

Cap $Z_{(36/32)}$, a Barbed End Actin-capping Protein, Is a Component of the Z-Line of Skeletal Muscle

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Abstract. Various biological activities have been attributed to actin-capping proteins based on their *in vitro* effects on actin filaments. However, there is little direct evidence for their *in vivo* activities. In this paper, we show that Cap $Z_{(36/32)}$, a barbed end, actin-capping protein isolated from muscle (Casella, J. F., D. J. Maack, and S. Lin, 1986, *J. Biol. Chem.*, 261:10915-10921) is localized to the barbed ends of ac-

tin filaments by electron microscopy and to the Z-line of chicken skeletal muscle by indirect immunofluorescence and electron microscopy. Since actin filaments associate with the Z-line at their barbed ends, these findings suggest that Cap $Z_{(36/32)}$ may play a role in regulating length, orienting, or attaching actin filaments to Z-discs.

ACTIN-CAPPING proteins bind to one end of actin filaments and block actin monomer interactions at that end (for reviews, see references 25 and 29). These proteins are widely distributed and have been isolated from protozoans and a variety of nonmuscle tissues from vertebrate species. Since capping proteins regulate the number and length of actin filaments and thereby the strength of actin gels *in vitro*, it is often postulated that these proteins regulate the gel-sol characteristics of the cytoplasm. In addition, because capping proteins bind to the very end of the actin filament, it is also suggested that they may attach actin filaments to other structures, such as plasma membranes. So far, little progress has been made in testing these hypotheses *in vivo*.

In contrast to most cells, striated muscle cells have actin filaments that demonstrate a uniform polarity and regular arrangement; the barbed end of the actin filaments is apparently rooted in the Z-line, and the filaments are of equal length (15). This highly defined structure facilitates ultrastructural and immunolocalization studies and implies the presence of a precise system for regulating both orientation and length of actin filaments. The *in vitro* properties of actin-capping proteins suggest that they could organize, regulate the length, and attach the actin filaments present in the actomyosin contractile apparatus, or mediate the interaction of actin filaments with other proteins in the cytoskeleton of muscle that surrounds the contractile apparatus.

To examine the role of actin-capping proteins in the organization of the muscle cytoskeleton and contractile apparatus, an actin-capping protein was identified and isolated from skeletal muscle (3). This protein, now referred to as Cap $Z_{(36/32)}$, binds with high affinity to the barbed end of actin filaments. Cap $Z_{(36/32)}$ appears to be a heterodimer consisting of subunits of M_r 36,000 and 32,000. Like other capping proteins, Cap $Z_{(36/32)}$ can accelerate the rate of actin poly-

merization by acting as a nucleus or enhancing the production of effective nuclei for subsequent polymerization, thereby favoring conversion of actin to its filamentous form. As expected for a protein that caps the barbed end of actin filaments, Cap $Z_{(36/32)}$ also raises the critical concentration of actin under physiological salt conditions. Like other capping proteins, Cap $Z_{(36/32)}$ also markedly reduces the low-shear viscosity of actin filaments by a mechanism that has not been clearly elucidated. Unlike some actin-capping proteins but similar to others, Cap $Z_{(36/32)}$ does not appear to be calcium regulated. To determine how the location of the protein in muscle tissue correlates with its *in vitro* effects on actin filaments, we have localized Cap $Z_{(36/32)}$ at a molecular and ultrastructural level using affinity-purified antibodies against the protein.

Materials and Methods

Production and Affinity Purification of the Anti-Cap $Z_{(36/32)}$ Antibodies

Antisera against the M_r 32,000 (beta) subunit of Cap $Z_{(36/32)}$ was prepared as previously described (3). Affinity purification of the antibody was carried out essentially as described by Davis and Bennett (6). Pooled immune sera was diluted with an equal volume of 0.15 M NaCl, 10 mM sodium phosphate, 1 mM NaEDTA, 1 mM NaN_3 , 0.2% (vol/vol) Triton X-100 and heated to 60°C for 20 min in the presence of 200 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride (PMSF) to minimize protease activity. All subsequent procedures were carried out at 4°C unless otherwise specified. 15 ml of the diluted antisera was then passed over a 1.5-ml Cap $Z_{(36/32)}$ -agarose column prepared using CNBr-activated Sepharose 4B and 0.5 mg of Cap $Z_{(36/32)}$ (purified by a previously described procedure [3], and dialyzed into 500 mM KCl, 10 mM potassium phosphate, pH 8 before coupling). The column was then washed serially with 20 ml of 0.5 M NaCl, 10 mM sodium phosphate, 1 mM NaN_3 , 0.2% Triton X-100, pH 7.5, 10 ml of 2 M urea, 0.1 M glycine, 1% Triton X-100, and then 20 mM Na acetate, pH 5, until the absorbance at 280 nm was 0. The bound antibody was then eluted using 1 M acetic

