

Mechanisms of Thin Filament Assembly in Embryonic Chick Cardiac Myocytes: Tropomodulin Requires Tropomyosin for Assembly

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Abstract. Tropomodulin is a pointed end capping protein for tropomyosin-coated actin filaments that is hypothesized to play a role in regulating the precise lengths of striated muscle thin filaments (Fowler, V. M., M. A. Sussman, P. G. Miller, B. E. Flucher, and M. P. Daniels. 1993. *J. Cell Biol.* 120:411–420; Weber, A., C. C. Pennise, G. G. Babcock, and V. M. Fowler. 1994. *J. Cell Biol.* 127:1627–1635). To gain insight into the mechanisms of thin filament assembly and the role of tropomodulin therein, we have characterized the temporal appearance, biosynthesis and mechanisms of assembly of tropomodulin onto the pointed ends of thin filaments during the formation of striated myofibrils in primary embryonic chick cardiomyocyte cultures. Our results demonstrate that tropomodulin is not assembled coordinately with other thin filament proteins. Double immunofluorescence staining and ultrastructural immunolocalization demonstrate that tropomodulin is incorporated in its characteristic sarcomeric location at the pointed ends of the thin filaments *after* the thin filaments have become organized into periodic I bands. In fact, tropomodulin

assembles later than all other well characterized myofibrillar proteins studied including: actin, tropomyosin, α -actinin, titin, myosin and C-protein. Nevertheless, at steady state, a significant proportion (~39%) of tropomodulin is present in a soluble pool throughout myofibril assembly. Thus, the absence of tropomodulin in some striated myofibrils is not due to limiting quantities of the protein. In addition, kinetic data obtained from [³⁵S]methionine pulse-chase experiments indicate that tropomodulin assembles more slowly into myofibrils than does tropomyosin. This observation, together with results obtained using a novel permeabilized cell model for thin filament assembly, indicate that tropomodulin assembly is dependent on the prior association of tropomyosin with actin filaments. We conclude that tropomodulin is a late marker for the assembly of striated myofibrils in cardiomyocytes; its assembly appears to be linked to their maturity. We propose that tropomodulin is involved in maintaining and stabilizing the final lengths of thin filaments after they are assembled.

ASSEMBLY of myofibrils during striated muscle differentiation is a complex process that requires coordinate expression of the constituent proteins and their association into highly organized sarcomeres. Multiple proteins appear to be responsible for specifically regulating the length, polarity, stability and spatial organization of thin filaments during myofibrillogenesis: properties which are required for efficient contraction. Proteins associated with the barbed (fast-growing) ends of muscle thin filaments at the Z disk assemble early during myofibrillogenesis; for example, the actin filament barbed end capping protein, capZ and the actin cross-linking protein, α -actinin (for example, see Sanger et al., 1986; Schultheiss et al., 1990; Schafer et al.,

1993). In contrast, the regulation of thin filament length and the assembly of proteins associated with the pointed (slow-growing) ends of thin filaments during myofibril assembly has not been studied. This is because no proteins that associate with actin filament pointed ends have been identified until recently.

Tropomodulin is a tropomyosin- and actin-binding protein that is specifically associated with the pointed ends of thin filaments in rat skeletal muscle (Fowler et al., 1993). Tropomodulin was originally identified in the erythrocyte membrane skeleton on the basis of its ability to bind tropomyosin; it binds to the NH₂-terminal end of tropomyosin and blocks tropomyosin head-to-tail associations along actin filaments (Fowler, 1987, 1990; Sung and Lin, 1994). Recently, it was found that tropomodulin is a pointed end capping protein; it blocks elongation and depolymerization at the pointed ends of tropomyosin-coated actin filaments. In the absence of tropomyosin, tropomodulin is a "leaky" cap,

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only partially blocking elongation and depolymerization (Weber et al., 1994). In this study, we used immunolocalization techniques and biochemical approaches to examine the mechanism of thin filament assembly at the pointed end in embryonic chick cardiomyocyte cultures. We were interested in whether tropomodulin was assembled in myofibrils prior to, coordinate with, or after other contractile proteins, and whether tropomodulin association with the pointed ends of thin filaments was coincident with the organization of thin filaments into I band periodicities.

Unexpectedly, our results indicate that tropomodulin is not assembled coordinately with tropomyosin or other thin filament proteins and is incorporated at the pointed ends of the thin filaments only after the appearance of periodic I bands. In fact, tropomodulin appears to assemble later than all other myofibrillar proteins studied including: actin, tropomyosin, α -actinin, titin, myosin, and C-protein. Unlike tropomyosin, a significant proportion of tropomodulin is not found in myofibrillar (Triton X-100 insoluble) structures, but is present as a soluble pool throughout myofibril assembly. This suggests that the absence of tropomodulin in some striated myofibrils is not due to the absence of the protein in the cell. Furthermore, tropomodulin appears to require tropomyosin for its assembly onto the pointed ends of thin filaments. Our results indicate that thin filament ends are not capped early in myofibrillogenesis and that pointed capping by tropomodulin may be involved in maintaining the final length of thin filaments in mature striated myofibrils.

Materials and Methods

Cell Culture

Cardiomyocytes were prepared from day 6 embryonic chick hearts by treatment with 0.045% collagenase (Worthington, Freehold, NJ) in calcium and magnesium-free Hanks' balanced salt solution for 5 min in a 37°C shaking incubator, followed by vortexing at low speed for ~10 s. After settling at 1 g, the solution containing dissociated cells was removed and fresh enzyme solution was added to the remaining tissue. This step was repeated six times. The first two supernatants were discarded; dissociated cells in the next four supernatants were spun down and resuspended in fresh nutrient medium (minimum essential medium with Earl's salts plus 5% "selected" fetal bovine serum, 2 mM glutamine, 50 U ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin; Lin et al., 1989). Dispersed cells were preplated for 1 h at 37°C to remove fibroblasts. Cells were plated at an initial density of 1×10^5 ml⁻¹ on autoclaved glass coverslips (Fisher Scientific, Springfield, NJ) for the permeabilized cell model and immunofluorescence analysis or in 35 mm tissue culture petri dishes for the biochemical studies. The day after plating, the cells were fed with glutamine-free nutrient medium and every other day thereafter.

Purified Proteins and Antibodies

Rabbit skeletal muscle tropomyosin and human erythrocyte tropomodulin were purified as previously described (Bailey, 1948; Fowler, 1990). Antibodies to purified human erythrocyte tropomodulin and tropomyosin were generated in rabbits and affinity purified as described (Fowler et al., 1993; Ursitti and Fowler, 1994). Antibodies to human erythrocyte tropomodulin cross-react specifically with a 43,000 M_r polypeptide on immunoblots of embryonic cardiomyocytes or perfused adult chicken heart muscle (data not shown). Furthermore, immunoprecipitated cardiac muscle tropomodulin, like immunoprecipitated skeletal muscle tropomodulin, was shown by an ¹²⁵I-tropomyosin blot overlay to be a specific tropomyosin-binding protein (data not shown: see Fowler, 1987 and Fowler et al., 1993). For some studies, purified tropomodulin and tropomyosin or anti-tropomodulin antibodies were biotinylated according to the manufacturer's instructions (Zymed, San Francisco, CA). Monoclonal anti-sarcomeric myosin heavy

chain (MHC)¹ antibodies (F4G3) were a generous gift from Dr. F. Pepe (University of Pennsylvania, Philadelphia, PA); these antibodies recognize MHC from cardiac and skeletal muscle cells only (for example see Lin et al., 1989). Anti-C-protein (clone MFI; Reinach et al., 1982) and anti-chicken muscle tropomyosin (CHI; Lin et al., 1985) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) under contract NOI-HD-6-2915 from the NICHD. Anti- α -actinin (clone BM-75.2) and anti-titin T11 monoclonal antibodies, and rhodamine-labeled phalloidin (rho-phalloidin) were purchased from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes Inc. (Eugene, OR), respectively. Anti-titin T12 antibodies and all other immunoreagents were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Indirect Immunofluorescence Microscopy

Cardiac muscle cells were fixed and stained as described (Gregorio et al., 1988). Briefly, coverslips were rinsed in PBS, fixed in 1.7% formaldehyde/PBS for 5 min, washed in PBS, and permeabilized in 0.2% Triton X-100/PBS for 15 min. To minimize non-specific binding of antibodies, the coverslips were pre-incubated in 1 mg/ml⁻¹ BSA/PBS for 30 min. For double staining of tropomodulin and other sarcomeric components, coverslips were incubated with anti-tropomodulin antibodies (7 μ g/ml⁻¹), followed by fluorescein-conjugated goat anti-rabbit IgG (1:200), followed by monoclonal anti- α -actinin (1:200), anti-myosin (1:100), anti-T11 (1:1,000), anti-T12 (1 μ g/ml⁻¹), or anti-C-protein antibodies (1:200) and rhodamine-conjugated sheep anti-mouse IgG (1:50). For double staining of tropomodulin and F-actin, cells were stained for tropomodulin as described above, followed by incubation in rho-phalloidin (1:400). To double stain for tropomodulin and tropomyosin, coverslips were incubated sequentially with anti-tropomyosin antibodies (10 μ g/ml⁻¹), rhodamine-conjugated goat anti-rabbit IgG (1:200), normal rabbit serum (1:100), biotinylated anti-tropomodulin antibodies (1:100), and fluorescein-conjugated avidin (1:400). Reconstituted biotinylated proteins were detected using avidin-FITC (1:400). Secondary antibodies alone, or in combination with pre-immune sera, consistently showed negligible fluorescence. The pattern of immunofluorescence staining did not depend on the sequence of antibodies used. Stained coverslips were observed with a Zeiss Axiophot and micrographs were recorded on Kodak T-MAX or Tri-X (400 ASA) film or a Biorad MRC 600 confocal microscope and stored as digital images.

