

# Cellular Titin Localization in Stress Fibers and Interaction with Myosin II Filaments In Vitro

Kenneth J. Eilertsen,\* Steven T. Kazmierski,† and Thomas C. S. Keller III\*†

\*Molecular Biophysics Program and †Department of Biological Science, Florida State University, Tallahassee, Florida 32306-3050

**Abstract.** We previously discovered a cellular isoform of titin (originally named T-protein) colocalized with myosin II in the terminal web domain of the chicken intestinal epithelial cell brush border cytoskeleton (Eilertsen, K. J., and T. C. S. Keller. 1992. *J. Cell Biol.* 119:549–557). Here, we demonstrate that cellular titin also colocalizes with myosin II filaments in stress fibers and organizes a similar array of myosin II filaments in vitro. To investigate interactions between cellular titin and myosin in vitro, we purified both proteins from isolated intestinal epithelial cell brush borders by a combination of gel filtration and hydroxypatite column chromatography. Electron microscopy of brush border myosin bipolar filaments assembled in the presence and absence of cellular titin revealed a cellular titin-dependent side-by-side and end-to-end alignment of the filaments into highly ordered arrays. Immunogold labeling confirmed cellular titin associa-

tion with the filament arrays. Under similar assembly conditions, purified chicken pectoralis muscle titin formed much less regular aggregates of muscle myosin bipolar filaments. Sucrose density gradient analyses of both cellular and muscle titin–myosin supramolecular arrays demonstrated that the cellular titin and myosin isoforms coassembled with a myosin/titin ratio of  $\sim 25:1$ , whereas the muscle isoforms coassembled with a myosin:titin ratio of  $\sim 38:1$ . No coassembly aggregates were found when cellular myosin was assembled in the presence of muscle titin or when muscle myosin was assembled in the presence of cellular titin. Our results demonstrate that cellular titin can organize an isoform-specific association of myosin II bipolar filaments and support the possibility that cellular titin is a key organizing component of the brush border and other myosin II-containing cytoskeletal structures including stress fibers.

THE brush border array of microvilli at the apical end of intestinal epithelial cells can be isolated with its actin-based cytoskeleton intact in amounts sufficient for biochemical analysis. This property makes the isolated brush border an excellent model system with which to identify and characterize cytoskeletal components (for review see references 1, 3, 18, 28, 29). Many of the well-characterized brush border cytoskeletal components are related to proteins of actin-based structures in other cells and muscle. For example, brush border actin, myosin, tropomyosin, and  $\alpha$ -actinin are isoforms of proteins that comprise the muscle sarcomere. Recently, we discovered a large, fibrous component of the brush border cytoskeleton that we named T-protein (7). On the basis of criteria including molecular weight, molecular morphology, and immunocrossreactivity, we proposed that T-protein is a cellular isoform of titin (7), a protein that was first identified as a component of muscle sarcomeres. Hereafter, we will refer to T-protein as cellular titin.

In sarcomeres, individual muscle titin molecules span the distance between the M-line and each Z-line (10, 11, 34,

41–44). Titin associates with muscle myosin at an estimated ratio of between 6 and 12 titin molecules per thick filament (45). It has been proposed that the thick filament-associated segment of each titin molecule could serve as a template for filament assembly (45). The remainder of each titin molecule forms an elastic link to the Z-line, which maintains alignment of the thick filaments and resists overstretching of the sarcomere (15, 16, 26, 27, 33, 39, 41).

In brush borders, cellular titin is localized in the terminal web region (7), where it could mediate the association of myosin II with the cytoskeleton. The brush border terminal web is composed of two subdomains, both of which contain myosin II. In the interrootlet subdomain, myosin II filaments and molecules of the brush border form of fodrin, TW 260/240 (13), cross-link core bundles of parallel actin filaments extending into the region from the overlying microvilli. In the circumferential ring subdomain, myosin II filaments can interact with anti-parallel actin filaments to constrict the ring (2, 6, 14, 18, 19, 28). Although the pattern of myosin filament distribution of the interrootlet domain remains unclear, myosin filaments are distributed in a periodic array around the ring (6). Other cytoskeletal structures, including stress fibers, display a similar periodic pattern of myosin filament distribution (24).

Address all correspondence to T. Keller, Molecular Biophysics Program and Department of Biological Science, Florida State University, Tallahassee, FL 32306-3050. Ph.: (904) 644-5572. Fax: (904) 644-0481.