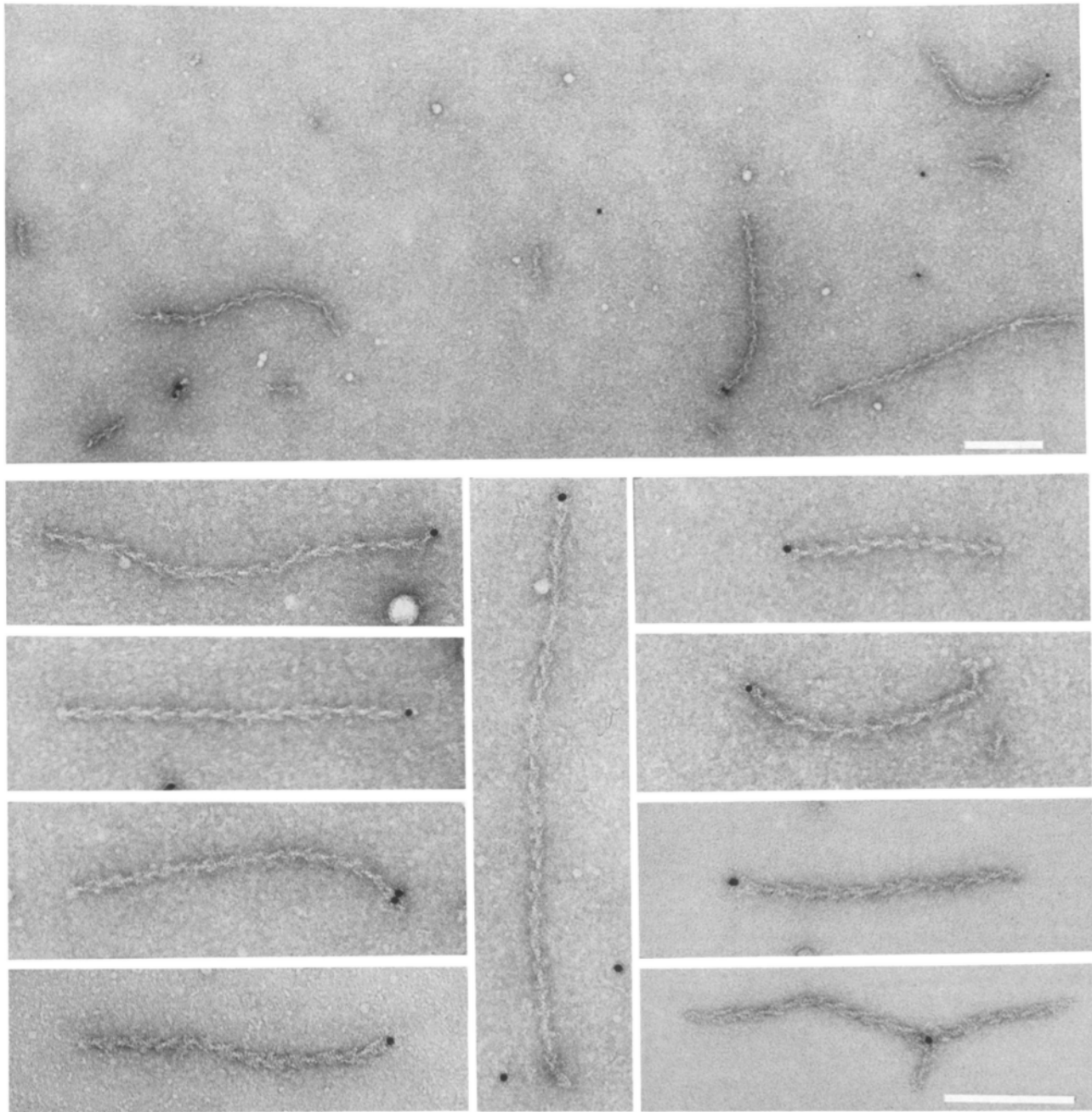


Figure 2. Immunolocalization of Cap $Z_{(36/32)}$ on SI-decorated actin filaments. Examples of protein A–colloidal gold labeling of the barbed ends of actin filaments exposed to Cap $Z_{(36/32)}$ and affinity-purified anti-Cap $Z_{(36/32)}$ antibodies as described in Materials and Methods are shown at representative low (*top*) and high (*bottom*) power views. Bar, 0.2 μm .

bound Cap $Z_{(36/32)}$ were then diluted to $\sim 65 \mu\text{g protein/ml}$, and 10 μl of this solution was incubated with 6 μl of protein A-coated 10-nm colloidal gold particles (a kind gift of Dr. Douglas Murphy) and 10 μl of 15 $\mu\text{g/ml}$ affinity-purified anti-Cap $Z_{(36/32)}$ or nonimmune IgG at the same concentration. 5 μl of this solution was then injected into 2 μl of the nuclei suspension buffer described above on a glow-discharged, carbon-coated, 400-mesh copper electron microscopy grid. After 1 min, the grid was washed and stained by touching the grid to three successive drops of the nuclei suspension buffer, three drops of distilled water, and three drops of 0.1% uranyl acetate. Excess stain was removed by blotting the edge of the grid on filter paper and gentle suctioning of the edge of the grid using a finely drawn pasteur pipet. The grids were then photographed at 5,000 \times , 60 kV in a Zeiss EM 10 A microscope. Negatives were then examined in a microfiche reader

(at 22 \times ; Atlantic Microfilm Corp., Spring Valley, NY) to determine the direction of SI labeling and the percentage of filaments labeled. The photographs of actin filaments shown in Fig. 2 were taken at 20,000 \times , 60 kV.

