Inhibition of CapZ during Myofibrillogenesis Alters Assembly of Actin Filaments

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Abstract. The actin filaments of myofibrils are highly organized; they are of a uniform length and polarity and are situated in the sarcomere in an aligned array. We hypothesized that the barbed-end actin-binding protein, CapZ, directs the process of actin filament assembly during myofibrillogenesis. We tested this hypothesis by inhibiting the actin-binding activity of CapZ in developing myotubes in culture using two different methods. First, injection of a monoclonal antibody that prevents the interaction of CapZ and actin disrupts the non-striated bundles of actin filaments formed during the early stages of myofibril formation in skeletal myotubes in culture. The antibody, when injected at concentrations lower than that required for

disrupting the actin filaments, binds at nascent Z-disks. Since the interaction of CapZ and the monoclonal antibody are mutually exclusive, this result indicates that CapZ binds nascent Z-disks independent of an interaction with actin filaments. In a second approach, expression in myotubes of a mutant form of CapZ that does not bind actin results in a delay in the appearance of actin in a striated pattern in myofibrils. The organization of α-actinin at Z-disks also is delayed, but the organization of titin and myosin in sarcomeres is not significantly altered. We conclude that the interaction of CapZ and actin is important for the organization of actin filaments of the sarcomere.

ACTIN filaments of myofibrils of striated muscle comprise part of one of the most highly ordered macromolecular structures found in cells. The mechanism for the assembly of actin filaments of the sarcomere requires regulation of the location, polarity and length of the actin filaments, as well as coordinated assembly with other components of the sarcomere. We hypothesized that the barbed-end actin-binding protein, CapZ, functions to organize sarcomeric actin filaments by specifying the location of the barbed end of actin filaments, hence, specifying the polarity of the actin filaments of the I-band. Indirect evidence in support of this hypothesis includes: (*a*) CapZ and the barbed end of sarcomeric actin filaments are located at Z-disks (5, 17, 18); (*b*) there is sufficient CapZ in muscle to bind all the actin filaments of the sarcomere (30); (*c*) CapZ can nucleate and cap the barbed end of actin filaments in vitro (2, 6); and (*d*) CapZ is assembled at Z-disks of nascent myofibrils before the appearance of striated actin filaments in myotubes in culture (30). Together, these data support the idea that CapZ functions to organize actin filaments during sarcomere formation and to stabilize actin filaments by preventing either polymerization or depolymerization from the barbed end, both of which would alter sarcomere function. These data

also predict that CapZ may bind to nascent Z-disks independent of an association with actin.

To obtain direct evidence for the participation of CapZ in organizing actin filaments during myofibrillogenesis, we have inhibited the actin-binding activity of CapZ in chick skeletal muscle myotubes in culture. Inhibition of CapZ was accomplished in two ways. In one approach, a monoclonal antibody (mAb 1E5) that blocks the interaction of CapZ and actin in vitro was microinjected into chick skeletal muscle myotubes at an early stage of myofibril formation. Binding of mAb 1E5 to CapZ inhibits both the capping and actin-nucleating activities of CapZ in vitro (16). In addition, mAb 1E5 binds the β1 subunit of CapZ within a region implicated to comprise part of the actin-binding site. In a second approach to inhibit CapZ in vivo, we transfected chick muscle cultures with cDNAs that provide expression of a mutant form of the CapZ β1 subunit. Deletion of 12 amino acids from the COOH terminus of the β1 subunit of CapZ removes the epitope bound by mAb 1E5 and also abrogates the ability of CapZ to interact with actin (16). The truncated β1 subunit associates with an α subunit to yield a stable heterodimer that is a dominant-negative mutant form of CapZ. Thus, the COOH terminus of the CapZ β1 subunit is a critical part of the actin-binding site for this actin-binding protein. Results from both sets of experiments indicate that alterations in the amount of functional CapZ in myotubes alter the actin cytoskeleton and delay the organization of actin in sarcomeres.

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Materials and Methods

All reagents were reagent grade and purchased from Sigma Chem. Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA) unless stated otherwise. Fertile chicken eggs were purchased from Spathos (Roanoke, IL). CapZ was purified from adult chicken pectoralis muscle as described (2). Polyclonal goat anti–chicken CapZ was prepared as described (29). Antibodies to *E. coli* β-galactosidase were elicited in rabbits using standard protocols by Cocalico Biological Inc. (Reamstown, PA). Monoclonal antibody (mAb) C4, which is specific for actin was a gift of Dr. James Lessard of the University of Cincinnati (Cincinnati, OH) (25). mAb EA-53, which is specific for sarcomeric α-actinin (11) was purchased from Sigma Chem. Co. mAb 9D10, which is specific for titin (34) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA).

Cell Culture Methods

Muscle cell cultures were prepared from 11-d chick embryos as described (27) and maintained in Earle's MEM supplemented with 10% horse serum, 2.5% chick embryo extract, glutamine, and antibiotics. Cells were plated in 35-mm diam, collagen-coated plastic dishes at 4×10^5 cells/dish for the microinjection experiments or at 5×10^5 cells/dish for the transfection experiments. Cultures were fed fresh media containing 1 μg/ml cytosine arabinoside on the third or fourth day after plating; fresh media without cytosine arabinoside was provided on the sixth day after plating.

Purification of Antibodies and Microinjection

mAb 1E5 (16), mAb 1B11 (16), and mouse immunoglobulins (Sigma Chem. Co) were purified on protein A–agarose as described (14). Proteins eluted from the protein A–resin were concentrated by addition of an equal volume of saturated $(NH_4)_2SO_4$ followed by incubation at 4°C overnight. The precipitated protein was dissolved in a small volume of injection buffer (75 mM KCl in 10 mM potassium phosphate, pH 7.5) and the proteins were dialyzed against two changes of injection buffer. Protein concentrations were determined by the method of Bradford (1). Before microinjection, the antibody solutions were centrifuged at 100,000 g for 20 min in a tabletop ultracentrifuge.

Microinjection was performed using a constant flow pipette constructed from a micrometer syringe that was guided by a micromanipulator (Narishige, Tokyo, Japan) on a Zeiss Axiovert microscope. During microinjection, cell cultures were maintained at room temperature in a medium containing 10% horse serum in Earle's MEM salt solution buffered at pH 7.4, with 20 mM Hepes; this solution also lacked $NaHCO_3$ and phenol red. To achieve a high concentration of injected antibody, several myotubes in a small region of the culture dish were injected at multiple sites along the length of each cell. In most experiments, the concentration of protein in the injection pipette was 10 mg/ml; in experiments using low concentrations of antibody, the protein concentration in the injection pipette was 2 mg/ml. After microinjection, the normal muscle cell culture medium was added and the cultures were returned to the CO_2 incubator. Seven microinjection experiments were performed using mAb 1E5; four experiments included mouse immunoglobulins as a control and four experiments included mAb 1B11, which binds the α subunit of CapZ, but does not inhibit actin binding.