Electron Microscopy

Chick cardiomyocyte cultures were plated directly on plastic tissue culture dishes. After 7 d in culture, the cells were washed in PBS, fixed in periodate-lysine-paraformaldehyde fixative (PLP) (McLean and Nakane, 1974) for 3 h at room temperature and immunostained for tropomodulin according to a modified procedure used to immunostain for other membrane skeletal proteins described by Black et al. (1988) and Gregorio et al. (1994). Briefly, after a wash in PBS, the fixed cells were incubated in 50 mM NH₄Cl in PBS for 30 min (to quench free aldehyde groups) and then permeabilized in PBS/0.1% BSA/0.02% saponin (PBS/BSA/SAP) with gentle agitation. Cells were incubated overnight with affinity purified anti-erythrocyte tropomodulin antibodies (15 μ g/ml⁻¹ in PBS/BSA/SAP). After extensive washing in PBS/BSA, the cells were incubated in peroxidase-conjugated goat anti-rabbit IgG (1:50 in PBS/BSA/SAP) for 1 h. Following several washes in PBS/BSA, the cells were fixed for 1 h in 2% glutaraldehyde/0.1 M phosphate buffer, pH 7.4, containing 7.5% sucrose, and washed extensively (three changes in phosphate buffer/7.5% sucrose, followed by three changes in 50 mM Tris-HCl, pH 7.6/7.5% sucrose, for a total of 90 min) prior to incubation in 0.2% diaminobenzidine in Tris-HCl/7.5% sucrose for 30 min. To visualize the sites of antibody binding, H₂O₂ was then added (to a final concentration of 0.01%) and incubation was continued for 15–30 min. The cultured cells were washed, post-fixed in 1% OsO₄, dehydrated in a graded alcohol series and embedded in Spurr's resin. Sections were cut parallel to the plate and viewed on a Hitachi-HU12A electron microscope.

1. *Abbreviations used in this paper:* MHC, myosin heavy chain; NSMF, non-striated myofibrils; PLP, periodate-lysine-paraformaldehyde; rho-phalloidin, rhodamine-conjugated phalloidin; SMF, striated myofibrils; TM, tropomyosin; Tmod, tropomodulin.

Metabolic Labeling and Immunoprecipitation

In vitro labeling of adherent cardiomyocytes with Tran^[35S]-label (ICN Pharmaceuticals, Inc., Irvine, CA) was performed in triplicate. Each petri dish was washed three times and preincubated for 20 min in 2 ml of methionine-free medium (MEM without methionine (GIBCO BRL, Gaithersburg, MD), supplemented with 5% heat-inactivated, dialyzed FBS). The cells were then incubated in 0.5 ml of methionine-free medium containing 1 mCi/ml of Tran^{35S}-label for 10 min (pulse-chase experiments) or in 1 ml of medium containing 20–200 $\mu\text{Ci/ml}^{-1}$ for 1–24 h at 37°C (steady state labeling experiments). At the completion of the labeling period, the cells were either solubilized directly in 0.4% SDS and processed for immunoprecipitation as described (Fowler et al., 1993) or chased with 2 ml of complete minimal essential nutrient medium containing an additional 2 mM of unlabeled methionine (GIBCO BRL) for varying periods of time at 37°C. The chase was stopped by a wash with ice-cold Ca^{2+} and Mg^{2+} -free PBS containing the following protease inhibitors: 100 $\mu\text{g/ml}^{-1}$ each of tosyl-L-lysyl chloromethyl ketone and phenylmethylsulfonyl fluoride, 5 $\mu\text{g/ml}^{-1}$ each of leupeptin and pepstatin A and 1 $\mu\text{g/ml}^{-1}$ aprotinin. The labeled cells were then extracted in a Triton X-100-containing cytoskeletal (myofibril) stabilization buffer (CSK buffer: 10 mM Pipes, pH 6.8, 100 mM KCl, 300 mM sucrose, 2.5 mM MgCl_2 , 0.5% Triton X-100 plus the protease inhibitors described above) for 5 min on ice on a rocking platform. The soluble fraction was removed and the insoluble fraction was collected by scraping the cells off of the petri dish with a cell lifter (Costar Corp., Cambridge, MA). The soluble and insoluble fractions were prepared and quantitatively immunoprecipitated as described (Fowler et al., 1993) with the exception that they were treated with 50 μg of diisopropyl fluorophosphate-treated DNase I (type II; Boehringer-Mannheim Biochemicals) for 20 min followed by the addition of EDTA (1 mM final) at room temperature prior to adding the extract to the beads. 1/25 of the extract was removed and added to protein A–trisacryl beads to which 4 μl of anti-muscle tropomyosin (CHI) antibodies (ascites fluid) had been adsorbed. The remainder of the extract was added to beads to which 2 μg of affinity-purified anti-human red cell tropomodulin antibodies had been adsorbed. These concentrations of antibodies were determined to quantitatively immunoprecipitate all tropomodulin and tropomyosin in the extracts as ascertained by sequential immunoprecipitation and autoradiography. Samples were electrophoresed on 12% polyacrylamide gels with a pH of 8.8 and a 3% stacking gel with a pH of 6.8 (Dreyfuss et al., 1984). After electrophoresis, gels were stained with Coomassie blue, processed for autoradiography with Enhance (New England Nuclear, Boston, MA), and dried under a vacuum. A quantitative measure of the level of protein associated with each fraction was obtained on a scanning phosphorimager using Image-Quant software (Molecular Dynamics, Sunnyvale, CA) and then exposed to Kodak XAR-5 x-ray film at -80°C .

Reconstitution of Tropomodulin onto Thin Filaments

Day 5 cardiomyocyte cultures grown on glass coverslips were permeabilized with 0.2 mg/ml^{-1} saponin in relaxing buffer (0.12 M KCl, 4 mM MgCl_2 , 20 mM Tris-HCl, pH 6.8, 4 mM EGTA, 4 mM ATP) for 5 min at 0°C. "Ghost myofibrils" were prepared by extraction of thick filaments in high salt buffer (0.5 M KCl, 5 mM MgCl_2 , 10 mM K-pyrophosphate, 1 mM EGTA, 10 mM Tris-HCl, pH 6.5, 0.2 mg/ml^{-1} saponin) for 10 min at room temperature (Huxley and Hanson, 1957; Ishiwata and Funatsu, 1985). The ghost myofibrils were then incubated in buffer containing 20 mM KCl, 5 mM Tris-HCl, pH 6.8, 0.1 mM CaCl_2 , 0.1 mM ATP, 0.2 mg/ml^{-1} saponin for ~ 15 s.

To reconstitute exogenous tropomodulin onto ghost myofibrils, the extracted cells were first incubated with or without tropomyosin at 0.25–0.5 mg/ml^{-1} in a buffer containing 0.1 M KCl, 10 mM Hepes, pH 7.5 (20 μl vol), for 10 min. The coverslips were subsequently rinsed and then incubated with biotinylated tropomodulin at 0.25–0.5 mg/ml^{-1} in a buffer containing 80 mM KCl, 2 mM MgCl_2 , 0.1 mM DTT, 20 mM Hepes, pH 7.3 (in a 20 μl vol), for 5–15 min at room temperature on a rocking platform. After rinsing the coverslips briefly in rigor buffer (60 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 10 mM Tris-HCl, pH 6.8, 0.2 mg/ml^{-1} saponin), the cells were fixed in formaldehyde and stained by immunofluorescence as described above. To rule out the possibility that the biotinylation of tropomodulin or tropomyosin affected the outcome of the assay, we also performed the assay with unlabeled proteins. The unlabeled proteins were detected using anti-tropomodulin or anti-tropomyosin antibodies as high probes: identical results were obtained (data not shown).

Results

Localization of Tropomodulin in Embryonic Chick Cardiomyocytes

Due to their flat, well-spread morphology, primary cultures of chick cardiomyocytes are particularly useful for detailed immunolocalization observations of the spatial relationships of assembling myofibrillar proteins. During approximately the first 2 d in culture, the preexisting myofibrils in isolated myocytes are disassembled. Meanwhile, as the myocytes attach and spread on the substrate, the cells concomitantly begin de novo synthesis and assembly of contractile proteins into new sarcomeres (e.g., Lin et al., 1989; Handel et al., 1991; Lu et al., 1992). At the earliest stages of assembly, microfilament bundles appear which resemble non-muscle stress fibers (referred to here as non-striated myofibrils; NSMFs) while, at later stages, these NSMFs are replaced by mature striated myofibrils (SMFs) (e.g., Peng et al., 1981; Dlugosz et al., 1984; Antin et al., 1986).

Tropomodulin had previously been localized by immunofluorescence microscopy to the pointed ends of thin filaments in isolated rat psoas skeletal myofibrils (Fowler et al., 1993). Due to the intrinsic difference between skeletal muscle and cardiac muscle (for example, skeletal muscle, but not cardiac muscle contains nebulin, a protein believed to be involved in specifying the length of thin filaments; Itoh et al., 1988), immunofluorescence confocal microscopy was used to localize cardiac muscle tropomodulin in fully assembled myofibrils in cultured cardiomyocytes (at a time when the cells were beating). Double-staining for F-actin (Fig. 1 *a*) and tropomodulin (Fig. 1 *b*) demonstrates that cardiac muscle tropomodulin is localized within the A bands at the ends of the thin filaments (merged image; Fig. 1 *c*). This staining is specific since a 40-fold molar excess of purified erythrocyte tropomodulin included in the primary antibody incubation eliminated all tropomodulin staining (data not shown).