Immunofluorescence Microscopy of Myofibrils

Glycerinated chicken muscle was prepared as described by Knight and Trinick (18). Myofibrils were prepared from this muscle in the presence of protease inhibitors (0.4 mM diisopropyl fluorophosphate, 0.5 mM PMSF, 5 $\mu\text{g/ml}$ leupeptin, 5 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin A, and 5 mM EDTA) as previously described (3). The rest of the procedure was carried out at 24 $^{\circ}\text{C}$. 10- μl vol of a fresh concentrated myofibrillar suspension was allowed to settle on glass slides for 2 min. The slides were then rinsed with PBS

and fixed for 5 min in 0.1% glutaraldehyde in PBS. The fixation reaction was then quenched with two 5-min washes with 0.1% sodium borohydride in PBS. The slides were then subjected to two 10-min washes with PBS and exposed to the first antibody (10 μ l of 15 μ g/ml affinity-purified anti-Cap $Z_{(36/32)}$ antibody, nonimmune IgG, rabbit anti-keyhole limpet hemocyanin antibody, or affinity-purified anti-Cap $Z_{(36/32)}$ plus excess anti-Cap $Z_{(36/32)}$) for 30 min. After repeat PBS washes, the slides were incubated with the FITC-conjugated anti-rabbit goat IgG (Cappel Laboratories, Cochranville, PA) at a 1:100 dilution in 0.5% albumin for 30 min, followed by washing with PBS. The specimens were then mounted in 0.1 M NaCl, 50 mM Tris-HCl, 0.2% Na₂S₂O₃, containing 10% glycerol and 1 mg/ml paraphenylenediamine (final pH 9.0) to reduce quenching of the fluorescence.

Tissue Preparation for Immunolabeling

Tissue was prepared for both immunofluorescence and immunoelectron microscopy by the following method. 9-wk-old chickens were decapitated and a thin (~5-mm wide by 1-mm thick) strip of pectoralis muscle was removed from the surface of the muscle with a razor blade, making sure that the origin and insertion remained attached. Working quickly, the strip of muscle was tied firmly to a flat wooden splint with dental floss. The muscle was removed and submerged in 100 ml of 0.5% glutaraldehyde in PBS plus 10 mM EGTA, pH 7.4 on ice. After 20 min, the splint was removed, and the muscle was cut into 5-mm squares and left in fixative on ice for 40 min. Afterwards, the pieces were trimmed to 1-mm square and set in a fresh 100 ml of 0.5% glutaraldehyde in PBS at 0°C for 2 h. The muscle pieces were then infiltrated with 2.3 M sucrose in deionized water by rotating them at 4°C for 1 h in the sucrose solution. After infiltration, the muscle was oriented transversely or longitudinally, placed on copper pegs, and immersed in liquid nitrogen for storage and later ultracryomicrotomy.

Cryostat sections, 0.08–0.5- μ m thick, were made on an LKB ultratome U with an attached cryo kit. Glass knives were broken with a LKB knife-breaker using the Griffith setting, and then tungsten-coated (26). Sections for light microscopy were cut at –60°C and were ~0.5- μ m thick. Sections were mounted on slides that were previously coated with a solution of 0.5% gelatin and 0.05% chromium alum and allowed to dry. 0.5- μ m sections were picked up with a drop of 2.3 M sucrose suspended on a copper wire loop. The frozen sucrose drop with section was allowed to thaw and then was touched to the prepared slides. The slides were placed in PBS for 5 min, and then quenched in 0.05% sodium borohydride in PBS three times, for 5 min each wash. The slides were then washed in PBS for 2 min and incubated in 5% BSA for 5 min. Immunostaining of the tissue sections for light microscopy was carried out as described above for myofibrils.

For electron microscopy, sections were cut to 80 nm at –90°C. The sections were picked up and placed on parlodian- and carbon-coated 200-mesh copper grids. Grids with sections were floated on the solutions as described by Tokuyasu et al. (31). The grids were first floated on 5% BSA for 5 min. Sections were labeled for 1 h with goat anti-rabbit IgG adsorbed to 5- or 15-nm gold particles (Jannsen Life Sciences Products, Piscataway, NJ) diluted 1:20 in 5% BSA. The grids were washed twice in 5% BSA, 5 min per wash. This was followed by six washes for 1 min in PBS, and six washes for 1 min in 0.25 M sucrose (isotonic).

Staining of Sections with Heavy Metals

1 ml of 0.25 M sucrose was saturated with uranyl formate (~3%). The sections were stained for 5 min on a drop of uranyl formate (pH 7.0) for 5 min, and then on uranyl formate (pH 6.0) for 5 min. Grids were then embedded in methylcellulose as described by Tokuyasu (31).

Results

Specificity of the Anti-Cap $Z_{(36/32)}$ Antibodies

Affinity-purified antibodies prepared as described in Materials and Methods recognized only the beta (M_r 32,000) subunit of Cap $Z_{(36/32)}$ in immunoblots of myofibrils and whole muscle (Fig. 1 A). As in previous studies in which antibodies were affinity-purified on immunoblots (3), no crossreactivity between the alpha- and beta-subunits of Cap $Z_{(36/32)}$ was seen. Absorption of the antibodies against crude M-line extracts was necessary, however, since even antibodies prepared using Cap $Z_{(36/32)}$ affinity columns showed weak reac-

tivity with M-line components in immunoblots and immunofluorescence assays. Whether this M-line reactivity is a result of true crossreactivity between Cap $Z_{(36/32)}$ and other M-line proteins or the presence of contaminating antibodies has not been determined. We were unable to obtain antibodies against the alpha-band of Cap $Z_{(36/32)}$ suitable for immunofluorescence studies. The blot purified antibodies against only the beta-subunit of Cap $Z_{(36/32)}$ used in previous studies immunoprecipitated equal amounts of the alpha- and beta-subunits of radiolabeled native protein (Fig. 1 B), confirming previous physical data that indicate that they are associated in solution (3).

