 1982; Lin et al., 1987; Marks et al.,...
1991), as have retroviral oncogenes (Holtzer et al., 1975; Alemà and Tatò, 1994). In vitro transformation of replicating myogenic cells by a variety of oncogenes inhibits the ability of these cells to attain terminal differentiation. When conditional mutants are used, however, the competence of transformed myoblasts to differentiate is resumed, allowing us to address questions concerning the regulatory mechanisms involved in the induction and maintenance of the differentiated state (Gosset et al., 1988; Alemà and Tatò, 1994).

To elucidate the cellular signals that regulate the maintenance of sarcomeric myofibrils and their coordinated linkage to cytoskeleton and triad junctions, we have used myogenic cells expressing temperature-sensitive mutants of the v-src oncogene. Quail skeletal src-myoblasts exhibit transformation and block of differentiation at the permissive temperature for the tyrosine kinase encoded by the oncogene (v-Src). When shifted to the restrictive temperature, src-myoblasts promptly stop proliferating, synthesize muscle-specific products and fuse into multinucleate myotubes (Falcone et al., 1985, 1991). Activation of v-Src in myoblasts causes a marked increase in phosphorysine content and a selective reorganization of sarcomeres and cytoskeleton, most strikingly characterized by the appearance of F-actin-containing bodies (actin bodies), originating from the progressive dismantling of the I-Z-I segments (Castellani et al., 1995). This phenomenon commences in the ventral region of the myotubes shortly after activation of the tyrosine kinase and is completed within 18-24 hours. Actin bodies are made of filamentous sarcomeric actin, α-actinin and vinculin, a cytoskeletal protein associated with costameres. Indirect immunofluorescence with antibody to phosphorysine shows labeling of both forming actin bodies and assembled Z-discs (Castellani et al., 1995). In the present paper we investigate the fate of the cytoskeleton, the A-bands and the membrane systems specialized for calcium homeostasis, during and following the selective dismantling of the I-Z-I complexes operated by v-Src. By immunofluorescence and electron microscopy (EM) we show that intermediate filaments and dystrophin, a component of the subsarcolemmal cytoskeleton, do not participate in the formation of actin bodies and that fully assembled A-bands resist dismantling by v-Src-triggered biochemical events. The protein and membrane components of the triad junctions are differentially affected by v-Src activation, suggesting that the spatial configuration of membranes and protein components of the triads are determined, at least in part, by distinct mechanisms.

MATERIALS AND METHODS

Materials

Monoclonal antibodies to phosphotyrosine (PT-66), vinculin (VIN-11-5), dystrophin (MANDYS 8), titin (T11), desmin (D-1033) and FITC-phalloidin were purchased from Sigma. Lipophilic carbocyanine dye DiIC<sub>16</sub> was from Molecular Probes (Eugene, OR). The hybridomas 8e6 (talin) (Otey et al., 1990), and VN3-24 (vinculin) (Saga et al., 1985) were obtained from the Developmental Studies Hybridoma Bank. Antibody to skeletal α-actinin (mAb 9A2B8) was kindly provided by Donald Fischman (Cornell University Medical College, New York), to chicken skeletal muscle myosin (polyclonal antibody) by Susan Lowey (Brandeis University, Waltham, MA) and to desmin (rabbit serum) by Howard Holtzer (University of Pennsylvania, Philadelphia, PA). The polyclonal antibody to calsequestrin and the monoclonal antibodies to the α and β isoforms of the RyR protein (34C) and to the α1 subunit of the DHPR (311F) have been previously characterized (Airey et al., 1990, 1993). TRITC- and FITC goat anti-mouse- and anti-rabbit antibodies were from Cappel (West Chester, PA), and horseradish peroxidase-conjugated goat anti-mouse- and goat anti-rabbit antibodies from Bio-Rad (Richmond, CA).

Cell culture

Polyclonal populations of v-src-transformed quail myoblasts were established and propagated at the permissive temperature (35°C) as previously described (Falcone et al., 1991). Differentiation was induced by plating 10<sup>5</sup> cells on 35 mm collagen-coated dishes in growth medium (GM) and, the following day, by replacing GM with F14 medium, supplemented with 2% fetal calf serum (differentiation medium, DM), and shifting the cultures to 41°C (restrictive temperature) for up to 6 days. v-Src kinase was reactivated by shifting the cultures differentiated at 41°C in DM to 35°C. All experiments were carried out with cells between passages 9 and 16.