Unlike the doublet observed for tropomodulin staining in isolated rat psoas myofibrils (Fowler et al., 1993), tropomodulin staining is visualized as a single band in cardiac myofibrils (Fig. 1 *a*). Therefore, an immunoperoxidase staining procedure was used to localize tropomodulin at the ultrastructural level. The electron micrographs in Fig. 2, *a* and *b* depict myofibrils immunostained for tropomodulin. Arrows indicate a region of dense reaction product within the AH zone where the thin filaments terminate. Staining is never detected at the Z disk or at any other location within the sarcomere (e.g., along the length of the thin filaments or in early Z disk-like structures; see below). Broad stripes of tropomodulin staining are the result of diffusion of the horseradish-peroxidase reaction product. Interestingly, the reaction product separated into two bands with increasing sarcomere length (Fig. 2, *b*, *d*, and *e*). Specifically, in sarcomeres with a length of ≤ 1.7 μm the reaction product is visualized as a single band. In sarcomeres with a Z disk distance of ≥ 1.8 μm , two distinct bands of reaction product could be resolved. These observations suggest that the inability to resolve two components of staining by immunofluorescence microscopy is due to the contracted state of the myofibrils and is consistent with our previous immunofluorescence localization of tropomodulin to the pointed ends of thin filaments in rat skeletal muscle myofibrils (Fowler et al., 1993).

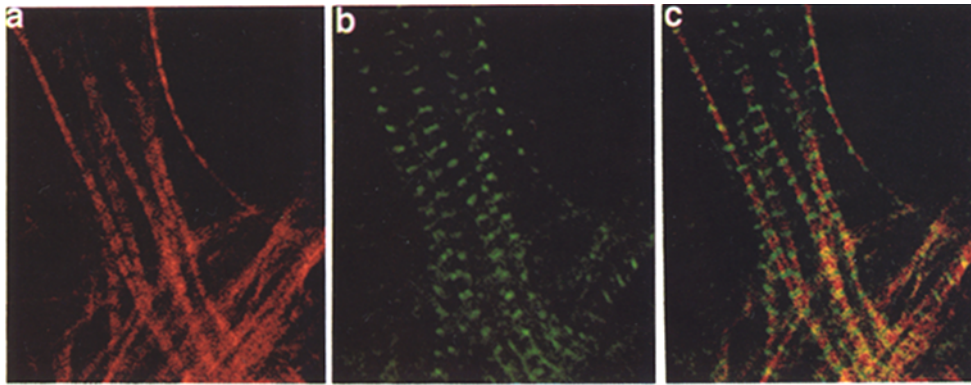


Figure 1. Confocal image of actin and tropomodulin in embryonic chick cardiomyocytes. Double fluorescence staining for (a) F-actin and (b) tropomodulin. (c) Merged image of a and b demonstrating that cardiomyocyte tropomodulin is localized within the A bands at the ends of the thin filaments. Failure to resolve each tropomodulin band into a doublet is likely due to the partial contraction of the myofibril. Note, the presence of green staining for tropomodulin (nonoverlap) in c is likely due to the size (~ 30 nm) and the limited accessibility/penetration of primary and secondary antibodies into the tightly packed thin filaments in I bands within intact sarcomeres.

ulin (nonoverlap) in c is likely due to the size (~ 30 nm) and the limited accessibility/penetration of primary and secondary antibodies into the tightly packed thin filaments in I bands within intact sarcomeres.

Assembly of Tropomodulin into Myofibrils with Respect to Thin Filament Components

To visualize the distribution of tropomodulin with respect to other sarcomeric proteins during cardiac myofibril assembly, days 3 through 7 cultures were double stained for tropomodulin and F-actin, α -actinin, tropomyosin, titin (T11 and T12 epitopes), myosin, or C-protein. Since the process of assembly is temporally irregular, a variety of structures are observed within the same cell and in different cells in the culture dish. Figs. 1 and 3 illustrate the range of structures that stain with rho-phalloidin. In some cells the majority of myofibrils are striated (Fig. 1 a); in others there is a mixture of SMFs which stain periodically and NSMFs which stain uniformly (Fig. 3, a and c). Many of the isolated cardiomyocytes that have contacted neighboring cardiomyocytes assemble intercalated disks (e.g., Fig. 3, c and d). Staining for tropomodulin and F-actin revealed that tropomodulin staining is not detected in NSMFs; this protein can only be detected in its characteristic sarcomeric location in SMFs (Figs. 1 b and 3, b and d, long arrows). Moreover, rho-phalloidin staining is observed in a sarcomeric striated pattern in many myofibrils which have no detectable tropomodulin staining (Fig. 3 c, short arrows). Consistent with this data is the observation that in early cultures which contain many more NSMFs than SMFs (days 3 and 4), very little if any tropomodulin staining could be detected. This is in contrast to late cultures (approximately days 5-7) where many myofibrils display tropomodulin in a striated pattern. Results from these experiments demonstrate that tropomodulin is

found at the pointed ends of thin filaments in only some of the myofibrils that contain actin in a striated pattern.

The absence of tropomodulin from NSMFs and some SMFs is not due to the absence of its other binding protein, tropomyosin, since tropomyosin is found associated with all nonstriated and striated myofibrils. In contrast, tropomodulin is detected at the pointed ends of thin filaments in only a subset of the tropomyosin-containing striated myofibrils (Fig. 4, a-d). In addition, although both nonmuscle and muscle tropomyosin isoforms are assembled into the same sets of microfilaments from skeletal myogenic cells, and chick cardiomyocytes contain nonmuscle and muscle tropomyosin isoforms in the same myofibrils (Lin et al., 1984; Handel et al., 1991), the presence or absence of tropomodulin staining does not appear to be related to the presence of different tropomyosin isoforms. This is based on the observation that similar results are obtained using either antibodies to erythrocyte tropomyosin which recognize both muscle and nonmuscle tropomyosins or using antibodies specific for sarcomeric tropomyosin (data not shown).

Peripheral areas of cells and leading edges have been hypothesized to be regions of active myofibril assembly. In these areas, α -actinin often displays a punctate or discontinuous pattern consisting of clusters of bead-like arrays, while F-actin appears continuous (non-striated) (these structures are referred to as ectopic patches or I-Z-I-like complexes). Other thin filament proteins such as tropomyosin and tropinin-I are also associated with these structures as well as titin and a non-muscle isoform of myosin (Schultheiss et al., 1990; Rhee et al., 1994). Fig. 5 (a and c) demonstrate

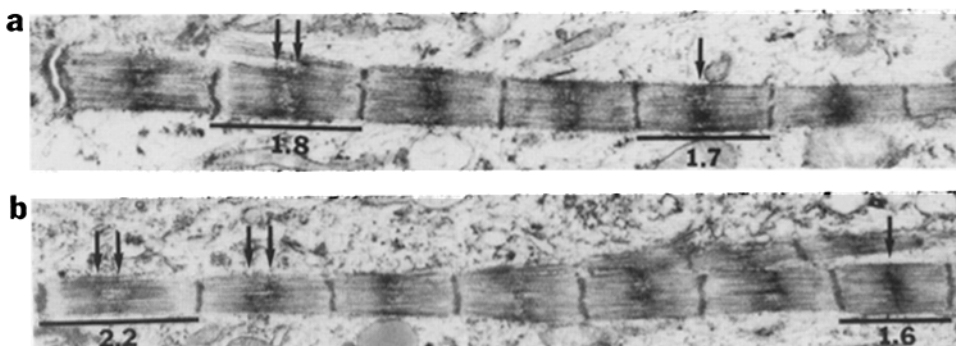


Figure 2. Ultrastructural immunoperoxidase localization of tropomodulin. (a-b) Cells immunostained for tropomodulin. Arrows indicate a region of dense immunoprecipitate. Note, the staining pattern separates into two bands with increasing sarcomere length (increasing distance between Z lines). Bar, 1 μ m.