Localization of Cap $Z_{(36/32)}$ to the Barbed End of Actin Filaments

Cap $Z_{(36/32)}$ was localized to the barbed end of cross-linked, S1-decorated actin–Cap $Z_{(36/32)}$ complexes using the affinity-purified antibodies and protein A-coated colloidal gold particles (Fig. 2). 17% of the filaments were labeled solely at the barbed end. The ratio of labeled barbed ends to labeled pointed ends was greater than 100:1. This finding corroborates previous biochemical data indicating that Cap $Z_{(36/32)}$ binds selectively to the barbed end of actin filaments and has the advantage of allowing evaluation of the association of the protein with the sides of actin filaments as well. The quantitative analysis of the labeling in this experiment (Table I) shows that slightly more gold particles were seen in association with the sides of actin filaments in samples containing anti-Cap $Z_{(36/32)}$ than in those containing preimmune antibodies. Although this difference is statistically significant ($P < 0.01$), the number of side-labeled filaments is small; the actual significance of this finding is unclear.

Immunolocalization of Cap $Z_{(36/32)}$ in Myofibrils and Frozen Sections of Muscle

Isolated myofibrils stained with the antibodies against the beta-subunit of Cap $Z_{(36/32)}$ showed fluorescence in the Z-line of myofibrils with no other staining above background (Fig. 3, A–D). The specificity of this reaction was established by the absence of Z-line staining when preimmune antibodies or an irrelevant IgG (rabbit anti-keyhole limpet hemocyanin) was substituted for the affinity-purified antibodies, and by obliteration of the immunofluorescent staining by preincubation of the affinity-purified antibodies with purified Cap $Z_{(36/32)}$. This pattern of localization was also observed in unfixed myofibrils and in myofibrils fixed with either 0.1%

Table I. Immunolocalization of Cap $Z_{(36/32)}$ to the Barbed End of Actin Filaments

Site of label	Barbed	Pointed	Side	None	Total filaments
Immune	116 (17.4%)	1 (0.15%)	19 (2.84%)	533 (79.8%)	668
Preimmune	1 (0.14%)	2 (0.28%)	10 (1.4%)	698 (98.2%)	711

S1-decorated actin filament–Cap $Z_{(36/32)}$ complexes were exposed to affinity-purified anti-Cap $Z_{(36/32)}$ antibodies and labeled with protein A–colloidal gold as described in Materials and Methods. Results are expressed as the number and percent (in parentheses) of filaments showing gold particles on their sides and on barbed and pointed ends.