Immunofluorescence and confocal analysis

Cultures were routinely fixed for 10 minutes with 4% paraformaldehyde in PBS at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and incubated with primary and secondary antibodies and/or FITC-phalloidin at the appropriate dilution. Antibody to skeletal α-actinin (mAb 9A2B8), although tolerating parafomaldehyde fixation of the cells, gave best results when the cultures were fixed with a methanol/acetone mixture (1:1, v/v; 10 minutes at −20°C). Both primary and secondary antibodies were diluted in PBS containing 1 mg/ml bovine serum albumin and the incubations were carried out at room temperature for 1-2 hours with agitation. After a final wash, cells were incubated with Hoechst 33258 (1 μg/ml) for 5 minutes before being mounted in Gelvatol. Labeling of the cultures with the lipophilic carbocyanine dye DiIC<sub>16</sub> was carried out in vivo as previously described (Flucher et al., 1991). Briefly, the cultures were rinsed in serum-free DM and incubated for 5 minutes with 4 μg/ml DiIC<sub>16</sub>. The myotubes were then rinsed and incubated for an additional 15 minutes with serum-free DM prior to viewing. In some experiments, DiIC<sub>16</sub>-labeled cultures were fixed with 4% paraformaldehyde, permeabilized and processed for immunofluorescence. The samples were routinely examined with a Zeiss microscope equipped with ×40 and ×50 objectives. Confocal analysis was carried out with a Leica TCS 4D system, equipped with ×40 1.00-0.5 and ×100 1.3-0.6 oil immersion lenses.

Electron microscopy

Myoblasts, plated on collagen-coated Thermonox coverslips in 35 mm dishes, were differentiated at 41°C and shifted to the permissive temperature (35°C) for increasing lengths of time before fixation. All myotubes were fixed in situ and processed for embedding as previously described (Castellani et al., 1995). Cross and longitudinal sections were cut on a Reichert OMEU ultramicrotome and stained with 2% KMnO<sub>4</sub> and Sato Lead (Reedy and Reedy, 1985). Sections were photographed on a Philips EM300 on Kodak SO163 EM film.

Polyacrylamide gel electrophoresis and western blot analysis

Cells were briefly rinsed with PBS containing 0.5 mM orthovanadate and collected in a buffer made up of: 8 M urea, 0.14 M β-mercaptoethanol, 0.1% SDS, 2.5 mM EDTA, 20 mM Tris-HCl, pH 7.2. Protein concentration was determined using the Bradford colorimetric method. SDS was then brought to 2% final concentration and the samples were boiled for 5 minutes. Equal amounts of total proteins were loaded on either 10% or 3-13% gradient polyacrylamide gels. SDS-PAGE and western blots were carried out as previously described (Castellani et al., 1995).
RESULTS

Cell shape and I-Z-I complexes are altered by v-Src activity in a reversible manner

Activation of the protein tyrosine kinase v-Src in src-myotubes, obtained by shifting the cultures to the permissive temperature (35°C), causes a sustained increase in phosphotyrosine content. Cell homogenates of myotubes incubated at 35°C for various lengths of time and analysed by western blot using a specific antibody to phosphotyrosine show a large number of phosphorylated polypeptides, migrating in SDS-PAGE with an apparent molecular mass ranging from 50 to 200 kDa (Fig. 1). The total phosphotyrosine content, very low in myotubes kept at 41°C, increases rapidly after temperature shift, reaching a plateau within 8-12 hours at 35°C. While the major phosphorylated protein species differ between src-myoblasts and myotubes shifted to 35°C, the pattern of polypeptides phosphorylated by v-Src in myotubes remains unchanged, although the extent of phosphorylation increases (Fig. 1). This is confirmed by western blot analysis of cell homogenates applied to SDS-PAGE in decreasing quantities to allow for a direct comparison of the phosphorylated bands.