Construction of the RSV-CapZ-lacZ Expression Vectors

The expression vector used in the transfection experiments provides the coexpression of cloned CapZ subunits and β-galactosidase as a marker for transfected myotubes under the control of an RSV-LTR promoter. The parent vector (pBJ-261) used to prepare the DNAs was a generous gift of Dr. John Majors of the Department of Biochemistry, Washington University (St. Louis, MO). Situated between the cloned CapZ subunit cDNA and the β-galactosidase cDNA is an internal ribosome entry site from encephalomyocarditis virus that allows ribosomes to bind to uncapped mRNAs (20), thus, two polypeptides are expressed from a single mRNA. Transfected myotubes were identified by immunolabeling with anti–β-galactosidase. The parent vector, pBJ-261, was linearized with BstE II and Xba I which also excises the gag-neomycin fusion protein cloned into these sites, and the CapZ subunit cDNAs were inserted by blunt-end ligation to form the following DNA constructs: pBJ-417, which encodes the full-length β1 subunit (277 amino acids) (3); pBJ-406, which encodes a truncated β1 subunit that lacks 12 amino acids at the COOH terminus of the full-length protein (265

amino acids) (16); and pBJ-410, which encodes the full-length α2 subunit (286 amino acids) (4). cDNA expression constructs were purified using Maxi-prep columns (Qiagen Inc., Chatsworth, CA) and their concentrations were determined by absorbance at 260 nm.

Transfection Protocol

Muscle cell cultures were transfected 1 d after plating by the calcium phosphate precipitation method. Briefly, cultures in 35-mm dishes were fed 1.0 ml fresh medium and incubated for 3 h. The calcium phosphate-DNA precipitate was prepared by mixing equal volumes of freshly prepared $2\times$ HSB (280 mM NaCl, 1.5 mM $NaHPO_4 \cdot 7H_2O$, 50 mM Hepes, pH 7.1) and DNA in 0.248 M $CaCl_2$ so that the final concentration of DNA after addition of the $2\times$ HSB is 20 μg/ml. (When a mixture of two DNA constructs was used, the final concentration of each DNA was 10 μg/ml). Mixing of the two solutions was performed by bubbling a gentle stream of air through the $2\times$ HSB while adding dropwise the solution of DNA. After incubating the mixture at room temperature for 30 min, 0.125 ml of the DNA suspension (2.5 μg DNA) was added dropwise to each 35-mm culture dish; cultures were incubated for 5–6 h, and then the DNA suspension was replaced with fresh muscle medium.

Phalloidin Staining and Immunolabeling of Muscle Cell Cultures

Cultures to be labeled with phalloidin were fixed according to the following method. Cells in 35-mm dishes were rinsed in 30 mM Hepes, pH 7.0, containing 70 mM KCl, 5 mM $MgCl_2$, and 3 mM EGTA, and fixed for 15 min at room temperature in freshly prepared 2% paraformaldehyde in the same buffer. Fixed cells were permeabilized by incubation in 0.1% Triton X-100 in the Hepes buffer for 15 min. Cultures to be labeled with mAb C4, to identify actin, or mAb EA-53, to identify α-actinin, were rinsed in the Hepes buffer, and fixed and permeabilized by addition of −20°C methanol and incubation at −20°C for 15 min. Fixed cells were blocked in 10% heat-inactivated calf serum and 3% BSA in TTBS (0.3 M NaCl, 20 mM TrisCl, pH 7.8, 0.1% vol/vol Tween-20 and 0.01% NaN₃). For microinjection experiments, cultures were incubated overnight with a mixture of 33 nM FITC-phalloidin (Molecular Probes, Eugene, OR) and rhodamine-conjugated rabbit anti–mouse IgG (Chemicon, Temecula, CA) at 1:200 dilution. Cultures were washed three times in TTBS and a 22 × 22-mm coverslip was applied using 50% (vol/vol) glycerol in 10 mM TrisCl, pH 8.0, containing 0.1% n-propylgallate as mounting medium. For transfection experiments, cultures were incubated overnight with a mixture of 33 nM FITC-phalloidin and antiserum to β-galactosidase at 1:2,000 dilution. Cells were washed three times in TTBS and incubated in rhodamine-conjugated donkey anti–rabbit Ig at 1:200 dilution. Cells were washed three times in TTBS and a coverslip was applied. For double immunolabeling to detect sarcomeric α-actinin and β-galactosidase, mAb EA-53 was used at 1:100 dilution and anti–β-galactosidase was used at 1:2,000 dilution. Bound antibodies were detected using a mixture of DTAF-conjugated donkey anti–mouse Ig at 1:200 dilution and rhodamine-conjugated donkey anti–rabbit Ig at 1:200 dilution.

Immunofluorescence microscopy was performed using a Zeiss Axioplan microscope equipped with a 100× (1.3 NA) plan-neofluar objective and a 63× (1.4 NA) planapochromat objective lens and filters for epifluorescence that excluded crossover between fluorescein and rhodamine; the performance of the filters was confirmed in control experiments. Photomicrographs were recorded on Kodak T-max 400 ASA film. In the analysis of the transfection experiments, an MRC-1000 Laser Scanning Confocal Microscope (BioRad Labs., Hercules, CA) was used to obtain images at the lower surface, the upper surface and at a middle focal plane for each myotube. Myotubes were first selected based in the expression of β-galactosidase and confocal images were collected using excitation and emission filters appropriate for detection of FITC-phalloidin. Confocal images were assembled as montages using Adobe Photoshop v. 2.5 and printed using a Kodak Colorease Printer.

Electrophoresis and Immunoblotting Procedures

Total extracts of the transfected muscle cultures were prepared using an extraction buffer containing 8 M urea, 2 M thiourea, 0.7 M β-mercapto-ethanol, 3% (wt/vol) SDS, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μM pepstatin, and 0.1 mM benzamidine in 0.05 M TrisCl, pH 6.8 (36). This buffer solubilizes myofibrillar proteins well. To prepare the extracts, cells from duplicate 35-mm dishes were trypsinized, washed, resuspended in 50 μl of

extraction buffer, and immediately boiled for 5 min; samples were stored at −70°C until use. Each sample was diluted with 4× Laemmli sample buffer (24) before loading on the gel and equal volumes of extract from each dish were loaded in each lane.

Proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels (24) and transferred to nitrocellulose (32). Blots were blocked in Super-block buffer (Pierce Chemical Co., Rockford, IL) and incubated overnight at 4°C with the primary antibodies diluted in 2% fish gelatin containing 5% heat-inactivated calf serum in TTBS. Affinity purified goat anti-CapZ was used at 1 μg/ml and rabbit antiserum to β-galactosidase was used at 1:10,000 dilution. Blots were washed in TTBS and bound antibodies were detected using an appropriate alkaline phosphatase–conjugated secondary antibody (Tago, Burlingame, CA) at 1:10,000 dilution. Blots were washed with TTBS and developed to yield the alkaline phosphatase reaction product (10).

Results

Injection of mAb 1E5 into Myotubes Alters the Distribution of F-actin in Myotubes

Binding of mAb 1E5 inhibits CapZ's ability to bind actin in vitro (16). To determine the effects of inhibiting CapZ activity during myofibrillogenesis in vivo, we injected mAb 1E5 into chick myotubes developing in culture and observed the organization of actin in the myotubes at varying times after injection. One limitation of this approach is that myoblasts continue to fuse with myotubes, contributing to increased cell volume and consequent decreased concentration of the injected antibody with time after injection. Thus, we injected myotubes on culture day 3, when myotubes have nearly reached their mature size but do not yet contain mature myofibrils (30). In addition, antibodies at high concentration were injected at multiple sites along the length of each myotube in order to obtain the maximum intracellular concentration of antibody. A second limitation is that the injected antibodies are degraded over time in culture, and the cells eventually recover as the concentration of antibody decreases. Nonetheless, we observed a dramatic effect on the organization of F-actin in myotubes for up to 24 h after injection of mAb 1E5.