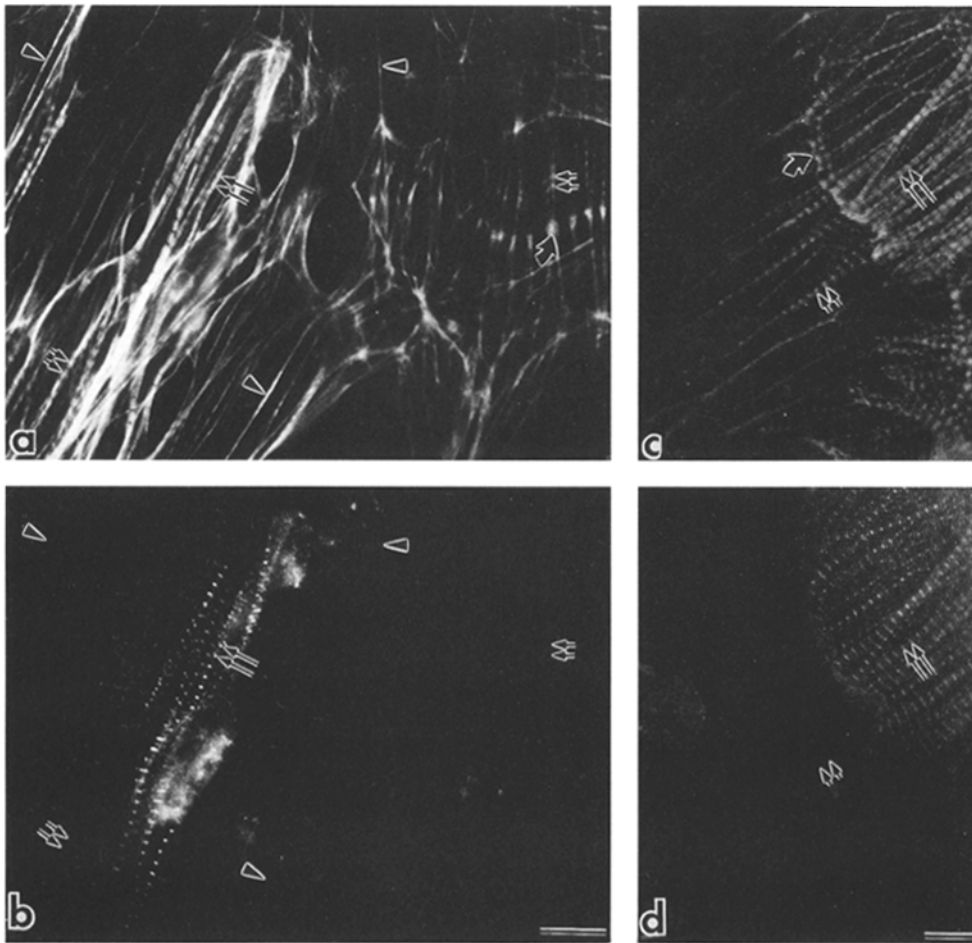


Figure 3. Tropomodulin is not coordinately assembled into myofibrils with respect to F-actin. Double fluorescence localization of (a and c) actin and (b and d) tropomodulin demonstrating that tropomodulin is only associated with *some* SMFs and is absent from all NSMFs. Long arrows indicate SMFs which demonstrate staining for tropomodulin, short arrows indicate SMFs which do not demonstrate staining for tropomodulin, arrowheads indicate NSMFs, and curved arrows indicate an intercalated disk. Anti-tropomodulin antibodies also stain some cardiomyocyte nuclei. (b). The significance of this staining pattern is uncertain; however, many antibodies have been reported to react non-specifically with nuclear components. For example, monoclonal antibodies to titin T11 epitope also stain cardiomyocyte nuclei (see Fig. 6 c). Bar, 10 μ m.

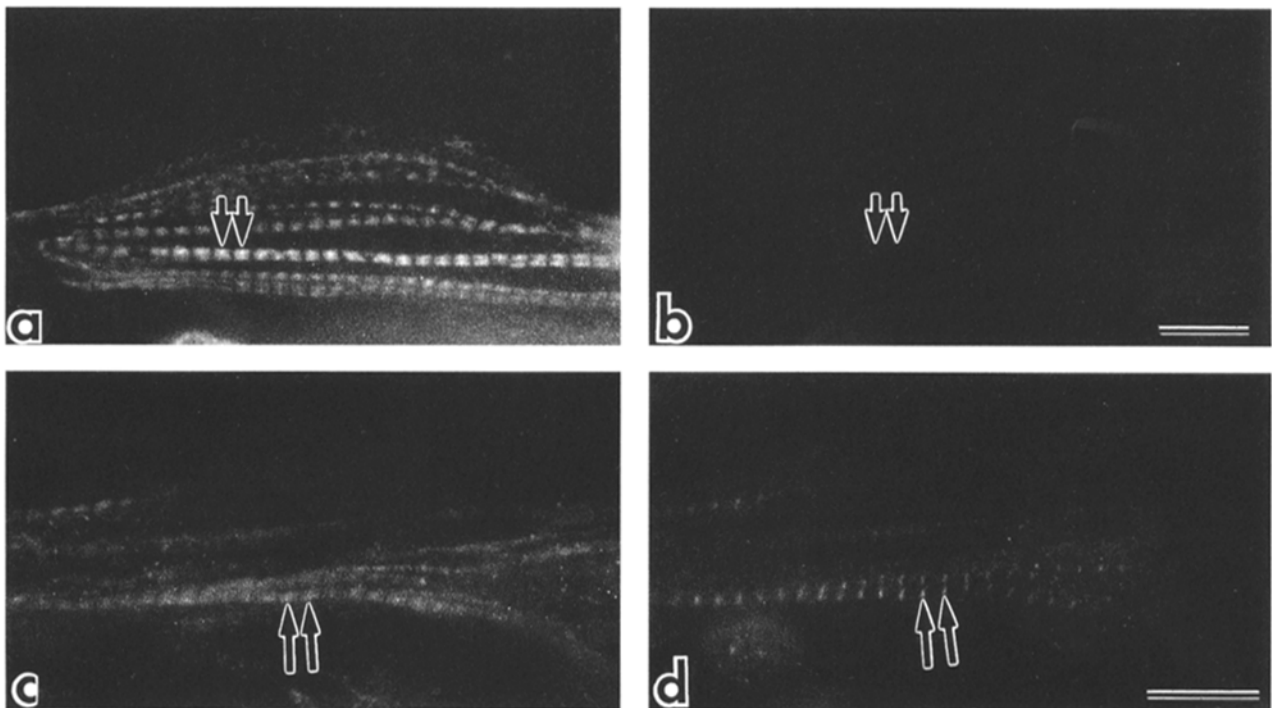


Figure 4. Tropomodulin is not coordinately assembled into myofibrils with respect to tropomyosin. Double immunofluorescence localization of (a and c) tropomyosin and (b and d) tropomodulin demonstrating that in some myofibrils tropomyosin can be found associated with periodic I bands in the absence of tropomodulin. Arrows indicate SMFs which demonstrate staining for tropomodulin and short arrows indicate SMFs which do not demonstrate staining for tropomodulin. Bar, 10 μ m.

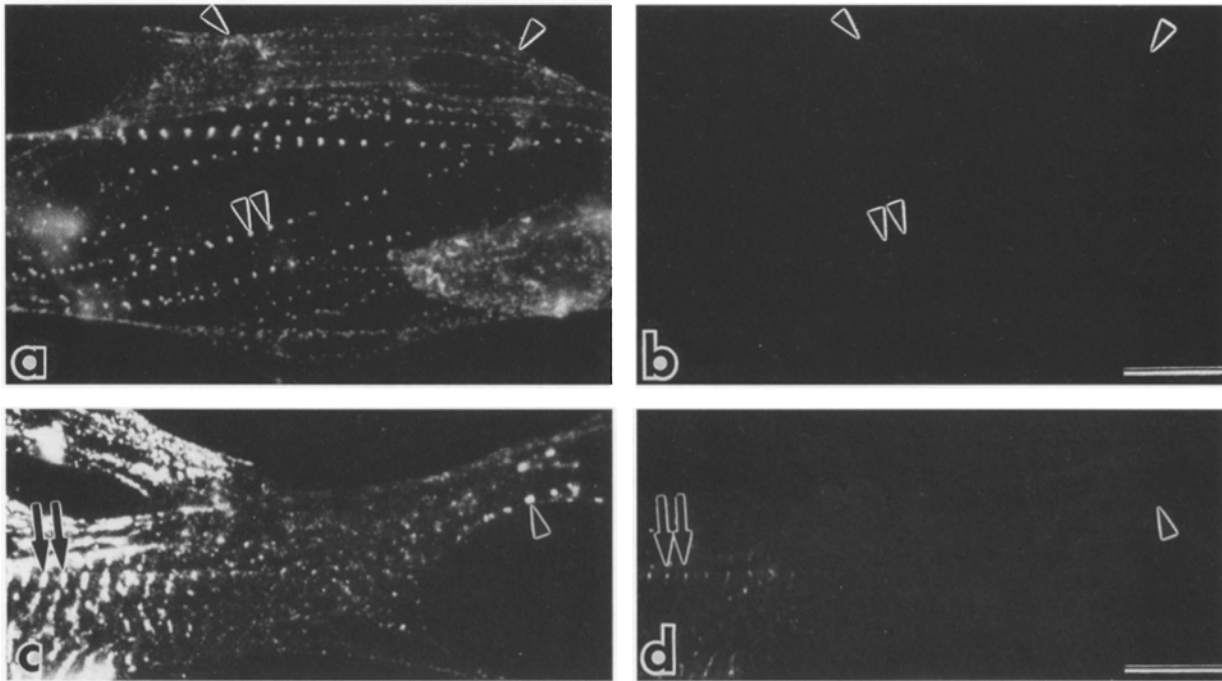


Figure 5. Tropomodulin is not associated with ectopic patches or leading edges. Double immunofluorescence localization of (a and c) α -actinin and (b and d) tropomodulin demonstrating that tropomodulin staining is only detected in SMFs. Arrows indicate SMFs and arrowheads indicate ectopic patches and/or leading edges rich in α -actinin aggregates. Bar, 10 μ m.

α -actinin staining in linear arrays of bead-like structures as well as staining at the edges of cells in ectopic patches. When cells are double-stained for α -actinin and tropomodulin, no tropomodulin staining could be detected in any of these structures; it is only present in striated areas (Fig. 5, b and d). In conclusion, tropomodulin appears to assemble later than all thin filament associated proteins studied.