Marked morphological changes characterized at the gross level by widening and flattening of the myotubes, and loss of alignment and rounding up of the nuclei accompany the activation of the kinase. As illustrated in electron micrographs of cross-sections, in addition to the flattened profile, myotubes kept at 35°C are characterized by a prominent filamentous meshwork under the sarcolemma, not observed in myotubes at the restrictive temperature (41°C), and by the lack of sarcomeric myofibrils (Fig. 2). The overall cellular remodeling of the myotubes induced by v-Src activation at the permissive temperature can be reversed by returning the cultures to 41°C. Since protein components of the I-Z-I segments are the first to be influenced by the activation of v-Src (Castellani et al., 1995), we used α-actinin as a marker of sarcomere organization in myotubes differentiated for two days (2d) at 41°C, shifted to 35°C for 2 days, and then returned to 41°C for 1-2 days (Fig. 3). The regular confinement of α-actinin to the Z-discs (Fig. 3A) is fully disrupted by incubation of the myotubes at 35°C for 2 days: pronounced fiber bundles, highlighted by a punctate distribution of stain, are observed at the periphery of the cells, while small fluorescent segments, reminiscent of fragmented Z-discs, accumulate in the center (Fig. 3B). The fiber bundles observed with staining for α-actinin are also labeled by phalloidin which appears uniformly distributed along the bundles (Fig. 3C). Labeling with antibody to phosphotyrosine highlights numerous adhesion plaques at the origin of the fiber bundles (Fig. 3D), which are not visible in myotubes at 41°C or incubated at 35°C for few hours. In addition, antibody to phosphotyrosine shows pronounced staining of sarcolemmal folds in the dorsal region of the myotubes (Fig. 3D, inset), also labeled by phalloidin, but no labeling of sarcomeric structures is observed. The overall morphology of the myotubes, as well as the periodic arrangement of the Z-discs, is progressively regained after returning the cultures to 41°C (Fig. 3E,F). During the first 24 hours at the restrictive temperature the Z-line pattern becomes apparent in...
the center of the cells, while punctate fiber bundles are still observed (Fig. 3E). After 48 hours the majority of the myotubes appear cylindrical with the nuclei aligned along the longitudinal axis and the sarcomeric pattern fully regained (Fig. 3F).

The involvement of cytoskeletal components in the formation of actin bodies

Activation of v-Src in myotubes induces the formation of actin bodies resulting from the selective disassembly of the I-Z-I segments (Castellani et al., 1995). The protein components of the actin bodies derive both from the I-Z-I and from the filamentous network made up of vinculin. To establish whether other proteins of the membrane skeleton were affected by v-Src activation, myotubes were double-labeled with FITC-phalloidin and antibody to talin or to dystrophin (Fig. 4). In myotubes at 41°C staining for talin is found along the sarcolemma (not shown), consistent with the localization of this protein to the subsarcolemmal lattice (Small et al., 1992). Upon temperature shift, a marked signal deriving from talin is observed at the periphery of the phalloidin-labeled actin bodies (Fig. 4A-C), reminiscent of the cortical distribution in the actin bodies of vinculin (Castellani et al., 1995). Labeling of myotubes with an antibody to the C terminus of dystrophin shows a diffuse distribution of stain localizing at the sarcolemma with occasional ‘hot spots’, both in cultures kept at 41°C or incubated at 35°C for several hours (Fig. 4D,E). Confocal analysis of myotubes double-labeled with phalloidin and an antibody to dystrophin clearly shows that dystrophin does not participate in forming actin bodies (Fig. 4E).

To ascertain whether intermediate filaments and microtubules participate in the formation of actin bodies, the distribution of desmin and tubulin was analysed in myotubes incubated at 35°C. Immunolabeling of desmin, the main protein constituent of intermediate filaments in muscle, shows a diffuse distribution in 3 day myotubes, which becomes
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organized into a regular pattern matching the periodicity of the Z-discs upon maturation of the myotubes for 4 to 5 days at 41°C (Fig. 5A). Activation of v-Src does not significantly alter the distribution of desmin in 3 day myotubes, while it disrupts the cross-striated pattern in 5 day myotubes (Fig. 5B). Independently of the degree of organization, however, desmin is excluded from phalloidin-labeled actin bodies (Fig. 5B and inset). The overall organization of the microtubules is not influenced by the activation of v-Src for several hours (not shown).