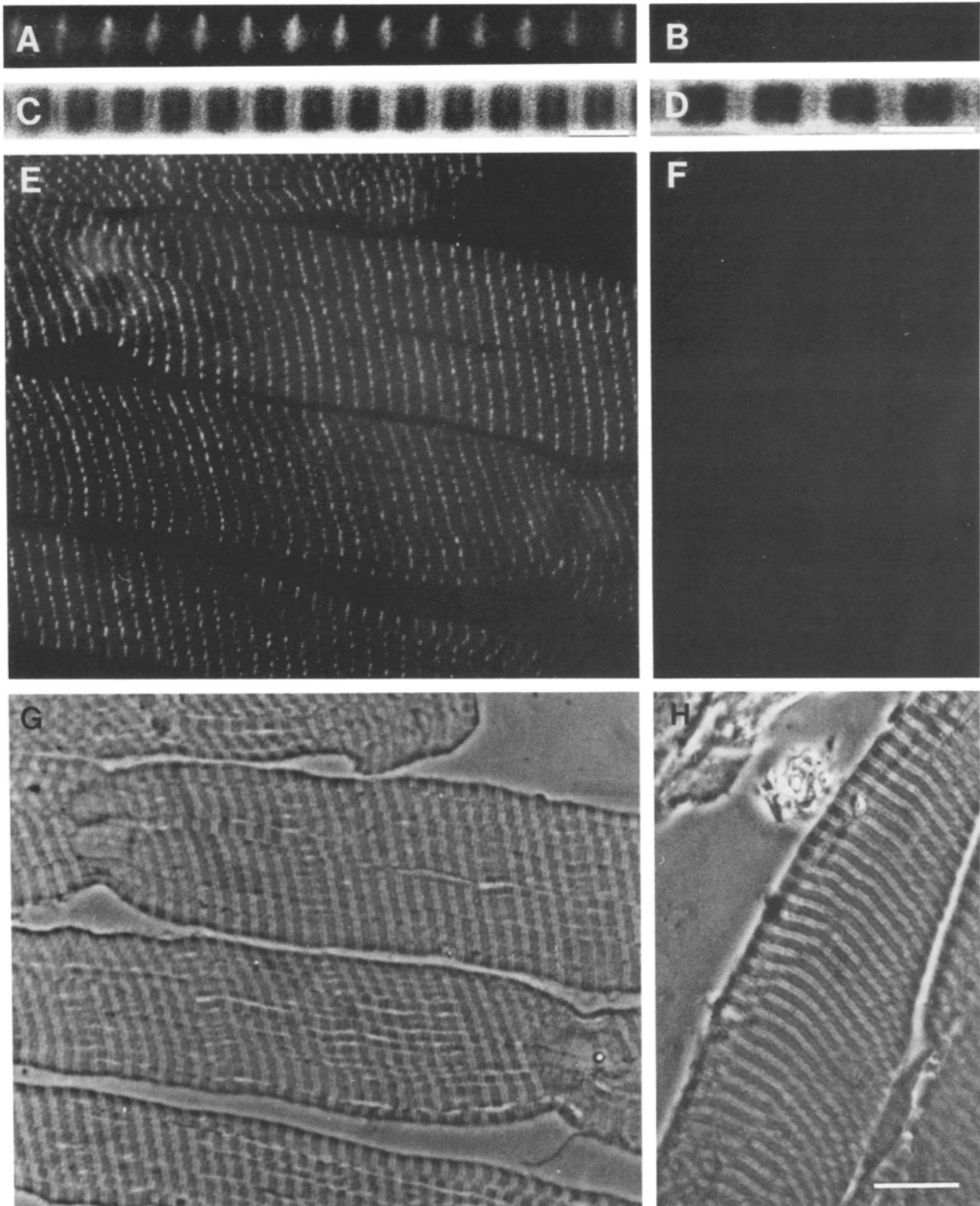
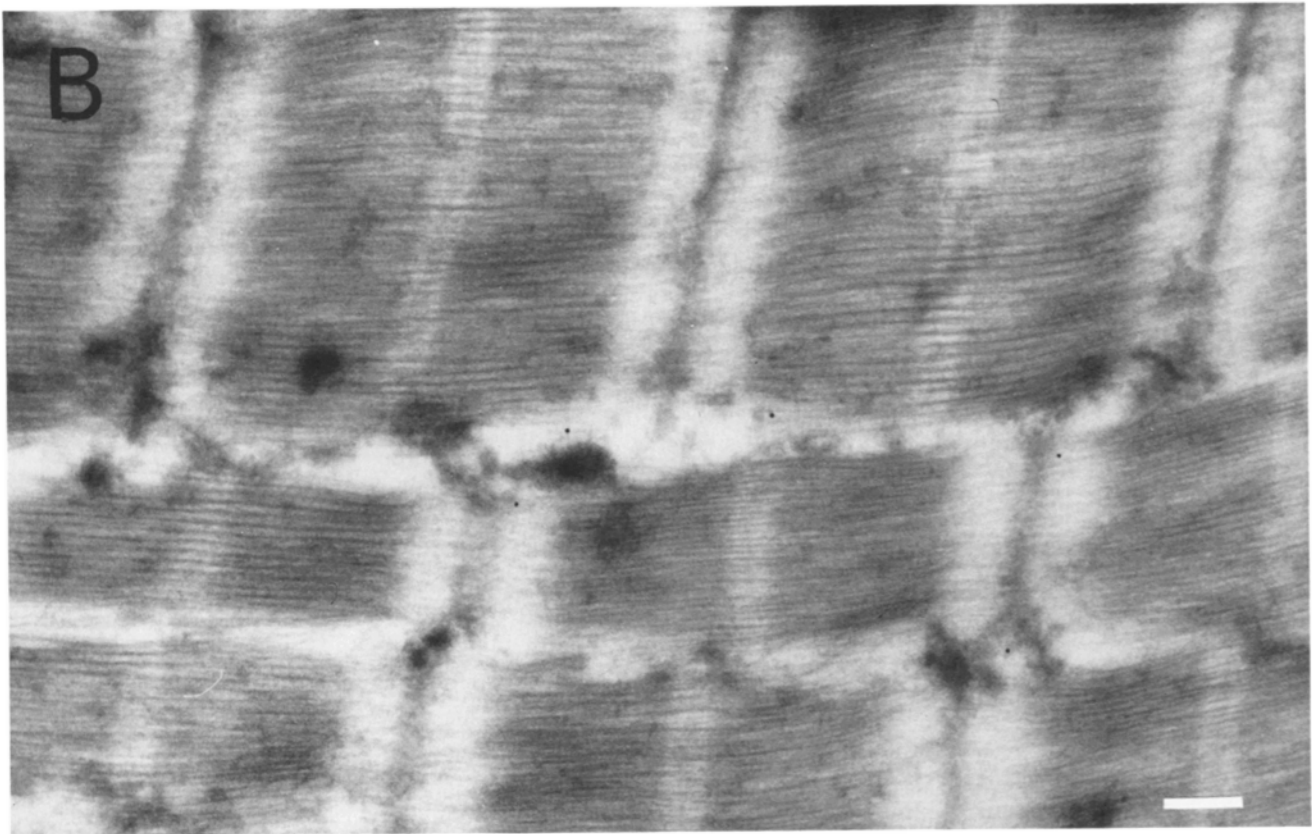