Three alterations of the actin cytoskeleton are apparent in myotubes 24 h after injection with mAb 1E5 on culture day 3 (Fig. 1, a–c). In many cells, the non-striated actin filament bundles which have been called stress fiber–like structures (SFLS) (9) or premyofibrils (28), and are the predominant F-actin structures in myotubes at this age in culture, are less numerous and much thinner than in myotubes injected with control mouse immunoglobulins (Fig. 1 d) or with mAb 1B11 (data not shown), an antibody specific for the α subunit of CapZ that does not inhibit the interaction of CapZ and actin in vitro (16). These effects of mAb 1E5 on F-actin were observed consistently in the majority of injected myotubes in seven different experiments. Some of the mAb 1E5-injected myotubes were much flatter and wider than control mouse immunoglobulin-injected or uninjected myotubes at this stage of development. Two unusual features of the F-actin in myotubes injected with mAb 1E5 were observed near the upper and lower surfaces of the myotubes. At the lower surface of the injected myotubes, F-actin was in amorphous patches and brightly stained foci (Fig. 1 c) and on the upper surface were numerous actin-containing projections that resembled microvilli (Fig. 1 b, arrows). Neither of these unusual features were observed in myotubes injected with mouse immunoglobulins or mAb 1B11. We presume that
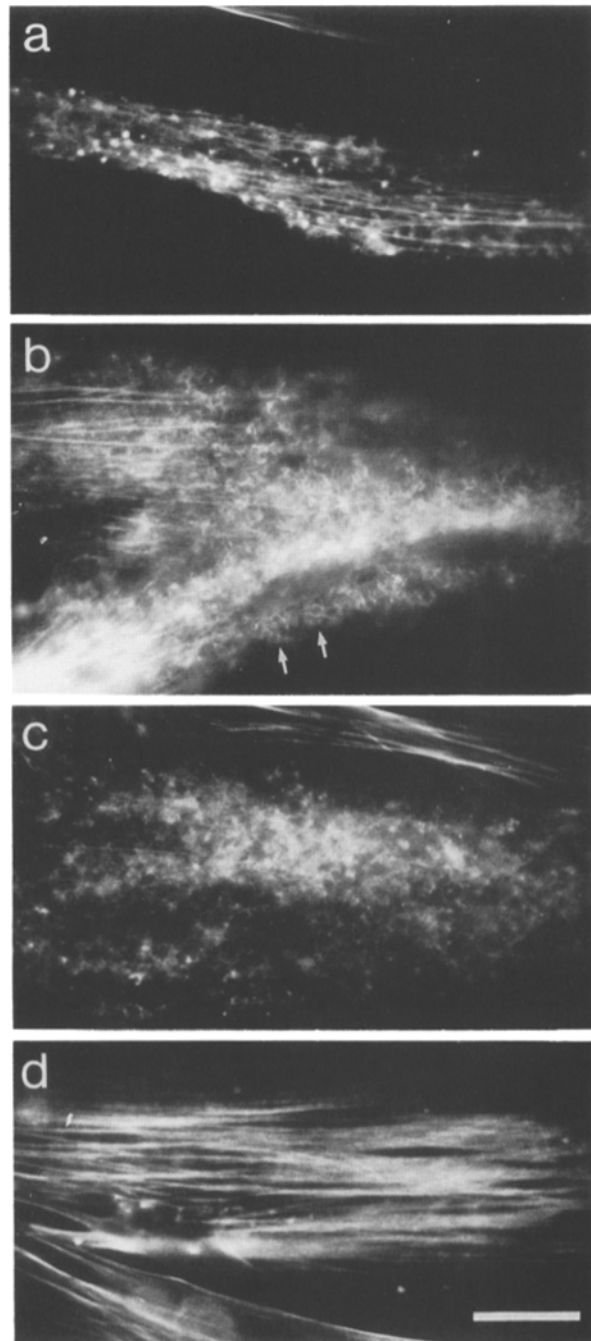
Figure 1. The actin cytoskeleton of young myotubes is altered by microinjection of inhibitory mAb 1E5. Myotubes were injected on culture day 3 with either mAb 1E5 (a–c) or mouse Ig (d), and actin was observed 24 h later. The major form of actin in control myotubes at this stage of development are non-striated filament bundles as observed in mouse Ig-injected myotubes (d). Few non-striated actin filament bundles are observed in the mAb 1E5-injected myotubes (a–c). Actin was in amorphous patches or bright foci near the lower surface and actin-containing projections were observed on the upper surface of the myotubes (arrows in b). Neither of these unusual actin-containing structures were observed in myotubes injected with mouse Ig. F-actin was observed using FITC-phalloidin; antibody-injected myotubes were identified by labeling with rhodamine-conjugated rabbit anti–mouse Ig (not shown). Bar, 20 μm.
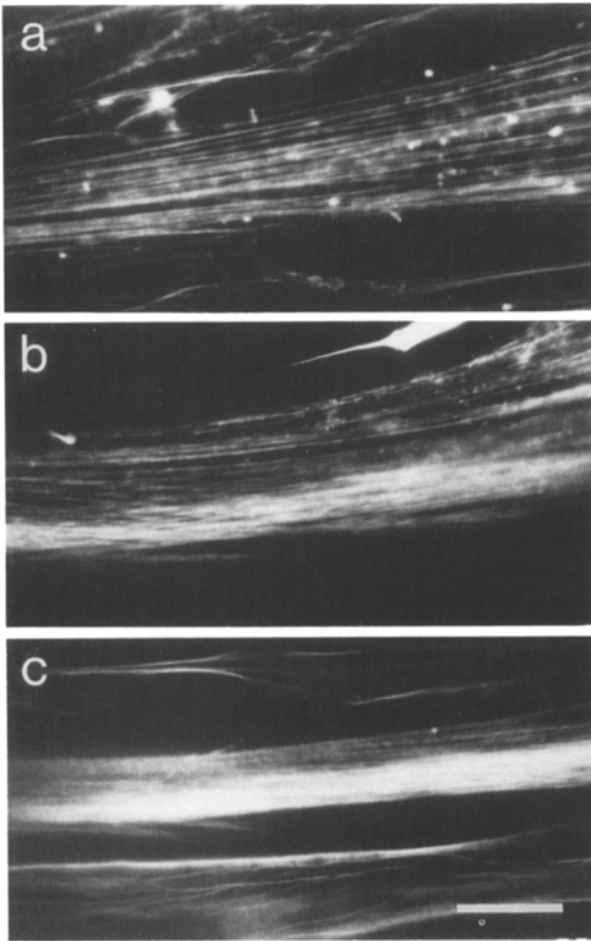
Figure 2. The actin cytoskeleton recovered in myotubes by 48 h after injection of mAb 1E5. Myotubes were injected on culture day 3 with either mAb 1E5 (*a* and *b*) or mouse Ig (*c*) and actin was observed 48 h later. Non-striated actin filaments were observed in mAb 1E5-injected myotubes but some of the bright foci that stain with phalloidin were still observed. In those myotubes injected with mouse Ig, non-striated actin filaments were abundant and some striated regions of nascent myofibrils were observed. F-actin was observed using FITC-phalloidin; antibody-injected myotubes were identified by labeling with rhodamine-conjugated rabbit anti-mouse Ig (not shown). Bar, 20 μm.

these F-actin–containing structures form from the actin displaced from the non-striated actin filaments that are normally abundant in these myotubes.