Dismantling of the A-band is temporally distinct from that of the I-Z-I complexes

The myofibrils of src-myotubes differentiated for 3 days at the restrictive temperature display clear sarcomeric banding of myosin and of titin, indicative of fully formed A-bands. Activation of v-Src for several hours does not alter the regular arrangement of the thick filaments. Immunofluorescence analyses of myotubes kept at 35°C for six hours, in fact, show phalloidin-labeled actin bodies together with the periodic pattern revealed by an antibody to titin which recognizes an epitope in the A-I junction region (Fig. 6). Sampling by thin section EM of myotubes shifted to 35°C also shows relatively intact A-bands in myofibrils lacking I-Z-I complexes (Fig. 7). Close inspection of the A-band edges reveals a fine network of filaments, likely to represent the loose ends of titin molecules detached from the I-bands (Fig. 7C). The immunofluorescence signal from the sarcomeric pattern of myosin and titin (Fig. 8) begins to show some misalignment of myofibrils after 18-24 hours at the permissive temperature (Fig. 8C,D), followed by loss of the sarcomeric band pattern at longer incubations at 35°C (Fig. 8F). After 36 hours at 35°C only a few myotubes retain limited areas displaying a distorted band pattern due to the thick filaments. The disassembly process of the A-band proteins is not characterized by the appearance of particular structures like the actin bodies and is temporally distinct from and later than that of the I-Z-I segments.

v-Src activity diminishes muscle-specific protein accumulation

The accumulation of muscle-specific protein isoforms was analyzed concomitantly to the assembly of sarcomeres used as a marker of muscle cell maturation in vitro. Western blot analysis of cellular extracts of src-myotubes cultured at 41°C for 3 to 5 days shows that protein constituents of the contractile filaments, such as myosin and α-actinin reach steady state levels of accumulation (Fig. 9A) and become organized into sarcomeres within 3 days (Fig. 7A). In contrast, dystrophin shows an increased accumulation with myotube maturation (up
to 5 days), whereas talin and vinculin show a decrease (Fig. 9A). Myotubes allowed to differentiate at 41°C for 3-5 days also express proteins associated with the triad junctions such as calsequestrin, RyR and the α1 subunit of the DHPR, as revealed by western blot analysis with specific antibodies (Fig. 9B). Western blots probed with mAb 34C, which recognizes the α and β isoforms of the RyR proteins typically observed in avian muscle (Airey et al., 1990), shows that the relative accumulation of the two isoforms follows the same time course in myotubes kept at 41°C for 3 to 5 days. The RyR protein isoforms, calsequestrin and the α1 subunit of DHPR, while present in cellular extracts of 3 day myotubes, reach steady state levels of accumulation at 5 days (Fig. 9B), suggesting that triad junctions mature later than the contractile filaments.

Extensive incubation of src/myotubes at the permissive temperature induces a pronounced reduction in the accumulation of muscle-specific proteins. Fig. 9 shows total cell extracts of 3 day myotubes shifted to 35°C from 12 to 48 hours and probed by western blotting with antibodies to sarcomeric (α-actinin and myosin heavy chain), triadic (calsequestrin, RyR and α1 subunit of DHPR) and cytoskeletal (dystrophin, vinculin and talin) proteins. A noticeable reduction in the accumulation of all muscle-specific proteins examined is observed after 24-36 hours incubation of the cultures at 35°C, especially when compared to the accumulation observed in sister cultures kept at 41°C (Fig. 9). A similar decrease of muscle-specific proteins accumulation is observed when the myotubes are allowed to mature in culture for up to 5 days prior to activation of the tyrosine kinase (not shown). In contrast, the accumulation of vinculin and talin increases with extended incubation at 35°C.