In this report, we demonstrate that cellular titin is localized in fibroblast stress fibers and that purified cellular titin aligns in vitro assembled brush border myosin II bipolar filaments into large, highly ordered supramolecular aggregates. Myosin filament distribution in these in vitro coassemblies with cellular titin is similar to filament distribution in stress fibers, and the coassemblies contain approximately one cellular titin molecule per myosin bipolar filament. In contrast, muscle titin organizes much less ordered arrays of large muscle myosin bipolar filaments in which there are multiple titins per thick filament. We also found that titin-myosin interaction is isoform specific; cellular titin interacts with cellular but not muscle myosin filaments, whereas muscle titin interacts with muscle but not cellular myosin filaments. Colocalization of cellular titin and myosin in stress fibers and the structure and isoform specificity their interaction in vitro strongly supports the possibility that cellular titin plays an important role in organizing nonmuscle myosin II bipolar filaments in a variety of actin-based cytoskeletons.

## Materials and Methods

### Culture of Chicken Embryonic Skin Fibroblasts and Immunolocalization

Fibroblasts were extracted from the skin of 10-d chick embryos by treatment with trypsin and collagenase and cultured on coverslips in MEM medium with Hank's salts, 10% horse serum, 5% chick embryo extract, L-glutamine, penicillin, streptomycin, and nystatin for 2–3 d at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air. For immunolocalization studies, the cells were fixed in 2% formaldehyde, permeabilized with 0.2% Triton-X-100 in 0.1 M PBS, and washed with 0.5 mg/ml sodium borohydride (in PBS). The cells were double labeled by incubation overnight in our anti-cellular titin antibody (reference 7; diluted 1:100 in a 1% BSA-PBS blocking solution) and a monoclonal antibody raised against chick brain myosin (reference 4; clone 25 diluted 1:50; kindly provided by Drs. A and G. Conrad, Department of Biology, Kansas State University, Manhattan, KS). Primary antibody binding was detected by incubation for 1 h in FITC-conjugated donkey anti-rabbit IgG antibody (diluted 1:200) and TRITC-conjugated donkey anti-mouse IgG antibody (diluted 1:200; both obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Control cell preparations were processed the same way but with either nonimmune serum as a control for FITC-antibody staining or no primary antibody as a control for TRITC-antibody staining. The coverslips were washed and mounted with medium containing para-phenylenediamine. Images were recorded with a Nikon Microphot microscope on T-Max 100 film.

### Isolation and Extraction of Brush Borders

Brush borders were isolated from chicken intestinal epithelial cells essentially by the method of Keller and Mooseker (17), with modifications as described by Eilertsen and Keller (7). This yielded isolated brush borders in brush border stabilization buffer (BBSB; 75 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2 mM DTT, 10 mM Imidazole, pH 7.0) containing the protease inhibitors PMSF (0.2 mM), aprotinin (0.1 mM), leupeptin (0.05 mM), and pepstatin (0.1 mM). All subsequent procedures were performed with the material on ice or at 4°C, unless otherwise indicated.

Isolated brush borders were extracted in 0.15 M KCl + 4 mM ATP solution for 10 min and pelleted by centrifugation at 15,000 *g* for 15 min as described previously except that the ratio of extraction solution to brush borders was kept low to minimize the supernatant volume to load on the subsequent gel filtration column (19). Brush border extraction was repeated, and the supernatants containing both cellular titin and brush border myosin II were combined for further purification of the proteins.