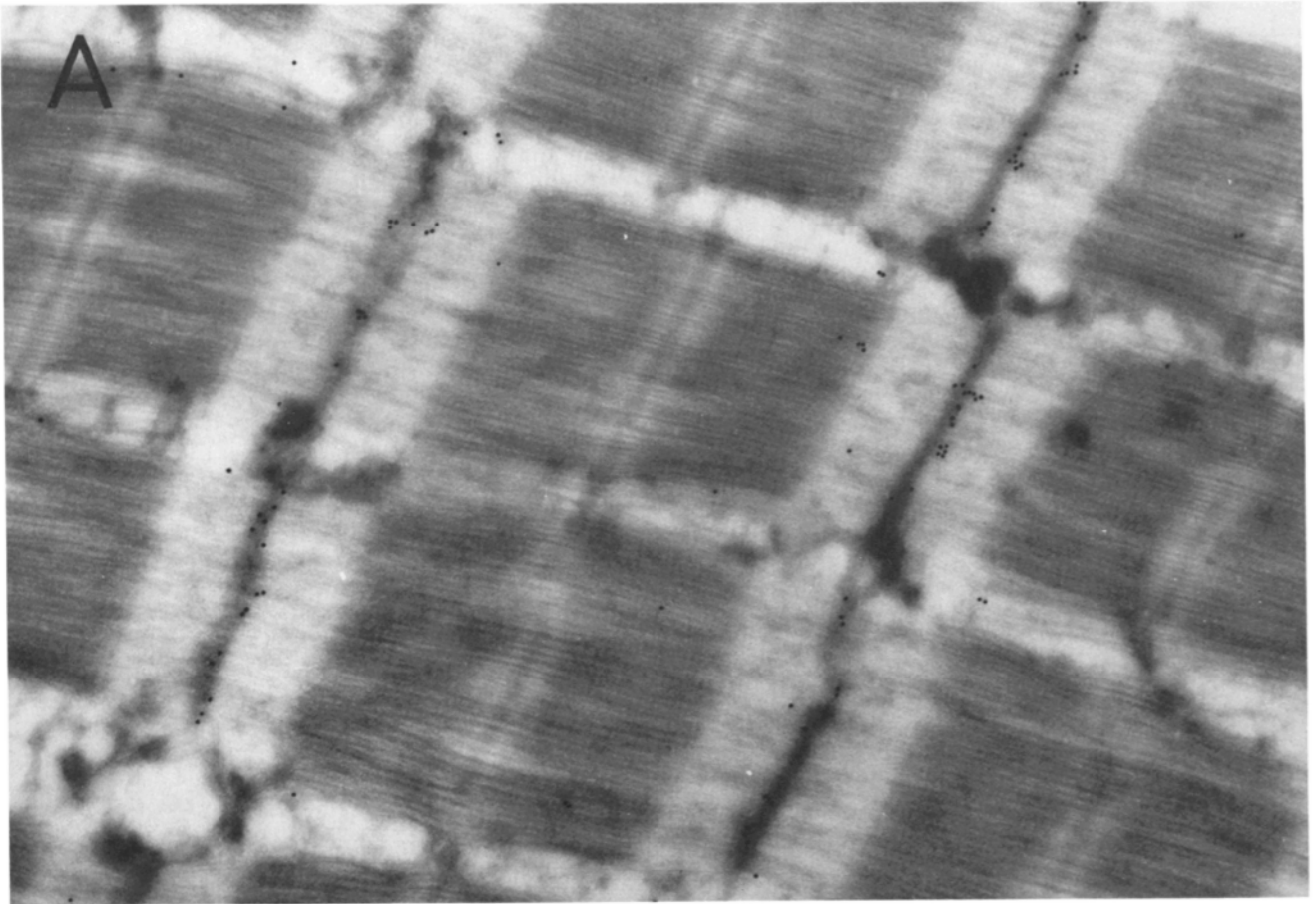