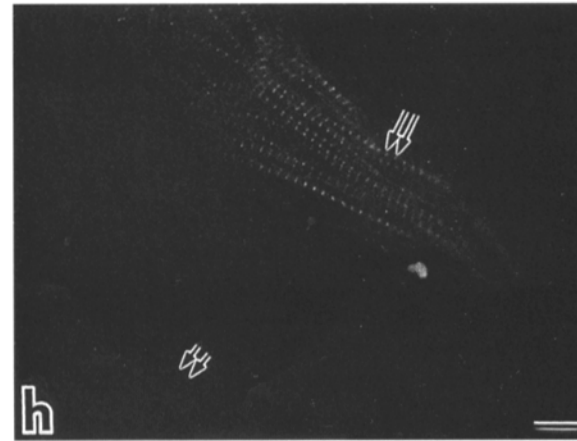
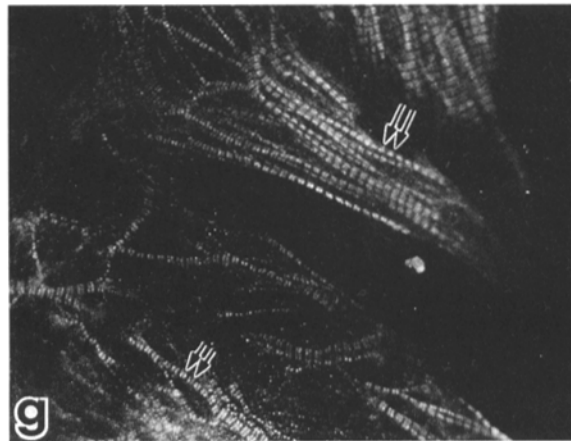
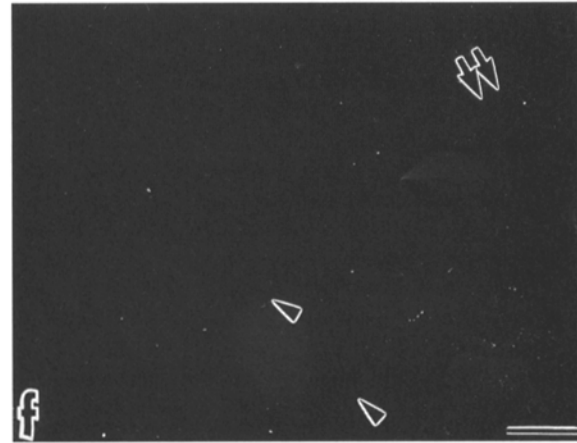
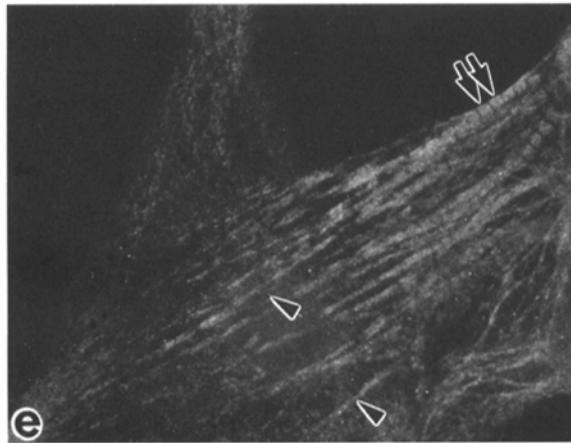
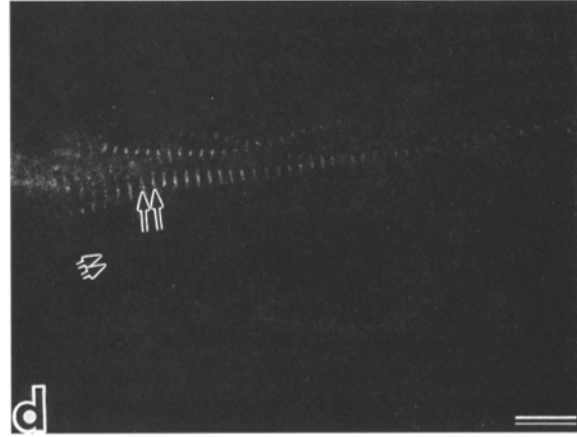
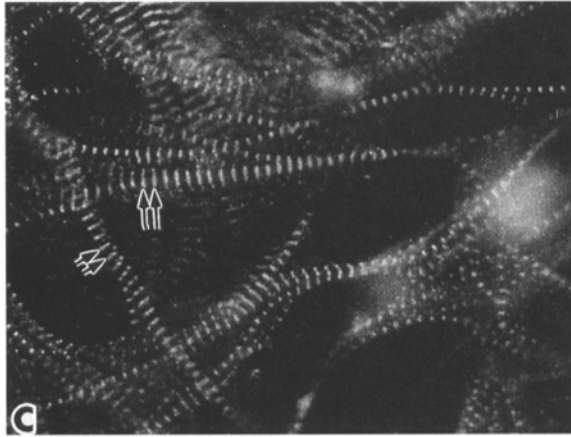
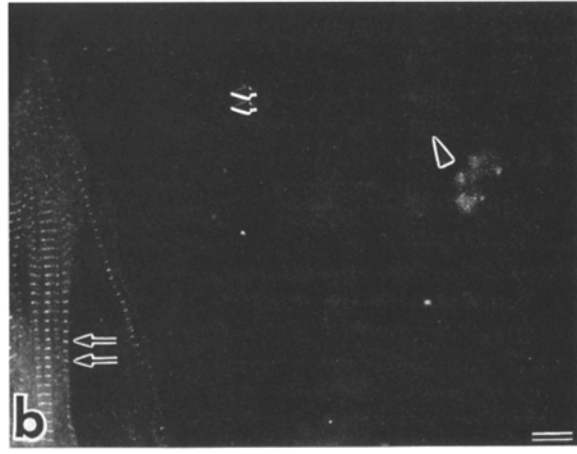
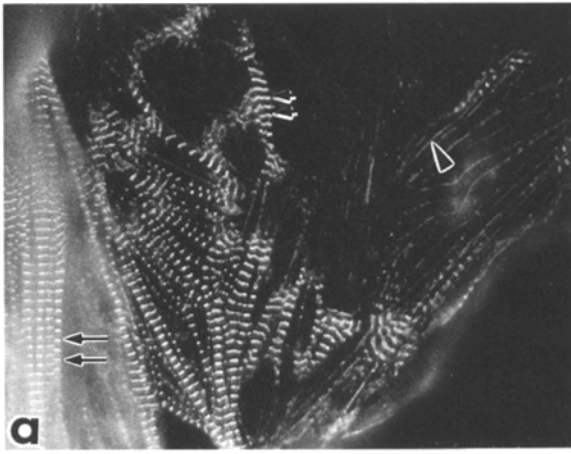
Assembly of Tropomodulin into Myofibrils with Respect to Titin and Thick Filament Components

We next determined the relationship between the assembly of tropomodulin and titin using the monoclonal antibodies T12 and T11 which are specific for titin epitopes close to the Z line (N-1 line) and the A-I junction, respectively (Furst et al., 1988). Titin is a major protein of the myofibrillar "third filament system" that extends from the M line to the Z disk (for review see Fulton and Issacs, 1991). Previous studies have demonstrated that epitopes of titin found in close proximity to the Z disk (e.g., T12 epitopes) can be detected in a sarcomeric pattern prior to titin epitopes which are located further from the Z disk (e.g., T11 epitopes; Furst et al., 1989; Schultheiss et al., 1990). Fig. 6 a demonstrates some of the patterns of titin T12 staining that are observed. Staining for titin T12 and tropomodulin reveals that tropomodulin is absent from interspersed nonstriated regions and NSMFs. Again, tropomodulin is only present in some striated myofibrils (Fig. 6, a and b); this observation is consistent with the data presented above. Although the titin T11 epitope is a later marker of myofibril assembly, immunostaining for titin T11 also demonstrates that T11 can be detected in a striated pattern in the absence of tropomodulin (Fig. 6, c and d). Interestingly, myofibrils immediately adjacent to one an-

other that stain for titin T11 in a striated pattern, often differed with respect to the presence of tropomodulin.

Lastly, we compared the subcellular distribution of tropomodulin with respect to thick filament proteins. Many studies have suggested that thin filaments are aligned into their mature sarcomeric pattern prior to the lateral association of thick filaments containing sarcomeric myosin into A bands (for a review see Epstein and Fischman, 1991 and references therein). These observations and that fact that tropomodulin is a thin filament-associated protein (Fowler et al., 1993), suggested that tropomodulin might be incorporated into sarcomeres before the appearance of thick filaments in A bands. Double immunofluorescence staining, however, revealed that myosin can also be found aligned into periodic A bands in some myofibrils in the absence of detectable tropomodulin staining (Fig. 6, e and f). Interestingly, in a recent study which documented the temporal appearance of ten muscle-specific proteins in post-mitotic myoblasts in primary chick skeletal muscle cultures, the thick filament associated protein, C-protein was detected in a striated pattern later than all other proteins studied (Lin et al., 1994). In our study, when cardiomyocytes are stained for tropomodulin and C-protein, many cells contained myofibrils that displayed both C-protein and tropomodulin in their appropriate striated pattern. However, a significant proportion of myofibrils that stained for C-protein did not stain for tropomodulin at the pointed ends of the thin filaments (Fig. 6, g and h).

In summary, the micrographs presented here illustrate the variations in spatial distribution and degree of maturation of myofibrillar structures that occur in chick cardiomyocytes. Our results indicate that tropomodulin is not present in NSMFs and is a late marker of striated myofibril assembly.



Identical staining patterns and results were obtained using a variety of fixation, enhancing and permeabilization procedures and using three different polyclonal anti-tropomodulin antibodies (data not shown). However, our data do not rule out the possibility that the pointed ends of thin filaments may be capped, in NSMFs or in striated myofibrils that do not contain any detectable tropomodulin, by a new isoform of tropomodulin (that our antibodies do not recognize) or by an unrelated pointed end capping protein.

Steady State Levels of Soluble and Insoluble Tropomodulin and Tropomyosin

The absence of tropomodulin staining in nonstriated and in many striated myofibrils could be due to insufficient quantities of the protein in the cell, or to the failure of it to assemble into myofibrils. To distinguish between these possibilities, the proportion of soluble or assembled tropomodulin during myofibrillogenesis in cultured cardiomyocytes was ascertained using [³⁵S]methionine labeling, followed by immunoprecipitation of cell fractions obtained after extraction in a 0.5% Triton X-100 containing buffer (CSK buffer). The results from this experiment reveal that at steady state, ~39% of the total tropomodulin is soluble in cultures labeled continuously for 1 h. The same proportion of soluble tropomodulin is also found when cells are labeled continuously for up to 24 h, indicating that 1 h of labeling is sufficient time to reach steady state (Fig. 7). In contrast, virtually all (~94%) of the cardiac tropomyosin is found in the insoluble fraction (Fig. 7). Immunoblot analysis of Triton X-100 soluble and insoluble fractions prepared from unlabeled cells also demonstrated that ~35% of the tropomodulin is soluble while all of the tropomyosin (~98%) is associated with the Triton X-100 insoluble fraction (data not shown). It is important to note that although there are contaminating fibroblasts in our cultures, these cells do not contain any detectable tropomodulin as determined by immunoblotting or immunoprecipitation from extracts of [³⁵S]methionine labeled primary chick embryo fibroblast cultures, as well as by immunofluorescence analysis (data not shown). Furthermore, cardiac tropomyosin was immunoprecipitated using an antibody (CH1) that is specific for muscle tropomyosin isoforms and does not recognize any fibroblast tropomyosins (Lin et al., 1985).