By 48 h after injection of mAb 1E5 into myotubes on culture day 3, the organization of actin more closely resembled that found in uninjected myotubes or in myotubes injected with mouse immunoglobulins (Fig. 2). Non-striated actin filaments formed; some myotubes still possessed the bright foci of phalloidin-stained material near the lower surface but these features were less apparent than at shorter times after antibody injection. Observations longer than 48 h after injection of antibody were difficult because, as the injected antibody was degraded, identification of the injected cells based on labeling with fluorescent anti–mouse immunoglobulin was often ambiguous.

Injection of mAb 1E5 into myotubes on culture day 6, many of which possessed mature, contractile myofibrils yielded a variable effect on the actin cytoskeleton among the injected myotubes (Fig. 3). In 28% of these myotubes, the effects of injected mAb 1E5 on the organization of actin were severe and resembled those seen in myotubes injected after 3 d in culture (Fig. 3 *a*). Actin filaments were absent or fragmented, amorphous, actin-containing patches were observed near the lower surface of the myotubes and microvilli-like structures were on the upper surface. However, many of these older myotubes appeared relatively unaltered by the presence of mAb 1E5 in the cytoplasm (Fig. 3 *b*); actin was
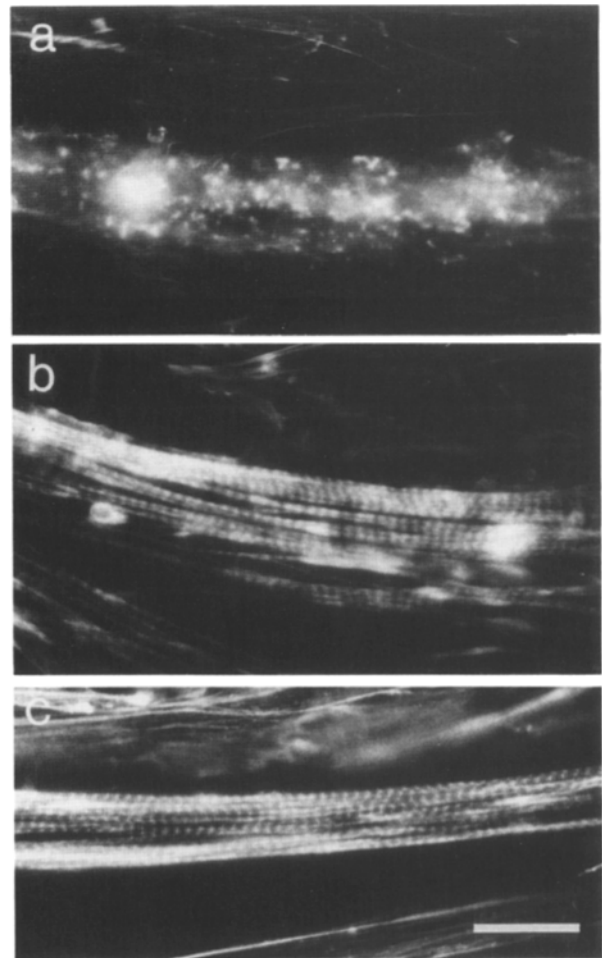
Figure 3. Myotubes of 6-d-old muscle cultures were injected with either mAb 1E5 (*a* and *b*) or mouse Ig (*c*) and actin was observed 24 h later. The actin in some myotubes injected with mAb 1E5 resembled that seen in myotubes injected on culture day 3; these myotubes contained few non-striated actin filaments and accumulated bright foci and amorphous patches of actin (*a*). Other myotubes, particularly those with mature, striated myofibrillar structures, were not severely affected by the presence of mAb 1E5 (*b*) and actin was observed in a striated pattern similar to that seen in control myotubes injected with mouse Ig (*c*). The different susceptibilities of the myotubes of older cultures to the effects on the organization of actin by mAb 1E5 likely results from heterogeneity in the maturity of the myotubes. F-actin was observed using FITC-phalloidin; antibody-injected myotubes were identified by labeling with rhodamine-conjugated rabbit anti–mouse Ig (not shown). Bar, 20 μm.

organized as non-striated filaments (33% of these myotubes) or was detected in a striated pattern (38% of these myotubes) that resembled the distribution of actin in myotubes injected with mouse immunoglobulins (Fig. 3 c). However, most of these injected myotubes also had actin-rich aggregates near their lower surface. The mild or severe effects of mAb 1E5 injection into these older myotubes may be related to differences in the amount of antibody delivered to individual myotubes, however, this was not apparent from the intensity of labeling with rhodamine-conjugated anti–mouse immunoglobulin. Alternatively, because the myotubes in older cultures are a heterogeneous population at varying stages of differentiation, the stability of the myofibrillar structures to mAb 1E5 may vary depending on the maturity of the myofibrillar structures. Those myotubes susceptible to the antibody may be less mature than those that are more resistant to injected mAb 1E5.
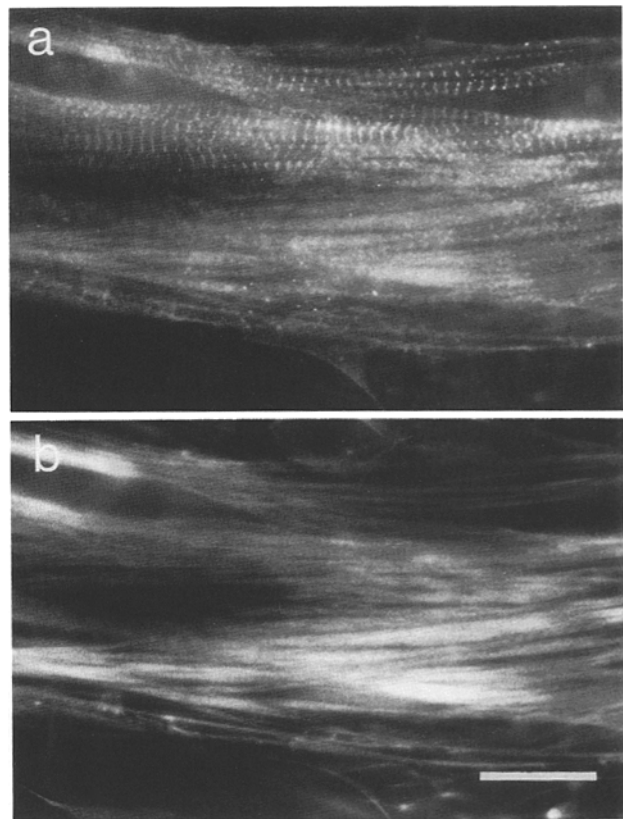
Interaction of CapZ with Nascent Z-Disks