**Time course of RyR, calsequestrin and DHPR accumulation matches sarcomere assembly**

Indirect immunofluorescence of 3 day myotubes double-labeled with antibody to RyR (mAb 34C) and to calsequestrin shows that these two proteins colocalize, giving rise to a punctate pattern of stain and to labeling of longitudinal elements (not shown). The distribution of DHPR, revealed by immunolabeling with an antibody to the α1 subunit (mAb 3F11), is spread throughout the 3 day myotubes in a finely punctate pattern (not shown). RyR, Cs and DHPR become organized in transverse rows at 5 day in culture, highlighting the mature configuration of triad junctions (Fig. 10, not shown for calsequestrin). Double-labeling of the myotubes with antibodies to RyR and to myosin shows that the rows of the RyR staining correspond to the location of the Z-discs (not shown). Occasionally, the transverse rows of the RyR protein labeling are resolved into doublets of dotted structures, indicative of the two SR terminal cisternae juxtaposed to the T-tubule (Fig. 10, not shown for calsequestrin).
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The arrangement of the T-tubule membranes was also analysed by in vivo labeling of the myotubes with the fluorescent membrane probe DiIC16 (Flucher et al., 1991). In 5 day myotubes this probe shows, in addition to staining of the plasmalemma and of the nuclear envelopes, a striated fluorescent pattern matching the periodicity of Z-discs (Fig. 10). Myotubes labeled with Dil in vivo and observed prior to fixation reveal a higher level of cross-striations than sister cultures fixed and permeabilized for immunocytochemistry with an antibody to a DHPR protein, suggesting that the T-tubule membranes, revealed by staining with the dye, reach their mature configuration before targeting of the DHPR to these structures is completed. A chemical fixation artifact is unlikely since sister cultures of 5 day myotubes, subjected to fixation and permeabilization, display periodic labeling of the RyR protein (Fig. 10). The localization of the Dil-labeled structures and of the RyR doublet in relation to the contractile filaments places the triads at the Z-lines, consistently with their localization in chick pectoralis muscle (Kidd and Yasumura, 1982; Takekura et al., 1993).

**Effect of v-Src on the organization of triad junctions**

In 3 day src-myotubes, RyR, DHPR and Cs proteins show an immature localization, at variance with the contractile filaments which are regularly organized in sarcomeres. Prolonged activation of v-Src in 3 day myotubes (up to 48 hours) alters the distribution of the triadic proteins, which appear condensed in large vesicles distributed throughout the myotube (not shown). This process is accompanied by a reduction of the fluorescence signal which matches the reduced accumulation of these

Fig. 7. Activation of v-Src induces the selective removal of I-Z-I segments, detailed by EM images of longitudinal thin sections of myotubes. (A) Differentiated myotubes show a high degree of sarcomeric ordering: A-bands, M- and Z-lines are laterally aligned and the SR cisternae (arrows) appear localized in the I-Z-I regions. (B) In myotubes shifted to 35°C for 19 hours, the I-Z-I segments are completely removed from the myofibrils, but the A-bands remain relatively intact, with prominent M-lines. The SR membranes remain localized in the region previously occupied by the Z-lines. (C) Higher magnification view of an A-band after removal of the I-Z-I segments shows no apparent thin filaments between thick filaments. A network of fine filaments (arrowheads in B and C) is instead seen at the edge of the surviving A-bands, possibly representing titin. Bars, 0.5 μm.
proteins (Fig. 9B). In order to determine whether the organization of the triads was influenced by v-Src activation prior to the reduction of their protein components, myotubes were differentiated at 41°C for 5 days before being subjected to temperature shift. Incubation of 5d myotubes at 35°C for 2-24 hours has a limited effect on the organization of the RyR protein since transversely oriented rows of stain are observed in at least 50% of the myotubes even after 24 hours of v-Src activity (Fig. 10). The pattern of immunostaining for Cs appears very similar to that of RyR (not shown). The cross-striated pattern of DiI membrane staining shows only modest alterations following v-Src activation also in myotubes with a highly flattened profile (Fig. 10). Confocal analysis of 5 day myotubes kept at 35°C for 6-12 hours and double-labeled for T-tubule membranes (DiI) and Z-discs (antibody to α-actinin) shows that the periodic pattern of DiI-labeling is maintained throughout the myotube, including the ventral region where α-actinin has migrated to the actin bodies (not shown). In contrast, the arrangement of the α1 subunit of the DHPR appears to be modified several hours after v-Src activation. The rows of the DHPR protein appear to be broadened and less defined in comparison to controls after 8 hours at 35°C (Fig. 10) and, after 12 hours, the labeling pattern reverts to that observed in immature myotubes (not shown). After 24 hours the DHPR staining is spread throughout the myotube with a dot-like appearance and occasional larger vesicular accumulations (Fig. 10).

**DISCUSSION**

Differentiated muscle cells are characterized by filaments specialized for movement and by a cytoskeletal network which permits dynamic cell adjustment to length and diameter changes during contraction. As early myofiber development proceeds, interactions between the sarcolemma and the intracellular cytoskeleton are likely to be involved in the remodeling accompanying myofiber assembly. As yet, we have little understanding of how morphogenetic changes
such as myosin (myo) and skeletal cytoskeletal proteins shows that the levels of myofibrillar proteins (A) Probing of the blots with antibodies to muscle and myotubes incubated at 35°C for increasing lengths of time (A,B, right panels). (B) Western blots monitored with antibodies to RyR protein, α1 subunit of DHPR and calsequestrin (Cs) show that these proteins increase with maturation in vitro. Note that the doublet observed with mAb 34C corresponds to the two RyR isoforms α and β. Prolonged activation of v-Src (A,B, right panels) induces a diminished accumulation of all muscle-specific proteins and augmented levels of talin, and to a lesser extent, of vinculin.