1. Abbreviations used in this paper: BBSB, brush border stabilization buffer; HAP, hydroxyapatite.

### Sephacryl S-500 and Hydroxyapatite Column Purification of Brush Border Cellular Titin and Myosin II

Both brush border cellular titin and myosin II were partially purified by Sephacryl S-500 column chromatography. The 0.15 M KCl + ATP extracts were raised to 10% sucrose by addition of solid sucrose to facilitate loading and loaded directly onto a Sephacryl S-500 column (2.5 × 90 cm) that was equilibrated and eluted with 150 mM KCl, 0.01 mM ATP, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM DTT, and 10 mM Imidazole, pH 7.0. Cellular titin eluted from the column near the void volume and was most readily detected by silver staining SDS gels of column fraction samples. Cellular titin fractions were contaminated with a high molecular weight glycoprotein, assumed to be mucin, that was best detected by Periodic acid-Schiff staining of the SDS gels (8). Other proteins that contaminated the cellular titin fractions included TW 260/240 (brush border fodrin) and an unidentified ~100-kD peptide. Fractions containing cellular titin were combined, dialyzed into 0.6 M KCl-BBSB (BBSB with 0.6 M KCl), and loaded onto a hydroxyapatite column (1.5 × 7.5 cm) equilibrated in 0.6 M KCl-BBSB. The column was washed extensively with 0.6 M KCl-BBSB containing 90 mM phosphate to elute contaminating mucin and TW 260/240. Cellular titin was collected and concentrated by eluting the column in the reverse direction with a step of 400 mM phosphate in 0.6 M KCl-BBSB. Purified cellular titin, occasionally still contaminated with a small and variable amount of the 100-kD peptide, was dialyzed into 0.6 M KCl-BBSB to yield a concentration of 1–20 µg/ml as determined by protein assay (Bio-Rad Laboratories, Hercules, CA).

Sephacryl S-500 column fractions containing brush border myosin II and free of high molecular weight proteins (>~800 kD) were pooled, dialyzed into 0.6 M KCl-BBSB, and loaded onto a hydroxyapatite column (2.5 × 16.5 cm) equilibrated in 0.6 M KCl-BBSB. The column was eluted with a 0–400 mM phosphate gradient made in equilibration buffer. Fractions containing pure myosin were pooled and dialyzed into 0.6 M KCl-BBSB to yield myosin at a concentration of 50–200 µg/ml.

### Extraction and Purification of Chicken Pectoralis Myosin and Titin

Both titin and myosin were extracted and purified from similar but separate preparations of chicken pectoralis muscle as described by Margossian and Lowey (25), with the following modifications. For titin, strips of freshly dissected muscle were incubated on ice for one hour prior to grinding and extracted for 10 min to maximize titin recovery. The tissue extraction was stopped by fourfold dilution with cold ddH<sub>2</sub>O. The muscle residue was removed by filtration through cheesecloth. The filtrate was further diluted with cold ddH<sub>2</sub>O to a final KCl concentration of 0.04 M. The precipitated actomyosin was removed by centrifugation at 16,000 *g* for 15 min, leaving a substantial proportion of the titin in the supernatant. The supernatant was diluted 1:1 with glycerol and stored at –20°C until use. For use, this supernatant was dialyzed into 0.6 M KCl-BBSB and subjected to hydroxyapatite (HAP) column chromatography as described above except that the column was eluted using a 0–400 mM phosphate gradient in equilibration buffer. Fractions containing the purified titin were pooled and dialyzed into 0.6 M KCl-BBSB to yield titin at a concentration of 10–100 µg/ml. Additional concentration and purification of titin, if necessary, were accomplished by ammonium sulfate precipitation as described by Trinick et al. (38).

For muscle myosin purification, pectoralis muscle was ground immediately on dissection from the animal and extracted for 5 min to minimize extraction of titin. The myosin resulting from subsequent purification steps also was purified further by HAP column (2.5 × 16.5 cm) chromatography and dialyzed into 0.6 M KCl-BBSB before use.

### Gel Electrophoresis

Electrophoresis was performed on high porosity SDS-polyacrylamide gels using the Laemmli buffer system (23). The 2.5–20% polyacrylamide gradient separating gels and 3% stacking gels were prepared from a stock solution of 30% acrylamide–0.4% bis-acrylamide. Peptide bands were visualized by staining with Coomassie blue (8) or silver (30). Periodic acid-Schiff staining (8) was used to detect glycoprotein contamination.

### Coassembly and Sucrose Density Gradient Centrifugation

Aliquots of purified brush border myosin, cellular titin, muscle titin, sarco-

meric myosin, or combinations of these proteins were dialyzed for 48–72 h with one change against an assembly buffer containing 0.1 M KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA (which decreases proteolysis of cellular and muscle titins), 0.2 mM DTT, 1 mM PMSF, and 10 mM Imidazole, pH 7.0. Samples then were negatively stained for EM or spun at 118,000 g for 30 h through a 5–60% sucrose gradient layered onto a pad of 65% sucrose. The gradients were collected as 25 drop fractions from the bottom to the top. Fractions were analyzed by SDS-PAGE and scanning densitometry of Coomassie blue-stained gels using Quantity One (pdi, Inc., Huntington Station, New York).