Even when very few SMFs are evident on days 2 and 3 of culture, between 35–40% of the tropomodulin is soluble. Therefore, the amount of tropomodulin available in cardiomyocytes does not appear to be limiting at any stage during myofibril assembly. The absence of tropomodulin staining in NSMFs or in some SMFs appears to be due to an additional factor(s) required for the assembly of tropomodulin.

Biosynthesis and Assembly of Tropomodulin and Tropomyosin

To compare the kinetics of assembly of tropomodulin and

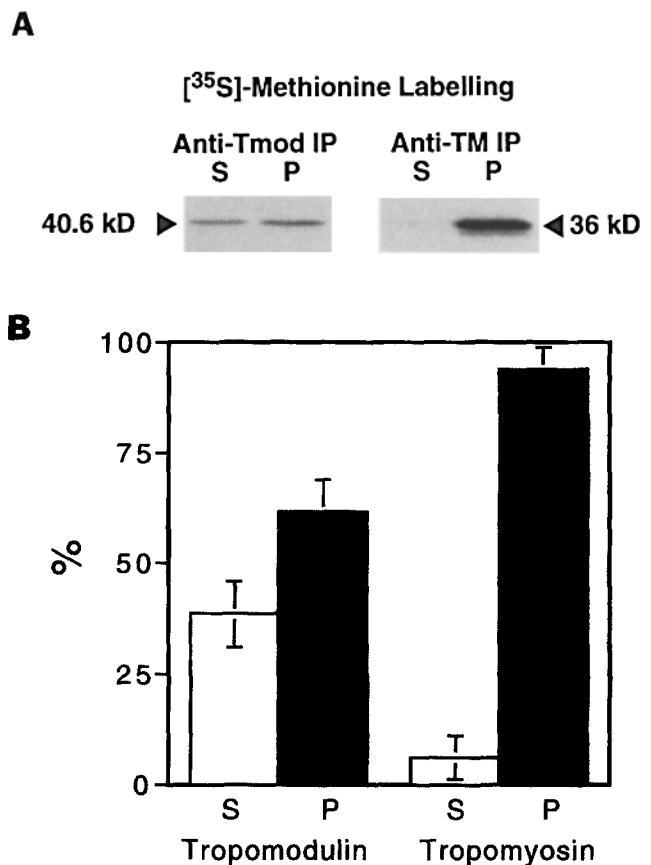


Figure 7. Steady state levels of soluble and assembled (myofibrillar) tropomodulin (Tmod) and tropomyosin (TM) in cardiomyocytes. Day 5 cardiomyocytes were metabolically labeled with 200 $\mu\text{Ci/ml}^{-1}$ [³⁵S]methionine for 2 h, extracted in CSK buffer and immunoprecipitated as described in Materials and Methods. (A) Autoradiogram of the soluble (S) and insoluble (P) fractions. One representative experiment is shown. (B) Percentage of soluble (S) and insoluble (P) tropomodulin and tropomyosin at steady state, calculated as percent of total (soluble plus insoluble) counts. Results are the mean of 15 tropomodulin and 11 tropomyosin immunoprecipitations. Bars, S.D.

tropomyosin, we examined their biosynthesis and assembly into the Triton-insoluble fraction (myofibrils) using pulse-chase analysis. Day 5 cardiomyocyte cultures were pulse labeled with [³⁵S]methionine for 10 min followed by various chase periods in the presence of excess unlabeled methionine. After extraction of labeled cells with CSK buffer, tropomodulin and tropomyosin were immunoprecipitated from soluble and cytoskeletal fractions, run on polyacrylamide gels and processed for autoradiography. Results from this experiment reveal that ~65% of tropomodulin synthesized during the 10-min labeling period is soluble and this proportion decreases to 35% within 1 h (65% insoluble; Fig. 8). The final proportions of soluble and insoluble tropomod-

Figure 6. Tropomodulin is a late marker of myofibril assembly with respect to titin, myosin, and C protein. Double immunofluorescence localization of (a) titin T12 epitope and (b) tropomodulin; (c) titin T11 epitope and (d) tropomodulin; (e) myosin and (f) tropomodulin; and (g) C-protein and (h) tropomodulin. Tropomodulin is only associated with a subset of striated myofibrils. Arrows indicate SMFs which demonstrate staining for tropomodulin, short arrows indicate SMFs which do not demonstrate staining for tropomodulin and arrowheads indicate NSMFs. Bar, 10 μm .

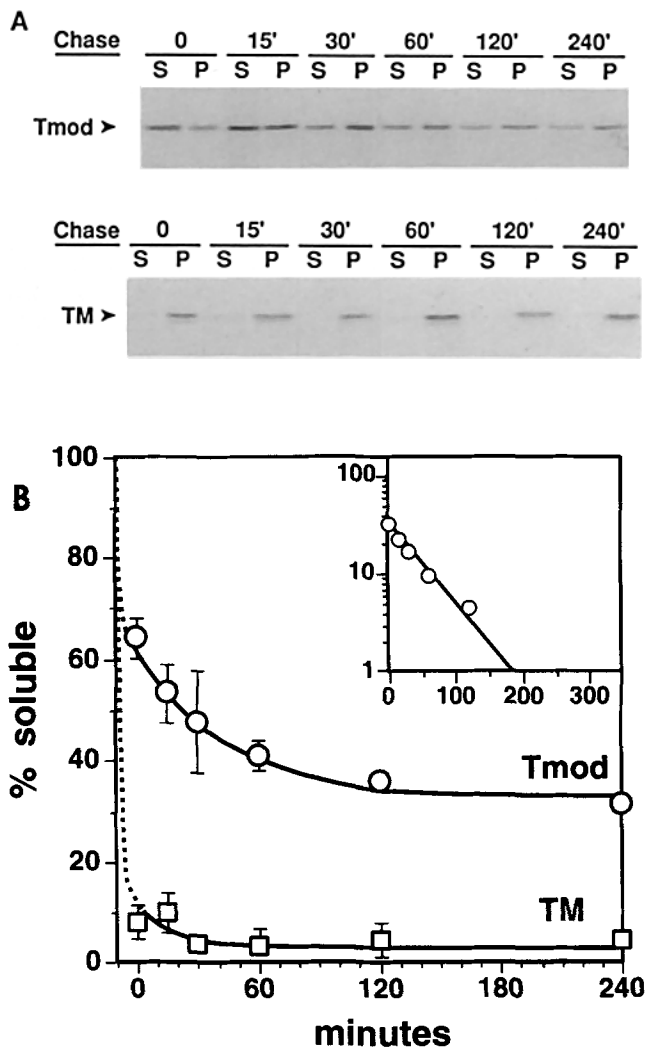


Figure 8. Assembly of newly synthesized tropomodulin (Tmod) and tropomyosin (TM) into myofibrils. Day 5 cardiomyocytes were pulse-labeled with [³⁵S]methionine for 10 min and subsequently chased with cold methionine for 0–240 min. At each time point, the labeled cells were extracted in CSK buffer and tropomodulin and tropomyosin were immunoprecipitated from the soluble (S) and insoluble (P) fractions and electrophoresed on SDS/polyacrylamide gels followed by autoradiography. Quantitation was performed on a scanning phosphorimager. (A) Autoradiogram of one representative experiment. (B) Percent soluble Tmod (○) and TM (□) as a function of chase time, calculated as a percent of the total (soluble plus insoluble). (B, inset) A semi-logarithmic graph of the percent soluble tropomodulin as a function of chase time. Results are averaged from triplicate determinations from one representative experiment out of four performed. Note that tropomodulin and tropomyosin were immunoprecipitated from the same extracts for each time point (24/25 of the extract and 1/25 of the extract, respectively). Bars, S.D.

ulin agree with its steady state levels (see above). The decrease in soluble tropomodulin is due to assembly of tropomodulin into the Triton X-100-insoluble fraction (myofibrils) and not to degradation, because the total amount of tropomodulin (raw counts: determined by adding the soluble plus insoluble counts) remained essentially constant throughout the chase period. Furthermore, a semi-logarithmic graph

of the data is a straight line and demonstrates that tropomodulin assembles randomly from solution with exponential kinetics (Fig. 8 B, inset). We estimated the half time of assembly of tropomodulin from this plot to be ~7 min. Similar results were obtained when cells were pulse labeled with [³⁵S]methionine for 2.5 min (data not shown). In summary, the kinetic data obtained from the pulse–chase experiments suggest that tropomodulin is synthesized as a soluble precursor and then is posttranslationally inserted into myofibrils. In situ hybridization experiments also suggest that tropomodulin is not targeted to thin filament pointed ends by localized translational machinery (Sussman et al., 1994).

In contrast, tropomyosin is incorporated into Triton-insoluble structures (myofibrils) considerably more rapidly than tropomodulin. Only 15% of the tropomyosin synthesized during a 10-min pulse is soluble and this decreases to ~6% within 30 min. Again, this percentage agrees with the percentages obtained by steady state labeling (see above). Since the kinetics of assembly for tropomyosin are so rapid, we can not conclude from our data whether tropomyosin is synthesized as a soluble precursor or whether some tropomyosin might be cotranslationally assembled into nonstriated and/or striated myofibrils, as has been proposed for some myofibrillar proteins, for example, vimentin, MHC, and titin (for review see Fulton and L'Ecuyer, 1993).