Activation of v-Src affects components of the cytoskeleton network in a differential manner

We have shown that both vinculin (Castellani et al., 1995) and talin, cytoskeletal proteins associated with costameres (Small et al., 1992), participate in the formation of actin bodies induced by v-Src. The redistribution of talin and vinculin triggered by activation of the tyrosine kinase is consistent with the observation by thin section EM of the forming of a prominent subsarcolemmal filamentous meshwork in myotubes at 35°C, indicative of cytoskeletal rearrangement. Dystrophin, shown to colocalize with vinculin in domains of the subsarcolemmal lattice (Porter et al., 1992) and to bind talin in vitro (Senter et al., 1993) is not present in the actin bodies suggesting that the dystrophin network is not significantly altered by v-Src-induced cytoskeletal rearrangement. Interestingly, these data on the different fate of dystrophin and vinculin in src-mytotubes are consistent with the previously reported finding that the distribution of vinculin is not modified by the lack of dystrophin in skeletal muscles of young mdx mice before the onset of necrosis (Massa et al., 1994).

Since intermediate filaments have been postulated to play a critical role in laterally linking Z-discs of adjacent myofibrils to one another and to costameres (Lazarides, 1982), we have also examined whether desmin participates in the formation of actin bodies. Immunolabeling of desmin is diffusely distributed in 3 day src-mytotubes which exhibit fully assembled sarcomeres, and only becomes organized into a pattern matching the periodicity of the Z-discs upon further myotube maturation. Incubation of myotubes at the permissive temperature disrupts the striated pattern of desmin concomitantly to the dismantling of the I-Z-I segments. No desmin, however, is found associated with actin bodies, suggesting either that intermediate filaments are only loosely associated with the Z-discs or that v-Src activity breaks a specific linkage between them.

**Uncoupling between the dismantling of I-Z-I segments and A-bands**

The complete disruption of the I-Z-I periodic pattern within the first 24 hours of v-Src activity precedes the disassembly of the A-bands. More specifically, is disassembly of A-bands under the control of v-Src or does it follow as a response to blocking of transcription of muscle-specific genes? Is removal of the I-Z-I segments a prerequisite for disposal of the A-bands? Our findings show that dismantling of the I-Z-I segments, resulting in the formation of actin bodies, is an early v-Src-induced event which parallels the phosphorylation of periodic structures matching the periodicity of Z-discs (Castellani et al., 1995). Western blot analysis shows that the marked increase in phosphotyrosine content induced by the kinase reaches steady state levels within 8 hours and no newly tyrosine-phosphorylated protein species are observed in myotubes kept at 35°C for longer periods, making unlikely a direct effect of v-Src on A-band proteins. The disassembly of the A-bands, instead, parallels the reduction of muscle-specific proteins observed upon activation of v-Src for up to two days. We had previously shown that the accumulation of muscle-specific mRNAs is down-regulated by the activation of v-Src for 24 hours (Falcone et al., 1991) suggesting that the temporal difference between diminished levels of muscle-specific mRNAs and proteins is due to the inhibition of accumulation, rather than acceleration of protein turnover by the kinase. The disassembly of A-bands may therefore be ascribed to both

![Image](86x461 to 263x724)
diminished protein accumulation and removal of the I-Z-I complexes, although our data do not allow us to distinguish between their relative contributions.

The concomitant disappearance of the titin and myosin band pattern after prolonged activation of the tyrosine kinase agrees well with the hypothesized binding of titin to the thick filaments (Trinick, 1994). The titin epitope recognized by the antibody used in this study maps to the edge of the A-band (Fürst et al., 1988), not allowing us to determine whether the portion of titin spanning the I-band region is disrupted during the dismantling of the I-Z-I segments. A network of fine filaments at the edge of the A-bands is, however, observed by EM, consistent with the possibility that the portion of titin spanning the I-band remains associated with the thick filaments. Nevertheless, we clearly show that the loss of periodicity of titin and myosin occurs later than that of the I-Z-I segments. These findings are in agreement with previous experiments on chick myotubes treated with TPA which show that phorbol ester treatment induces the disassembly of the I-Z-I, followed by the dissolution of the myofibrils (Lin et al., 1989). Together, these data support the view that thick filaments and I-Z-I complexes, once assembled in the sarcomere, are maintained by distinct mechanisms that can be selectively altered by v-Src-triggered cellular signals.