Figure 3. Immunolocalization of Cap $Z_{(36/32)}$ in sections of whole muscle and in myofibrils. Fluorescent (*A* and *B*) and phase (*C* and *D*) images of myofibrils exposed to affinity-purified anti-Cap $Z_{(36/32)}$ (*A* and *C*) and preimmune (*B* and *D*) antibodies. Fluorescent (*E*) and phase (*G*) images of a 0.5- μm frozen section of chicken pectoralis muscle exposed to affinity-purified anti-Cap $Z_{(36/32)}$ antibodies are compared with fluorescent (*F*) and phase (*H*) images of a similar section exposed to preimmune antibodies. Bar: (*A-D*) 3 μm ; (*E-H*) 20 μm .



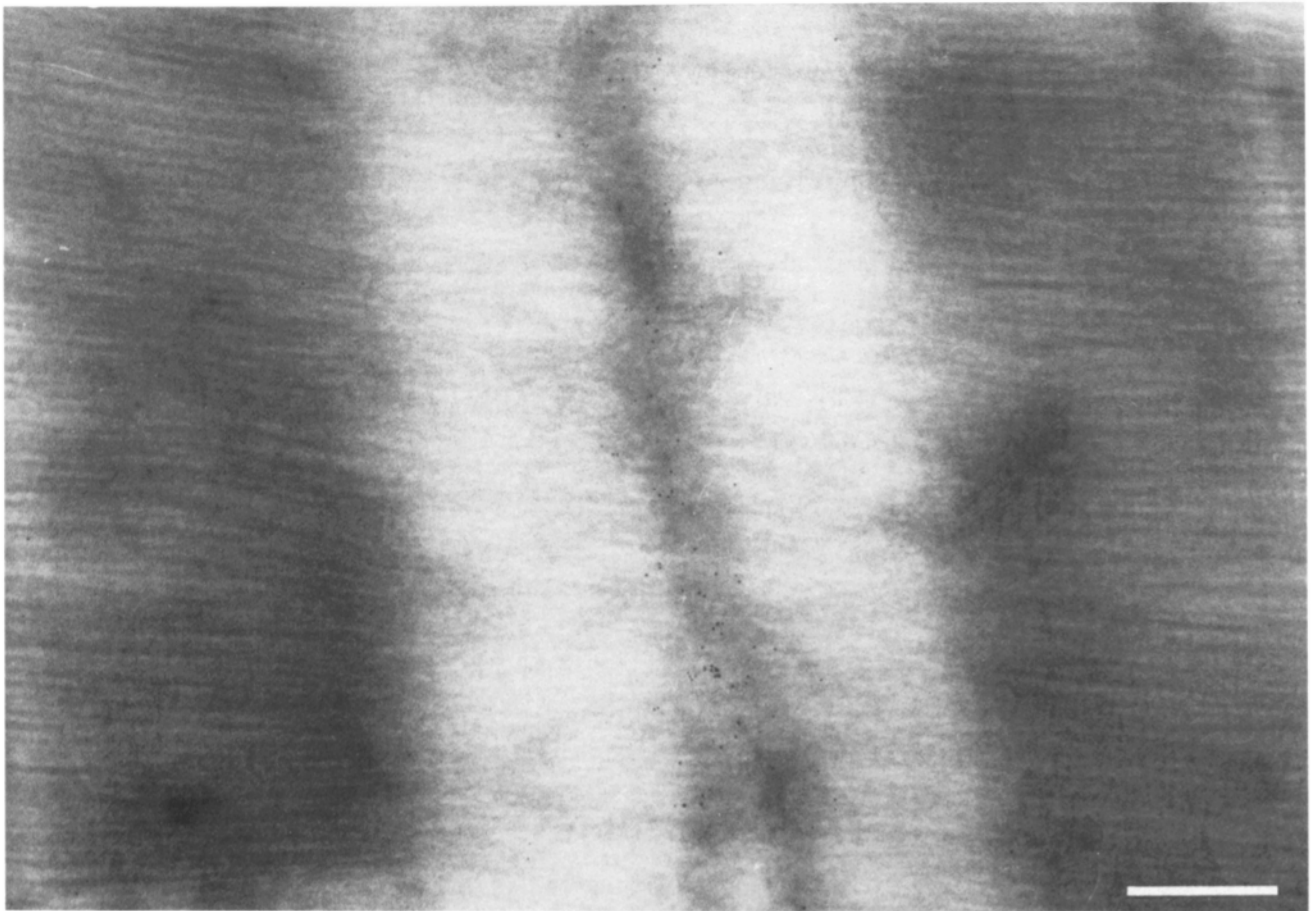


Figure 5. Immunolocalization of Cap $Z_{(36/32)}$ in frozen sections of muscle by electron microscopy (5-nm gold particles). Sections of skeletal muscle prepared identically to those shown in Fig. 4 were labeled with 5-nm gold particles, as compared with the 15-nm particles shown in the previous figure. Note the higher density of labeling of the Z-line. Bar, 0.3 μm .

glutaraldehyde, 3% paraformaldehyde, or Nakane's fixative, although all of these fixatives reduced the intensity of the Z-line staining.

Longitudinal cryostat sections of whole chicken pectoralis muscle showed a pattern of staining identical to that seen in myofibrils; in addition, there was no visible staining of sarcolemma or intracellular organelles (Fig. 3, *E-H*). Transverse sections of muscle (data not shown) showed no other staining as well. These findings suggest that Cap $Z_{(36/32)}$ is a constituent of, and possibly limited to the Z-line of skeletal muscle, the site of insertion of the barbed ends of actin filaments in the contractile apparatus.

Immunolocalization of Cap $Z_{(36/32)}$ in Ultrathin Frozen Sections of Muscle by Electron Microscopy

Cap $Z_{(36/32)}$ was also localized to the Z-line in ultrathin cryostat sections of chicken pectoralis muscle (Figs. 4 and 5). These ultrastructural studies confirm the result obtained at the level of light microscopy. In addition, the electron

microscopy results suggest that Cap $Z_{(36/32)}$ is distributed throughout the Z-disk, in contrast to some other Z-line proteins, e.g., desmin, synemin, and spectrin, which are found only at the periphery of the Z-disk (7, 9, 10, 13). As was the case in light microscopy studies, no other staining above background was observed. However, these results do not preclude the presence of Cap $Z_{(36/32)}$ at lower concentrations at other sites or in structures that may be poorly preserved by the techniques used in this study.