The fact that the binding of mAb 1E5 blocks CapZ binding to actin (16) allowed us to test whether CapZ associates with nascent Z-disks independent of an interaction with actin. We expected that injected mAb 1E5 would associate with CapZ at Z-disks if the CapZ were bound to the Z-disk via an interaction that does not involve actin. When a low concentration of mAb 1E5 (fivefold lower than required for disruption of F-actin) was injected into 3-d-old myotubes, no significant effects on the actin cytoskeleton were observed at 24 h after injection (Fig. 4 b). Nonetheless, the intracellular distribution of injected mAb 1E5 was instructive. Some of the antibody was found at Z-disks of nascent myofibrils (Fig. 4 a). Double immunolabeling for injected mAb 1E5 and titin using mAb 9D10, which binds near the Z-disk domain of titin (ref) confirmed that the periodic structures with bound mAb 1E5 were Z-disks (data not shown). A similar distribution of injected antibody was observed in myotubes of 5–6-d cultures. Since the interaction of CapZ with actin or mAb 1E5 in vitro is mutually exclusive, we conclude that at least some of the CapZ associated with nascent Z-disks is not yet bound to the barbed end of an actin filament and that CapZ interacts with other components of the Z-disk during assembly of the structure.

Expression of Mutant CapZ in Myotubes Delays the Appearance of Atriated Actin Filaments of Sarcomeres

A complementary approach to inhibit CapZ during myofibril formation in myotubes in cultures is to express a mutant form of CapZ that is unable to bind actin. The advantage of this approach over the antibody injection experiments is that the efficacy of the inhibitory reagents is not temporary but can persist throughout the entire culture period. The mutant protein used in these studies was a truncated form of the β1 subunit of CapZ. Loss of 12 amino acids from the COOH terminus of the β1 subunit results in loss of actin-binding activity, but retention of ability to bind an α subunit (16). Thus, we expect that a fraction of the $\alpha\beta$ heterodimer formed in myotubes transfected with the truncated β1-cDNA will be inactive.

Myoblasts were transfected with cDNA constructs that simultaneously express CapZ subunits and β-galactosidase,

Figure 4. The binding of CapZ to nascent Z-disks is independent of an interaction with actin. mAb 1E5 was injected at a low concentration (2 mg/ml in the injection pipette) into myotubes of 3-d cultures. Myotubes were fixed and stained with fluorescein-conjugated rabbit anti–mouse Ig (a) to observe the distribution of injected antibody and with rhodamine-phalloidin to observe F-actin (b). The injected mAb 1E5 was detected at periodically arrayed structures having the dimensions and spacing identical to that of nascent Z-disks (a). There was no significant effect of this concentration of mAb 1E5 on the distribution of actin in these myotubes (b). Because the binding of mAb 1E5 and actin to CapZ is mutually exclusive, this finding suggests that CapZ binds to a component of nascent Z-disks independent of an interaction with actin. Bar, 20 μm.
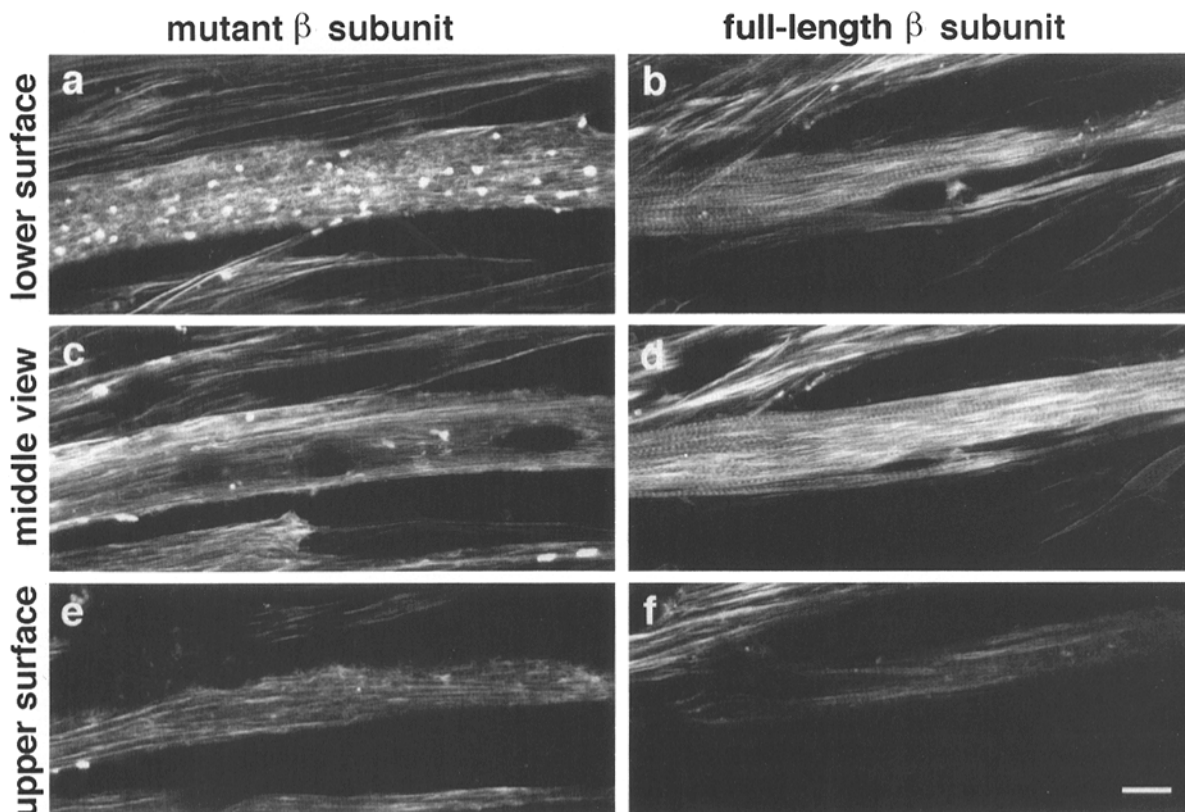
as a marker for transfected myotubes, under the control of an RSV-LTR promoter. The CapZ cDNAs included those encoding the full-length β1 subunit, the mutant β1 subunit that lacks 12 amino acids at the COOH terminus, and the α2 subunit. Additional controls included transfection with vector DNA that encodes a viral gag-neo fusion protein in place of the CapZ subunit and a mock transfection protocol with no DNA. Cotransfections using mixtures of the DNAs containing the α2-subunit cDNA with either the full-length or mutant β1-subunit cDNAs also were performed. No alterations in the growth and fusion of myoblasts were observed in any of the transfected cultures. By 3 d after transfection, nearly all of the myotubes expressed the β-galactosidase marker. The high level of transfection efficiency results from the fact that, although not all myoblasts were initially transfected, all the myotubes that subsequently form contain at least some nuclei that have incorporated the exogenous DNA.