Components of the triad junctions are selectively affected by v-Src activation

Quail src-myotubes are characterized by fully organized sarcomeres and a developed E-C coupling system. While the contractile apparatus is completely assembled after 3 days in
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culture, junctional SR and T-tubules acquire their mature location at the Z-line at 5 days. The structural maturation of the triad junctions parallels the accumulation of the RyR protein and of the α1 subunit of DHPR which reach steady state levels after 5 days in culture. The late maturation of junctional SR and T-tubules in relation to myofilibrils is in agreement with previous studies carried out in developing murine and avian muscle (Franzini-Armstrong, 1991; Flucher et al., 1993a). Prolonged activation of v-Src in 3 day myotubes blocks the maturation of the triads, both in terms of specific protein accumulation and of cellular localization. In 5 day myotubes displaying fully organized triad junctions, v-Src activation has a limited effect on the localization of the RyR protein at junctional SR. In contrast, the localization of the α1 subunit of the DHPR is disrupted relatively soon after tyrosine kinase activation, even though its host T-tubule membranes retain a regular disposition. The different fate encountered by the DHPR and the RyR proteins upon activation of v-Src is consistent with previous findings showing that the association of DHPR and RyR proteins may not be necessary to the positioning of the triad junctions in relation to the myofilibrils. It has been shown, in fact, that RyR and triadin, a component of junction SR (Caswell et al., 1991), coaggregate in clusters and are capable of attaining mature cross-striated distributions both in normal and dysgenic mouse myotubes, that lack the α1 subunit of the DHPR (Flucher et al., 1993b). Yet unidentified membrane-cytoskeleton interactions, however, are likely to exist in order to maintain junctional SR and T-tubule membranes at specific sites along the sarcomere. Indeed short cross-links between SR and Z-discs, the nature of which remain unknown, have been observed by thin sectioning EM (Nunzi and Franzini-Armstrong, 1980), but no direct linkages between myofilibrils and T-tubule membranes have been reported. Our findings that the location of junctional SR and T-tubule membranes is maintained in myotubes in which the I-Z-I have been dismantled and the regular disposition of the intermediate filaments is disrupted suggest that either a component of the cytoskeleton, not influenced by the activity of the tyrosine kinase, or the junctional SR (Flucher et al., 1993a) is responsible for the proper positioning of triad junctions.

Conclusions

In summary, our data point to a tyrosine kinase signaling cascade as a mechanism for destabilizing sarcomeres and their attachment to the sarcolemma. They also point to cytoskeletal reorganization as a contributor to these processes and, possibly, to the remodeling of the E-C coupling membrane system. It is reasonable to assume that, similar to what is known for the actin cytoskeleton in fibroblasts and other cell types, processes such as myofilament assembly into sarcomeres and their stabilization are also under the fine control of signaling molecules like protein kinases and phosphatases. Given the association of v-Src with the membrane cytoskeletal matrix (Burridge et al., 1988), one major target of the kinase is likely to be an unidentified component of the cytoskeleton devoted to the anchorage of sarcomeres to the sarcolemma at specific sites, such as costameres.

Little is known as to how muscle cells respond to mechanical stimuli, transmit the information to second-messenger systems and finally regulate gene expression. The mechanical stress applied to the cell surface is probably transmitted to the cytoskeleton and this signal may initiate activation of second-messenger systems and protein phosphorylation. We postulate that a number of protein kinases, including tyrosine kinases, localize at specialized domains of the sarcolemma such as costameres. The ultimate consequence of putative phosphotyrosine-regulated signaling pathways originating at costameres would be to lead to the phosphorylation of key components of the cytoskeleton and, by this mechanism, modulate the ability of these proteins to associate with other molecular components, thereby regulating the cytoskeleton architecture and sarcomere turnover even at long range. Such signals may be also critical during the reshaping of the cytoskeleton and sarcomeres occurring in development and muscle remodeling.

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REFERENCES


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