Discussion

The studies described in this paper provide a direct demonstration that an actin-capping protein localizes specifically to the barbed ends of actin filaments in vitro and to an anatomical site associated with the barbed ends of actin filaments in situ. Previous conclusions on the site of action of actin-capping proteins have been drawn from kinetic and stoichiometric data; however, attempts to localize capping proteins on purified actin filaments using immunological techniques

Figure 4. Immunolocalization of Cap $Z_{(36/32)}$ in frozen sections of muscle by electron microscopy (15-nm gold particles). Cryostat sections of muscle were exposed to affinity-purified anti-Cap $Z_{(36/32)}$ (*A*) or preimmune (*B*) antibodies followed by 15-nm protein A-coated colloidal gold particles as described in Materials and Methods. Bar, 0.3 μm .

have been unsuccessful (25). The success of the experiments reported herein is most likely due to experimental procedures involving cross-linking of the antigen to its binding site before exposure of the ligand-receptor complex to washes, thereby preventing inadvertent removal of bound protein. The frequency of labeling of actin filaments in these experiments was also markedly enhanced by removal of unbound protein by gel filtration before exposure to antibodies. These techniques supplement and extend available biochemical methods of determining binding of proteins to actin, in which small amounts of protein binding to the sides of actin filaments may be difficult to detect. It will be important to determine whether proteins that appear to sever as well as cap actin filaments (e.g., gelsolin [38], villin [1, 5, 12, 23], fragmin, [14] and severin [11]) show a higher incidence of binding to the sides of actin filaments than those that appear to bind only to the ends of actin filaments (e.g., the *Acanthamoeba* [4, 16], *Dictyostelium* [28], brain [17] capping proteins, and Cap $Z_{(36/32)}$).

In other studies of the localization of capping proteins in cells, gelsolin and the *Acanthamoeba* capping proteins have been localized to actin-rich areas of cells. Gelsolin has been immunolocalized to the area around actively phagocytic regions of the cytoplasm in leukocytes (37). Gelsolin has also been localized to actin-containing rosette structures in cells transformed by Rous sarcoma virus (34) and to the I-band of skeletal and cardiac muscle (27, 37). However, one recent study failed to localize gelsolin in the I-band of muscle and suggested that the previous results need to be re-evaluated (2). The *Acanthamoeba* capping protein has been localized to the actin-rich subcortical region of the amoeba (4). However, in none of these studies was the capping protein in question identified at a known site of termination of the barbed ends of actin filaments.

The present study defines Cap $Z_{(36/32)}$ as a new Z-line protein. Although a number of the Z-line proteins, including alpha-actinin (30), zeugmatin (20), Z-proteins (21, 24), and spectrin (22), have been implicated in anchorage of actin filaments at the Z-line by their location, only Cap $Z_{(36/32)}$ has been shown to bind to the barbed end of actin filaments in vitro. Alpha-actinin binds to the side of actin filaments in vitro. In fact, the molecular reason for restriction of alpha-actinin to the Z-line of intact muscle is uncertain, although evidence suggests that at 37°C tropomyosin is an effective competitor for side-binding to actin filaments (30). Nevertheless, no direct evidence exists to suggest that alpha-actinin binds directly to the ends of actin filaments. In contrast, Wilkins et al. (35) have recently reported that polyclonal antibodies to HA1, a heterogeneous group of proteins from avian smooth muscle, localized to the Z-line of striated muscle and to focal adhesive sites of nonmuscle cells. This finding is of interest in that HA1 was first identified by its ability to inhibit monomer association at the barbed end of actin filaments. The same study also showed that many of the HA1 polypeptides are most likely proteolytic fragments of larger (200 and 150 kD) polypeptides. As the authors indicate, however, it is not clear that the 200- and 150-kD polypeptides are responsible for the actin-capping activity of the HA1 preparation, since the 200- and 150-kD polypeptides have not yet been purified; although the ability of HA1 to affect the low-shear viscosity of actin was reduced after immunoprecipitation with the anti-HA1 antibodies, antibodies

affinity-purified against the 200- and 150-kD polypeptides did not have the same effect. Further characterization of the HA1 polypeptides and their parent proteins will be required before meaningful comparisons with Cap $Z_{(36/32)}$ can be made. However, the possibility that the HA1 polypeptides and Cap $Z_{(36/32)}$ represent proteins from smooth and striated muscle, respectively, with similar activities is an exciting one.

The presence of Cap $Z_{(36/32)}$ in the Z-line of skeletal muscle indicates that this protein is in an appropriate position to regulate the number, length, orientation, and attachment of actin filaments in muscle. Unfortunately, localization of Cap $Z_{(36/32)}$ with respect to the known substructure of the Z-line was not possible because antigenicity was lost in fixatives more stringent than 0.1% glutaraldehyde. Such fine structural localization of Cap $Z_{(36/32)}$ will be necessary to relate the localization of Cap $Z_{(36/32)}$ to existing models of Z-line structure, such as those proposed by Yamaguchi et al. (36), in which actin filaments of opposite polarity are tethered to one another by diagonal Z-filaments consisting primarily of alpha-actinin. One can speculate that Cap $Z_{(36/32)}$ might function in such a model by either (a) mediating Z-filament to actin filament connections, or (b) forming an additional site for attachment of actin filaments at their barbed ends. Further conclusions must await a systematic investigation of the interactions between Cap $Z_{(36/32)}$ and other Z-line constituents.

This study raises several interesting questions. For example, is Cap $Z_{(36/32)}$ a constituent of other sites where actin filaments terminate, such as the plasma membrane, adhesion plaques, or intercalated discs? Are there "Z-line equivalents" containing Cap $Z_{(36/32)}$ or other capping proteins in nonmuscle cells that allow force transduction between opposing actin filaments? Answers to these questions will require further investigation of Cap $Z_{(36/32)}$ at a cellular level.

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