A day 3 cultures
B day 5 cultures

β-gal

CapZ-β

CZ 1 2 3 4 5 6 7   1 2 3 4 5 6 7 CZ
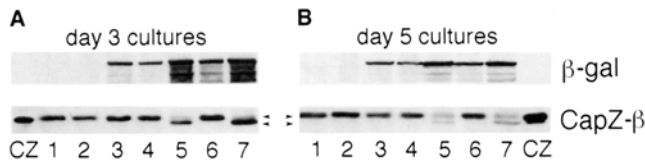
Figure 5. Expression of β-galactosidase and of CapZ β subunit in muscle cultures 3 d (*A*) and 5 d after transfection (*B*). Equal amounts of each culture extract, prepared as described in Materials and Methods, were loaded on a 10% SDS-PAGE gel, transferred to nitrocellulose, and the blots probed with either anti–β-galactosidase (*upper panel*) or goat anti-CapZ (*lower panel*). The intensity of the bands corresponding to the CapZ β subunits were similar in all culture extracts. The expression of the mutant β1 subunit in those cultures transfected with pBJ-406 is indicated by the faster mobility of the truncated protein (lanes 5 and 7); this protein also did not react with mAb 1E5, consistent with the absence of the epitope on the mutant β1 subunit (data not shown). β-Galactosidase was expressed only in transfected muscle cultures (lanes 3–7) and not in control untransfected or mock-transfected cultures (lanes 1 and 2). Lanes contain: lane *CZ*, purified chicken muscle CapZ as standard; lane *1*, extract from untransfected muscle culture; lane *2*, extract from mock-transfected muscle culture; lane *3*, extract from culture transfected with α2-subunit cDNA (pBJ-410); lane *4*, extract from culture transfected with full-length β1-subunit cDNA (pBJ-417); lane *5*, extract from culture transfected with mutant β1-subunit cDNA (pBJ-406); lane *6*, extract from culture transfected with a mixture of α2-subunit and β1-subunit cDNAs (pBJ410+pBJ417); lane *7*, extract from culture transfected with a mixture of α2 subunit and mutant β1-subunit cDNAs (pBJ-410+pBJ-406). Only the portion of the blots containing β-galactosidase or CapZ β subunits are shown. Coomassie blue staining of identical gels verified that similar amounts of protein were loaded in each lane.

Analysis by Western blot of the amounts of β-galactosidase and of the CapZ β subunit expressed in the transfected myotubes at 3 d and 5 d of culture is shown in Fig. 5. β-Galactosidase was expressed at nearly equal amounts in all the transfected cultures, except in those expressing the α2 subunit or the full-length β subunit, which was slightly lower. The total amount of CapZ β subunit expressed in all cultures, as determined by comparing the intensity of bands corresponding to the β subunits of all the culture extracts, was nearly identical (Fig. 5). Thus, the amount of CapZ expressed by the transfected myotubes is the amount normally expressed by these myotubes in culture and the effects we observe on the organization of actin in myotubes are not due to elevated levels of CapZ subunits. Expression of the mutant β1 subunit protein that lacks 12 amino acids at its COOH ter-

mutant β subunit   **full-length β subunit**

lower surface — a, b
middle view — c, d
upper surface — e, f

Figure 6. The actin cytoskeleton in myotubes 3 d after transfection with cDNAs that direct the expression of the mutant β1 subunit (*a*, *c*, and *e*) and the full-length β1 subunit (*b*, *d*, and *f*). Confocal microscopy was used to obtain images at three different focal planes for each myotube to visualize the lower surface (*a* and *b*), the middle of the myotube (*c* and *d*), and the upper surface (*e* and *f*). By this time in culture, myotubes expressing the full-length β1 subunit contained some striated actin (*d*), whereas those expressing the mutant β1 subunit were primarily non-striated (*c*). Actin also was observed in an amorphous meshwork with some brightly stained foci near the lower surface (*a*) of myotubes expressing the mutant β1 subunit and small actin-rich projections were observed on the upper surface of these myotubes (*e*). These unusual F-actin structures were not observed in myotubes expressing the full-length β1 subunit. The distribution of actin was observed using FITC-phalloidin; transfected myotubes were identified by anti–β-galactosidase immunostaining (not shown). Bar, 10 μm.

minus was detected by its faster migration in the SDS gel (Fig. 5, lanes 5 and 7) and by its lack of reactivity with mAb 1E5 (data not shown). The results from three independent transfection experiments indicated that the amount of mutant β1 subunit present in these myotubes is 50–80% of the total β-subunit protein normally expressed by the myotubes. It is likely that the mutant β1 subunit competes with the endogenous, full-length β1 subunit in binding an α subunit; excess β-subunit proteins are presumably not stable and are degraded. The ratio of truncated β1 subunit to endogenous, full-length β1 subunit was higher at 3 d after transfection than at 5 d after transfection (compare lanes 5 and 7 of Fig. 5, A and B). The change in the relative expression of the exogenous mutant β subunit may result from an increase in the number of untransfected nuclei in myotubes as myoblasts continue to fuse with existing myotubes.

Myotubes that express the mutant CapZ β subunit were delayed in the appearance of striated actin filaments. Representative views of the distributions of F-actin in myotubes 3 d and 5 d after transfection with the mutant β subunit and with the full-length β subunit are shown in Figs. 6 and 7 and quantitation of the number of striated myotubes in cultures 5 d after transfection is presented in Table I. By three days after transfection, actin was detected in a partially striated pattern in some myotubes transfected with the full-length β1-subunit cDNA (Fig. 6 d). Only non-striated actin was observed at this time in culture in myotubes transfected with the β1-subunit cDNA (Fig. 6 c). By five days after transfection, most myotubes in control cultures had striated actin (Table I and Fig. 7 d), whereas most myotubes expressing the mutant β1 subunit contained either non-striated or partially striated F-actin (Table I and Fig. 7 c). Myotubes transfected with a mixture of DNAs for the mutant β subunit and α2 subunit were similarly delayed in the formation of mostly striated actin; however, the defect was not as severe as in those myotubes expressing the mutant β subunit alone (Table I).