### **Solid Phase Binding Assay**

Aliquots of purified muscle or brush border myosin II (10 µg) in 250 mM KCl, 1 mM MgCl<sub>2</sub>, 0.2 mM DTT, 10 mM Imidazole, pH 7.0; were slot-blotted onto strips of nitrocellulose. The nitrocellulose strips then were blocked for 2 h in 10% nonfat dry milk made in TBS-Tween. The blocked strips were incubated overnight either in cellular titin or muscle titin (5 µg/ml) diluted from dialyzed HAP column fractions into block solution such that the final KCl concentration was 250 mM. Bound cellular titin and muscle titin were detected indirectly with a rabbit polyclonal antibody raised against cellular titin that crossreacts with muscle titin followed by a secondary anti-rabbit IgG conjugated to alkaline phosphatase.

### **Electron Microscopy**

Coassembled protein samples were negatively stained with or without immunogold labeling and examined by electron microscopy. For negative staining, glow-discharged, formvar- and carbon-coated 400 mesh Nickel grids were floated on drops of the samples for 30 s. The samples were stained with 2% uranyl acetate using three drops of stain. The first two drops were removed immediately after contact with the grid using a piece of filter paper. The grid was incubated for 30 s in the third drop and then washed with 5–10 drops of assembly buffer.

For immunogold labeling, grids were incubated on drops of sample for 5 min. The grids were transferred to a drop of 1% glutaraldehyde/PBS, pH 7.0, and incubated for 5 min followed by a 5-min incubation on a drop of 0.1 M Tris-HCl, pH 7.0. Grids were incubated for 5 min on a drop of anti-cellular titin polyclonal antibody that was diluted 1:10 or rabbit IgG (Sigma Chemical Co., St. Louis, MO) diluted to 7 µg/ml in 0.1 M Tris-HCl, pH 7.0, followed by a 5-min incubation on a drop of anti-rabbit IgG-conjugated 15-nm gold particles (Janssen Life Sciences, Beerse, Belgium; diluted 1:100 in 0.1 M Tris-HCl, pH 7.0). The immunogold-labeled samples were stained with 1% uranyl acetate using seven drops of stain. The first six drops were removed with filter paper after contact with the grid. The last drop was left on the grid for 30 s before wicking. Stained grids were washed with 10 drops of 0.1 M Tris-HCl, pH 7.0, and air dried. Grids were examined by transmission electron microscopy (1200 EX; JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

## **Results**

### **Colocalization of Cellular Titin and Myosin in Fibroblast Stress Fibers**

Double label immunofluorescence revealed colocalization of cellular titin (Fig. 1 *a*) and myosin (Fig. 1 *b*) in fibroblast stress fibers. The similar punctate pattern of staining for cellular titin and myosin indicates that each protein is regularly distributed along the length of the stress fibers with a periodicity of ~300 nm.

### **Purification of Cellular and Muscle Isoforms of Titin and Myosin**

To investigate whether cellular titin and brush border myosin interact *in vitro*, we extracted and purified both proteins from isolated brush borders. For this, a 0.15 M KCl + ATP extract of isolated brush borders that contained both proteins (Fig. 2, lane *B*) was loaded directly onto a Sephacryl S-500

column. Cellular titin eluted near the void volume in fractions that typically also contained other polypeptides including TW 260/240, a peptide of ~100 kD, and the glycoprotein mucin (Fig. 2, lane *C*). In SDS gels of the fractions, mucin stained poorly with Coomassie blue but was readily detected by silver staining and periodic acid–Schiff staining (data not shown). Cellular titin was separated from these contaminating proteins and concentrated by hydroxyapatite column chromatography (Fig. 2, lane *D*). Occasionally, a small amount of the ~100-kD peptide contaminated the pooled cellular titin fractions from the hydroxyapatite column.