Reconstitution of Tropomodulin onto Thin Filaments

A permeabilized cell model was developed to investigate whether the assembly of tropomyosin was required for the assembly of tropomodulin into myofibrils, as suggested by the immunofluorescence and pulse–chase kinetic data. In this thin filament assembly model, cardiomyocytes are permeabilized with saponin and extracted in 0.5 M KCl; conditions which we determined to remove all detectable tropomodulin. Coverslips were stained by immunofluorescence microscopy to visualize the presence or absence of particular sarcomeric components (i.e., actin, tropomyosin, tropomodulin, and myosin; Fig. 9). Essentially all detectable tropomodulin is removed under these conditions (Fig. 9 d) and tropomyosin staining is substantially reduced (i.e., the staining appeared as faint narrow doublets) or is not detectable (compare Fig. 9 c to typical I band staining depicted in unextracted cells in Fig. 4). As expected from previous reports of isolated myofibrils that were treated with high salt, all detectable myosin is removed (Fig. 9 a) and F-actin staining remained at a similar intensity and in a similar distribution as in the unextracted cells (compare Fig. 9 b with Figs. 1 a, and 3, a and c). These are referred to as “ghost myofibrils” (Huxley and Hanson, 1954) within the confines of an extracted cell.

Initially, we determined that exogenous tropomyosin bound well to ghost myofibrils since the intensity of tropomyosin staining after rebinding is similar to that present in unextracted cells for both striated and unstriated myofibrils (Fig. 10 d; compared to Fig. 4, a and c). To determine whether tropomodulin requires tropomyosin for assembly onto the pointed ends of thin filaments, ghost myofibrils were reconstituted with excess biotinylated tropomodulin with or without the prior addition of unlabeled tropomyosin. We found that tropomodulin assembles at the pointed ends of thin filaments (at levels similar to that present in unextracted cells) only after thin filaments are first reconstituted with tro-

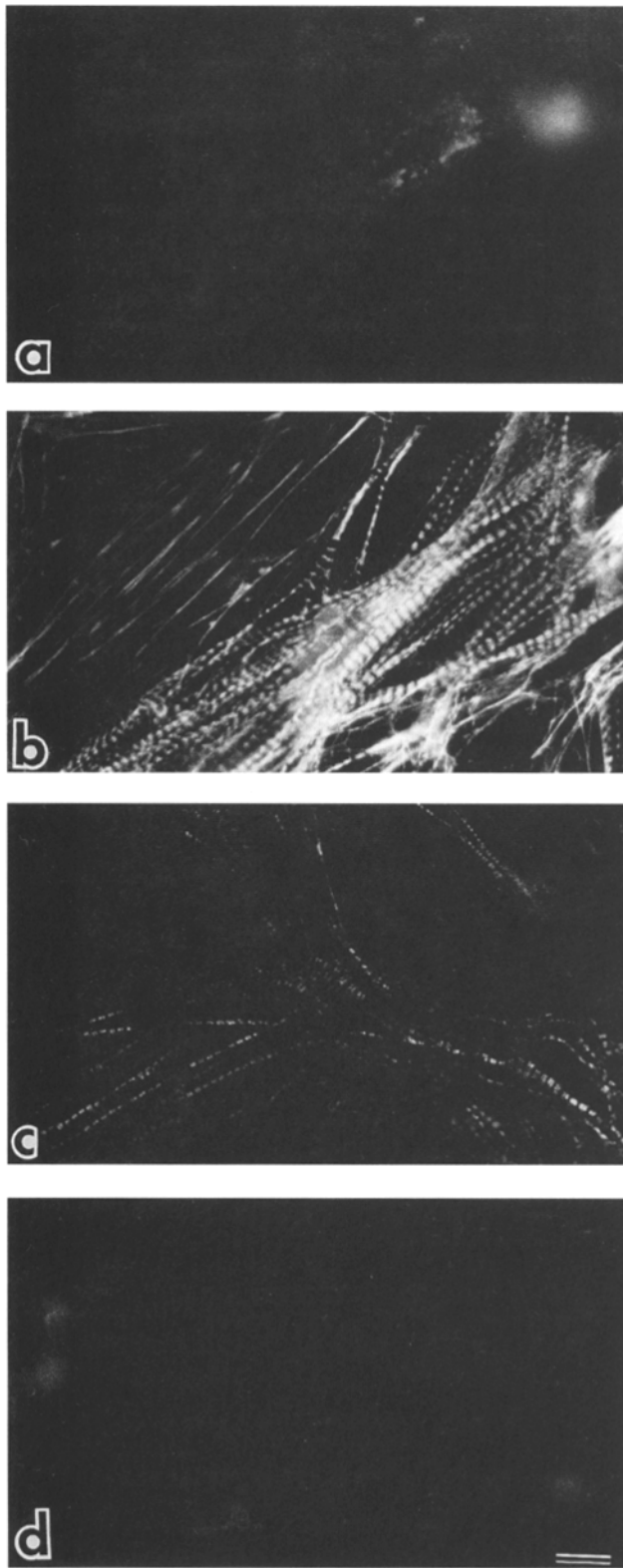


Figure 9. Characterization of a permeabilized cell model for thin filament assembly. Cardiomyocytes were permeabilized in saponin and extracted in 0.5M KCl. Immunofluorescence staining for (a) myosin, (b) F-actin, (c) tropomyosin, and (d) tropomodulin. Essentially all myosin and tropomodulin are removed under these conditions, tropomyosin staining appears disrupted or nonexistent, and F-actin staining appears at normal levels. Bar, 10 μ m.

pomyosin (Fig. 10, compare *b* to *f* and *h*). Furthermore, after reconstitution with tropomyosin, tropomodulin only associates with *some* SMFs but not with any NSMFs (Fig. 10, *f* and *h*); this is similar to the tropomodulin distribution observed in unextracted cells (Figs. 1, and 3–6). In addition, both native erythrocyte and recombinant chicken skeletal muscle tropomodulins (Babcock and Fowler, 1994) are competent to associate onto tropomyosin-reconstituted thin filaments (data not shown). In the absence of tropomyosin, however, rebinding of tropomodulin is essentially not detectable in most SMFs; although, very faint staining of the biotinylated tropomodulin can sometimes be detected on some SMFs (Fig. 10 *b*). This suggests that exogenous tropomodulin is competent to bind only a minor population of actin filaments or that it is binding to the residual tropomyosin remaining after high salt extraction.

In summary, results from this assay demonstrate that thin filament components that are removed with high salt-extraction can be reconstituted with exogenous proteins. Moreover, it appears that tropomodulin requires the prior assembly of tropomyosin onto thin filaments for its own assembly into mature myofibrils.

Discussion

Tropomodulin is the only known thin filament-associated protein that is not assembled coordinately with respect to tropomyosin and other thin filament proteins. Tropomodulin is not detected in assemblies of contractile proteins which are involved in early stages of myofibril assembly (i.e., non-striated myofibrils). Furthermore, titin as well as all thin filaments (α -actinin, actin and tropomyosin) and thick filament (myosin and C-protein) proteins studied are observed in a sarcomeric striated pattern in many myofibrils which have no detectable tropomodulin staining at thin filament pointed ends. Based on the temporal ordering of assembly of these well characterized sarcomeric components, our data suggests that tropomodulin may be the latest marker of striated myofibril assembly yet described. The delayed appearance of tropomodulin with respect to other contractile proteins suggests that this protein is unnecessary for both the initial (formation of nascent Z bands) and subsequent (appearance of I and A band periodicities) phases of myofibrillogenesis. Moreover, since myofibrils in chick cardiomyocytes may also be disassembling in culture (see for example Lin et al., 1989), tropomodulin may not only be the last protein to enter myofibrils, but it may also be one of the first proteins to leave the myofibrils as they disassemble in these cells.

Although tropomodulin does not assemble coordinately with tropomyosin, it does require tropomyosin to assemble. Specifically, results from a saponin-permeabilized cell model for thin filament assembly demonstrate that the prior assembly of tropomyosin is necessary for the assembly of tropomodulin onto ghost myofibrils. These observations are supported by the pulse-chase kinetics analysis of assembly which demonstrates that tropomyosin assembles into the Triton X-100-insoluble fraction considerably more rapidly than tropomodulin, as well as by the immunofluorescence data which demonstrates that tropomodulin is never found associated with thin filament pointed ends in the absence of tropomyosin. Additionally, these data are consistent with previous findings from in vitro assays demonstrating that