The unusual F-actin structures observed in myotubes injected with mAb 1E5 also were observed in myotubes that express the mutant β1-subunit protein. Actin was detected in a meshwork at the upper and lower surfaces (Fig. 6, a and
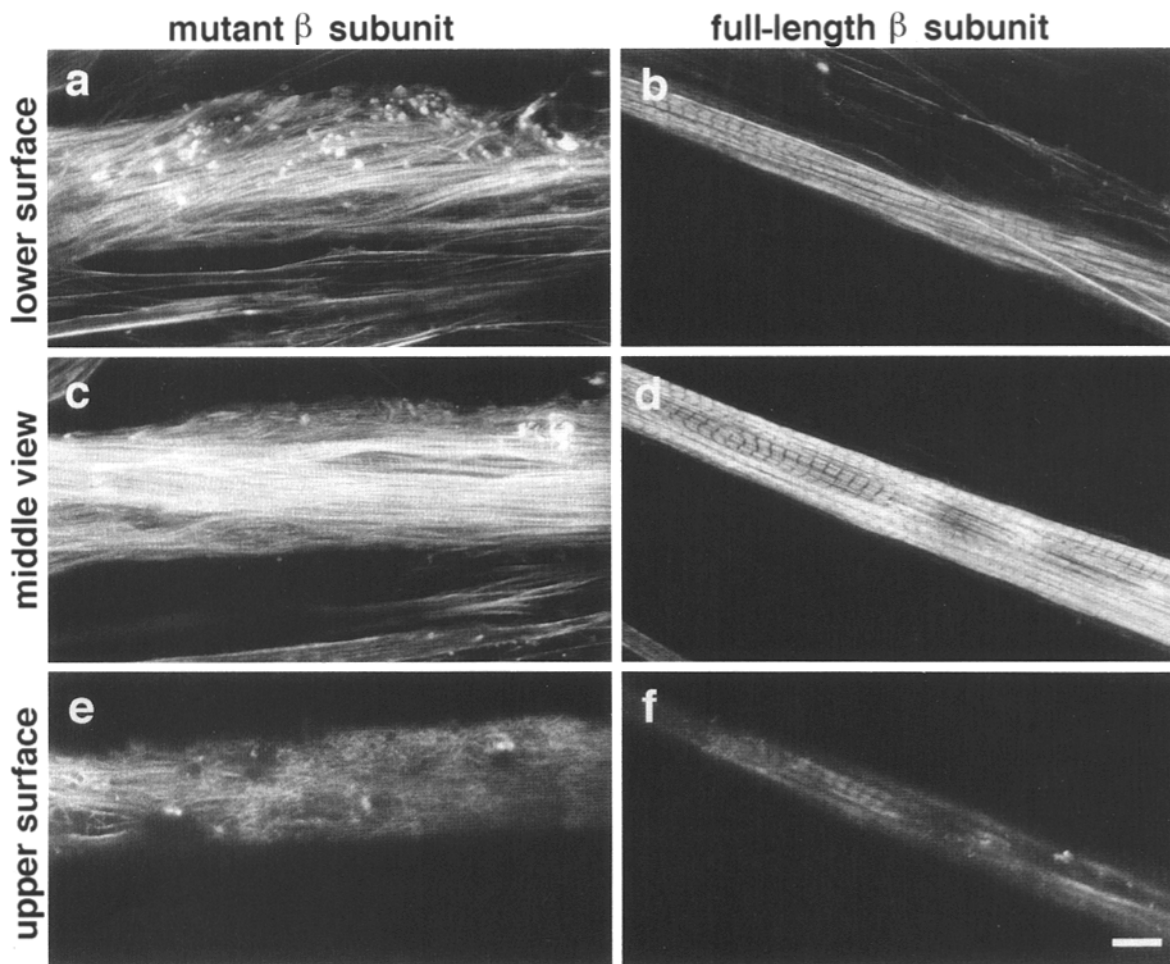
Figure 7. The actin cytoskeleton in myotubes 5 d after transfection with cDNAs that direct expression of the mutant β1 subunit (*a, c,* and *e*) and the full-length β1 subunit (*b, d,* and *f*). Confocal microscopy was used to obtain images at three different focal planes for each myotube to visualize the lower surface (*a* and *b*), the middle (*c* and *d*), and the upper surface of myotubes (*e* and *f*). By this time in culture, myotubes expressing the full-length β1 subunit possess I-bands (*d*), whereas myotubes expressing the mutant β1 subunit were either non-striated or partially striated (*c*). Myotubes expressing the mutant β1 subunit also possess actin meshworks and bright foci of actin at their lower (*a*) and upper (*e*) surfaces. The distribution of actin was observed using FITC-phalloidin; transfected myotubes were identified by anti–β-galactosidase immunostaining (not shown). Bar, 10 μm.

Table I. Quantitation of the Actin Organization in Myotubes 5 d after Transfection with Control and Mutant CapZ β1-Subunit Proteins

|	Actin organization		
Protein expressed	only non-striated	partially striated	mostly striated
none	26%	52%	22%
gag-neo fusion	39%	27%	34%
β1 subunit	29%	46%	25%
mutant β1 subunit	73%	27%	0%
α2 subunit + β1 subunit	15%	53%	32%
α2 subunit + mutant β1 subunit	49%	44%	7%

Myotubes in phalloidin-stained cultures 5 d after transfection were scored according to the following criteria. If only uniformly stained actin filaments were observed throughout the length of the myotube, it contained only non-striated actin. If at least one region was observed where the actin was in a striated pattern, it was scored as partially striated. If the majority of the actin in a myotube was in a striated pattern, it was scored as mostly striated. Between 100 and 129 myotubes in each dish were scored, and the percentage of myotubes in each class was calculated. Similar results were obtained if mAb C4 was used to detect the actin (not shown). Quantitation of two independently performed transfection experiments yielded similar results. DNA constructs used for exogenous protein expression were pBJ-261 (gag-neo), pBJ-417 (β1 subunit), pBJ-406 (mutant β1 subunit), and mixtures of pBJ-410 (α2-subunit) and pBJ-417 or pBJ-406 (full-length or mutant β1 subunit). Sample labeled "none" is tabulated from an untransfected muscle culture.

e and Fig. 7 *e*) and bright, phalloidin-stained foci were observed at the lower surface of the cells expressing the mutant β1-subunit protein (Figs. 6 *a* and 7 *a*). A small number of similar phalloidin-stained foci occasionally were observed in myotubes of control cultures subjected to the transfection protocol, however, they were especially abundant in those myotubes transfected with the mutant β1-subunit cDNA. Some of the myotubes also formed actin-rich projections on their upper surfaces which were observed by transmission electron microscopy (data not shown), however, these were not as prominent as those observed in myotubes injected with mAb 1E5. We presume these F-actin–containing structures form from actin displaced from sarcomeres by the mutant CapZ.

The delay in the appearance of actin in a striated pattern in myotubes 5 d after transfection with the mutant β1 subunit also was reflected in the distribution of sarcomeric α-actinin in myotubes by 3 d after transfection (Fig. 8). Sarcomeric α-actinin was primarily observed at Z-disks in the periodic pattern characteristic of mature sarcomeres in control myotubes or those transfected with the full-length β1-subunit cDNA by this time in culture. The distribution of sarcomeric α-actinin in myotubes expressing the mutant β1 subunit was primarily in a stippled pattern. This pattern is typical of the distribution of sarcomeric α-actinin in myotubes at earlier times in culture. Thus, the alterations in myofibril assembly elicited by a mutant CapZ β1 subunit includes an effect on other proteins of the sarcomere that interact with actin. In contrast, the distributions of titin and myosin were not dramatically altered by the expression of the mutant β1 subunit (data not shown).

Discussion

Inhibition of the barbed-end actin-binding protein, CapZ,
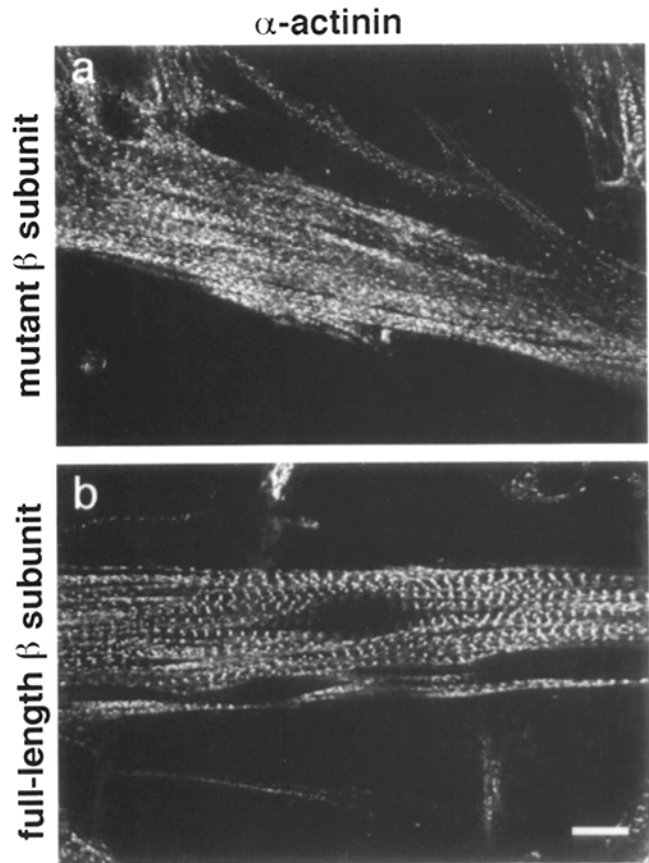
α-actinin