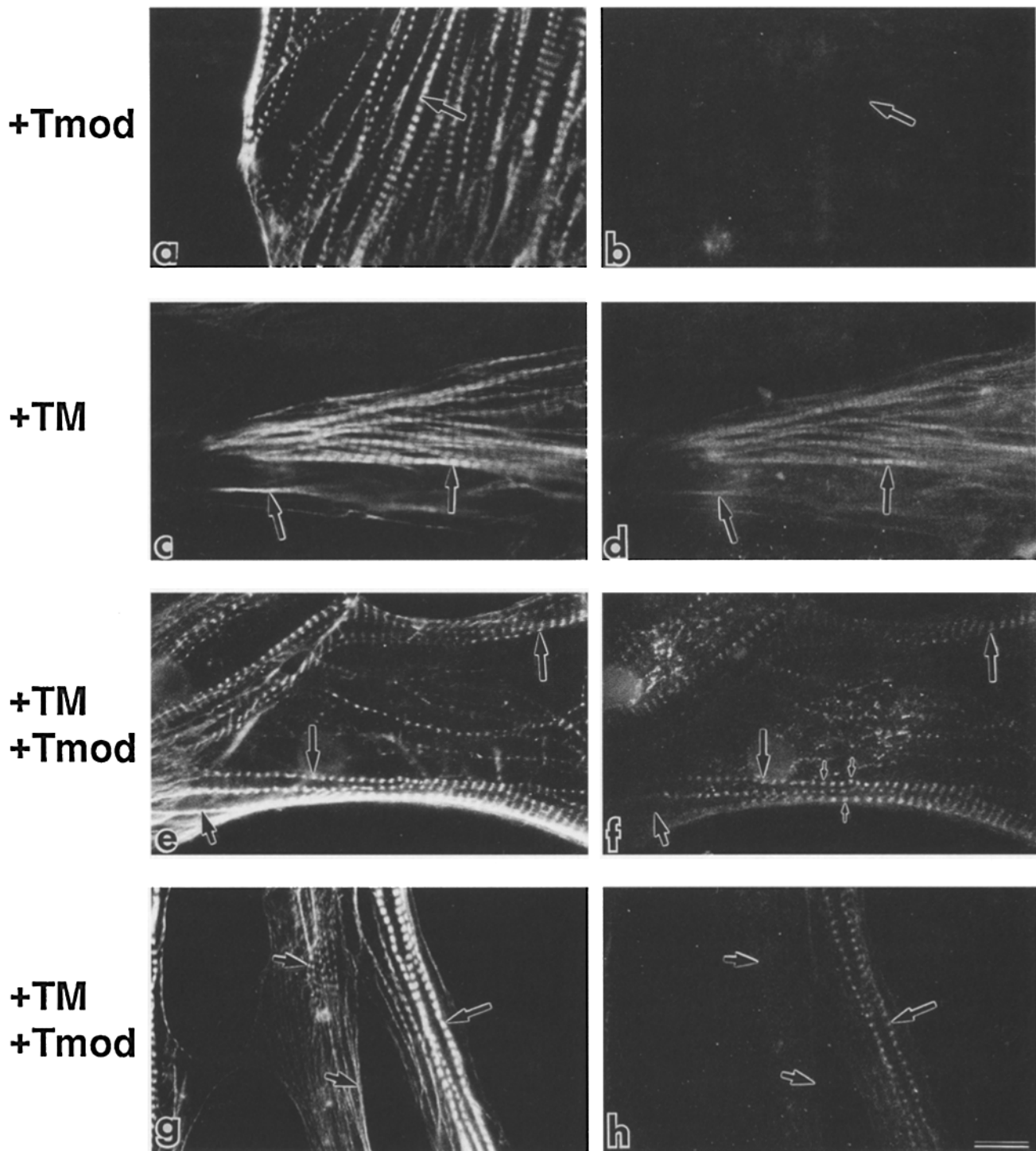


Figure 10. An *in vitro* thin filament assembly model reveals that tropomodulin requires tropomyosin for assembly into mature myofibrils. Ghost myofibrils were reconstituted with biotinylated tropomodulin (*a* and *b*) or tropomyosin (*c* and *d*) and double stained for (*a*) actin and (*b*) tropomodulin; and (*c*) actin and (*d*) tropomyosin. Alternatively, ghost myofibrils were reconstituted with biotinylated tropomodulin after preincubation with tropomyosin (*e*–*h*) and double stained for (*e* and *g*) actin and (*f* and *h*) tropomodulin. Small arrows (*f*) indicate doublets of tropomodulin staining at the pointed ends of thin filaments. Short arrows indicate NSMFs or SMFs that do not contain detectable tropomodulin staining (*e*–*h*). Double exposure immunofluorescence microscopy demonstrated that the exogenous tropomodulin is indeed being reconstituted to its proper sarcomeric location at the pointed ends of thin filaments (data not shown). Bar, 10 μm .

tropomodulin caps the pointed ends of tropomyosin-coated actin filaments very tightly (K_d of ≤ 1 nM), but is a weak cap for pointed ends of pure actin filaments ($K_d = 0.1$ – 0.4 μM ; Weber et al., 1994).

The presence of tropomyosin on thin filaments in sarcomeres is, however, not sufficient for tropomodulin's assembly since tropomodulin associates with thin filament pointed ends only in a subset of striated myofibrils and does not as-

sociate with any nonstriated myofibrils, all of which contain tropomyosin. Thus, assembly of tropomodulin into myofibrils appears to require an additional factor (or factors): for example, a posttranslational modification or the prior assembly of an additional thin filament component(s) other than actin or tropomyosin.

The presence of a large soluble pool of tropomodulin at a time when I bands have already formed also suggests that the absence of tropomodulin staining in some striated myofibrils is not due to the absence of the protein in the cell. Furthermore, the pulse-chase experiments demonstrate that most (65%) of the newly synthesized soluble tropomodulin is competent to assemble into myofibrils. The remaining newly synthesized tropomodulin (35%) remains soluble for hours and is not degraded. It is attractive to speculate that the soluble tropomodulin is in dynamic equilibrium with the assembled tropomodulin at the pointed ends of thin filaments and that the amount of assembled tropomodulin reflects the K_d of tropomodulin for the pointed ends of thin filaments. Other investigators have also suggested that contractile proteins of myofibrils are in dynamic equilibrium with a monomer pool of uncertain size, based on for example, analysis of contractile protein exchange in living cells using microinjection of fluorescent proteins and photobleaching techniques (e.g., McKenna et al., 1985a,b; Mittal et al., 1987). Similarly, in vitro experiments, which include incubation of permeabilized cells or isolated glycerinated myofibrils with fluorescently labeled proteins, or incorporation of in vitro translated proteins into myofibrils, have also supported the idea that the dynamic exchange of contractile proteins may be a common phenomenon (Sanger et al., 1984, 1986; Bouché et al., 1988).

The presence of a soluble pool of tropomodulin during differentiation appears not to be specific to chick cardiomyocytes. Preliminary data from studies on primary chick skeletal muscle cultures indicate that in myotubes, tropomodulin is present in both the Triton X-100 soluble and insoluble fraction (assembled into myofibrils) (Almenar-Queralt, A., C. C. Gregorio, and V. M. Fowler, unpublished results). A significant soluble pool of tropomodulin is also present in other non-muscle tissues in the process of active differentiation. For example, a large soluble pool (60%) of tropomodulin is present in the fiber cells of rat lens (Woo and Fowler, 1994). In contrast, in terminally differentiated cells (e.g., erythrocytes and skeletal muscle) no soluble tropomodulin is detected (Fowler, 1987; Fowler et al., 1993). Perhaps the pool of tropomodulin that exists in chick cardiomyocytes is available to be readily recruited according to the changing requirements of the cell (e.g., newly created "mature" thin filament pointed ends during cell growth). In support of this idea, it was previously found that activation of beating in cultured adult feline ventricular myocytes triggers myofibrillar reassembly in the presence or absence of cycloheximide; implying the existence of an assembly competent pool of cytoskeletal proteins (Simpson et al., 1993).

In vitro assays demonstrate that tropomodulin blocks both elongation and depolymerization at the pointed ends of tropomyosin-coated actin filaments, thus functioning as a pointed end capping protein (Weber et al., 1994). Based on data presented here together with that of others, it is possible to envision the following mechanism for thin filament assembly. (a) Nascent Z disks containing cap Z and α -actinin are

assembled and are associated with thin filaments containing actin, tropomyosin, and the troponins (e.g., Schultheiss et al., 1990). (b) Thin filaments which extend across the sarcomere and thus are incorrectly polarized with respect to the myosin cross-bridges are selectively disassembled at the pointed ends and become organized in an anti-parallel distribution in striated myofibrils (however, the mechanism by which this would occur is unknown). Alternatively, premyofibrils are formed into minisarcomeres, composed of short thin filaments which are proposed to grow in length during the maturation process (Rhee et al., 1994). (c) Thin filaments become mature in the presence of a putative "third factor" and are subsequently stabilized (capped) at their pointed ends by tropomodulin. In this capacity, tropomodulin would be required to maintain the final length of thin filaments. Thus, the formation of I bands may be contingent upon the availability of uncapped pointed ends in nascent myofibrils. We surmise that in the absence of tropomodulin during early stages in the assembly of sarcomeres, the absolute length of individual polarized thin filaments might be less well defined than those found in mature myofibrils. These ideas are supported by ultrastructural observations of myofibril assembly which showed that some thin filaments initially extend all the way across the sarcomere. Restriction of filament length and separation of thin filaments into two half sarcomeres (as evidenced by defined H zone and M lines) occurred late in myofibril assembly, after interdigitation of thick and thin filaments (Peng et al., 1981; Shimada and Obinata, 1977; Legato, 1972; Markwald, 1973; Brooks et al., 1983).

Since pointed end capping by tropomodulin does not appear to depend on filament length in vitro (Weber et al., 1994), tropomodulin alone can not specify the length of thin filaments. The specification of thin filament length in striated muscle is likely to require multiple interacting components. Tropomyosin, for example, has been shown in in vitro assays to reduce actin filament depolymerization at pointed ends (Broschat, 1990; Weigt et al., 1990) and has been proposed to function in thin filament length determination through a vernier-like mechanism (Huxley and Brown, 1967). Since tropomodulin binds to one end of tropomyosin (Fowler, 1990), it appears likely that together, tropomyosin and tropomodulin function to maintain and stabilize actin filament length at the pointed ends of the thin filaments. Additionally, although cardiac muscle does not contain nebulin (Itoh et al., 1988), this protein is proposed to function as a template to specify thin filament length in skeletal muscle (Wang and Wright, 1988; Kruger et al., 1991). Interestingly, nebulin also appears late during myofibrillogenesis (Furst et al., 1989; Komiyama et al., 1992). Perhaps nebulin and tropomodulin interact together to specify and maintain the appropriate length of thin filaments in skeletal muscle.

In summary, the results presented here indicate that tropomodulin may be responsible for maintaining the final length of thin filaments in mature striated myofibrils. The temporal order of the appearance of tropomodulin into sarcomeres also suggests that tropomodulin might be required for efficient contraction.

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