Figure 8. The assembly of sarcomeric α-actinin at Z-disks was delayed in myotubes expressing the mutant β1 subunit. Sarcomeric α-actinin was distributed in a stippled, fibrillar pattern in myotubes 3 d after transfection with pBJ-406 (mutant β1-subunit cDNA) (*a*). In contrast, by this time in culture, sarcomeric α-actinin was distributed in a striated pattern at nascent Z-disks in myotubes transfected with pBJ-417 (full-length β1-subunit cDNA) (*b*). Confocal micrographs were collected at a focal plane through the middle of the myotubes. Sarcomeric α-actinin was detected using mAb EA-53 and transfected myotubes were identified by anti–β-galactosidase immunostaining (not shown). Bar, 10 μm.

during myofibrillogenesis results in a delay in the organization of actin in I-bands. Similar effects on the actin cytoskeleton of myotubes were observed using two independent approaches to inhibit CapZ-actin binding in vivo: blocking CapZ's interaction with actin using the inhibitory antibody mAb 1E5 and expression of a mutant form of CapZ that does not bind actin (16). Even though neither approach leads to inhibition of all the endogenous CapZ in the myotubes, we consistently observed these effects on the organization of actin of myofibrils. These data support the hypothesis that CapZ is required for the organization of actin filaments of sarcomeres in striated muscles.

Most significantly, the appearance of striated actin was delayed in myotubes expressing a mutant form of CapZ. We propose that the delay in the appearance of striated actin results from the loss of actin-binding activity of CapZ. In our hypothesis for myofibril formation (Fig. 9), the mechanism for assembly of polarized actin filaments of I-bands is that CapZ binds to nascent Z-disks, and then either nucleates ac-

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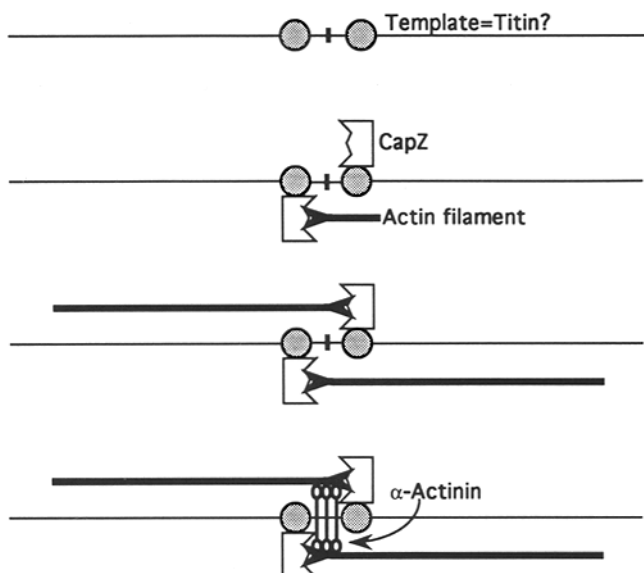
Figure 9. Model for the role of CapZ in organizing actin filaments during myofibrillogenesis. This model is based on the experiments reported here, together with results of previous experiments (30). First, a "molecular template" aligns along the longitudinal axis of the myotube in an array that defines the spacing of sarcomeres and the sites where Z-disks form. Titin is a likely candidate for this molecular template. The orientation of the titin fibrils along the longitudinal axis of the myotube may be guided by either non-striated actin filaments or microtubules. CapZ interacts with the template at sites where nascent Z-disks form, and then either nucleates actin polymerization to form the actin filaments of the I-band or, alternatively, CapZ may capture preformed actin filaments and anchor them at the Z-disk. Either case would yield an array of actin filaments with all the barbed end capped and situated at the Z-disk. Finally, α-actinin assumes its mature distribution in the sarcomere by binding selectively to the region where overlapping, anti-parallel actin filaments occur at Z-disks and stabilizes those structures.

tin polymerization or captures the barbed end of actin filaments of the non-striated stress fiber-like structures to form the actin filaments of the I-band. Capture of actin filaments could occur when a reptating or sliding actin filament collides with CapZ bound at a Z-disk or when a filament elongating by polymerization at its barbed end interacts with CapZ at a Z-disk.

This hypothesis for CapZ action during myofibrillogenesis predicts that CapZ interacts with nascent Z-disks before actin of sarcomeres is organized. CapZ is localized at Z-disks before actin is observed in a striated pattern (30). Since the binding of mAb 1E5 and actin to CapZ are mutually exclusive (16), our observation, that injected mAb 1E5 binds CapZ at Z-disks, is consistent with the idea that CapZ assembles at Z-disks independent of its interaction with actin. We propose that CapZ binds at nascent Z-disks whose position is specified by a molecule that functions as a template for the assembly of sarcomeres. One likely candidate for such a template is titin. Titin has been proposed to act as a "molecular scaffold" that guides the coordinated assembly of myofibrillar proteins (12, 26, 33). The alignment of titin in the head-to-head, tail-to-tail distribution could define the spacing of sarcomeres and the positions of nascent Z-disks. Myo-

sin thick filaments may organize concomitantly, perhaps guided by interactions with titin (23). The early assembly of titin in a sarcomeric distribution during myofibrillogenesis supports this role for titin as a scaffold for the sarcomere (7, 13, 15, 31, 35).

A second, though less likely, candidate for binding CapZ to Z-disks is nebulin. Nebulin, however, is one of the last myofibrillar proteins to assemble in the distribution seen in mature sarcomeres (21) and also is absent from cardiac myofibrils (19), which do contain CapZ, thus, nebulin is neither necessary for CapZ binding to Z-disks nor for sarcomere formation. On the other hand, nebulin binding to CapZ at a later stage of myofibrillogenesis may serve to correctly position the Z-disk domain of nebulin and allow nebulin to specify the length of the thin filaments of skeletal muscle sarcomeres (22).

In addition to slowing the appearance of striated actin in myotubes, both experimental approaches resulted in the formation of unusual F-actin–containing structures that are not normally observed in abundance in muscle cells. We propose that the amorphous patches, foci, and membrane projections that contain F-actin form because the actin subunits that would normally assemble as sarcomeric actin filaments of I-bands add to other actin structures. Some of these displaced actin filaments may enter a degradative pathway. Indeed, the numerous, bright, actin-rich foci seen near the lower surface of myotubes that express the mutant β subunit or have been microinjected with mAb 1E5, resemble macules, which are proposed to comprise part of a degradative pathway for disposal of β- and γ-actin during myofibril formation (8).

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