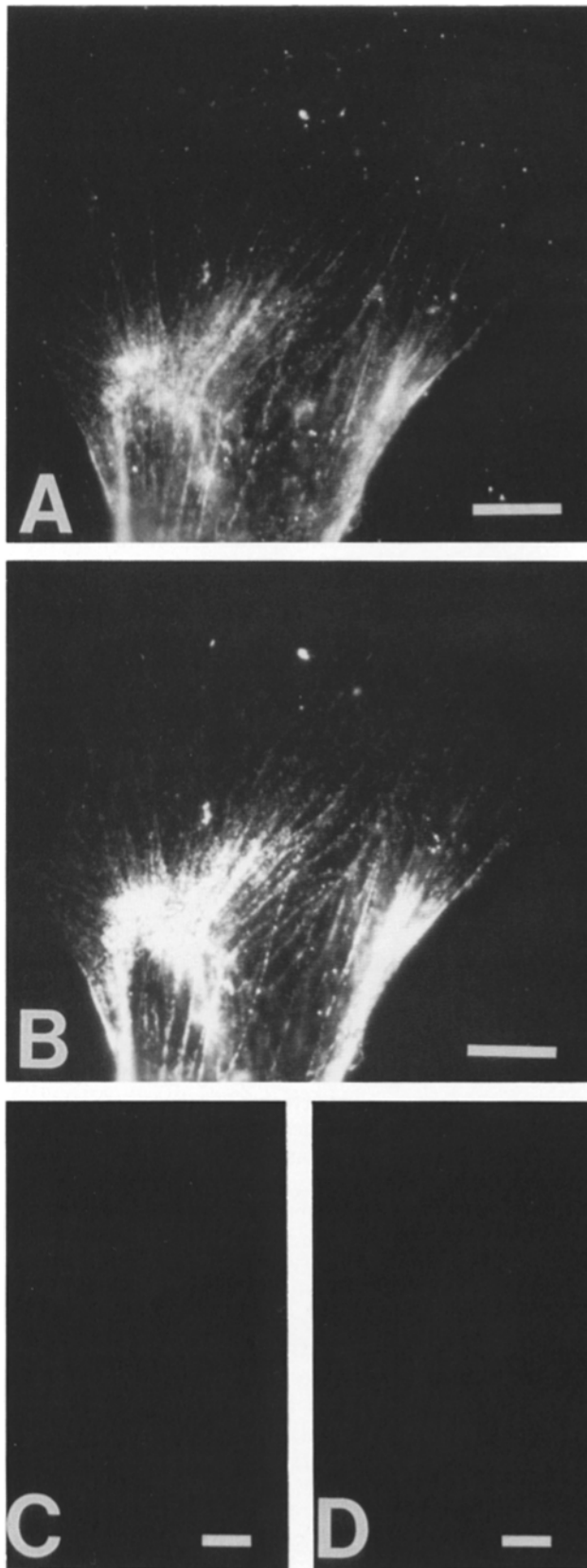
Brush border myosin II eluted from the Sephacryl S-500 column after the cellular titin, consistent with the size differential of the molecules. In some preparations, small and variable amounts of a high molecular weight protein (~1,200-kD) contaminated the first fractions of myosin eluted from the column. This contaminant reacted positively with our anti-cellular titin antibody and thus likely was a cellular titin proteolytic fragment. Subsequent hydroxyapatite column chromatography failed to separate this protein from the bulk of the myosin, so only the myosin fractions lacking the 1,200-kD polypeptide (Fig. 2, lane *E*) were used for subsequent myosin purification by HAP chromatography (Fig. 2, lane *F*).

To compare and contrast interactions between muscle and nonmuscle components, we also purified titin (Fig. 2, lanes *H* and *I*) and myosin (Fig. 2, lane *J*) from extracts of chicken pectoralis muscle (Fig. 2, lane *G*). Separate extracts were used for these purifications to maximize yields of titin and myosin.

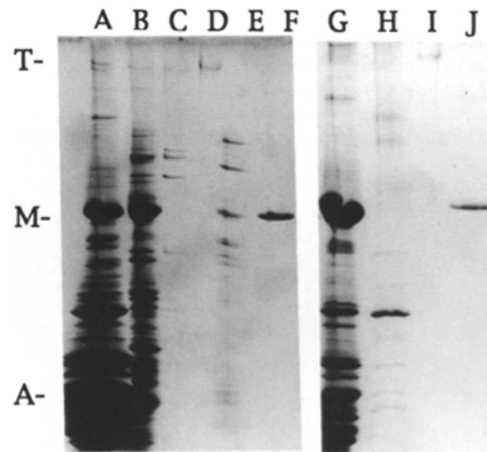
### **Brush Border Myosin II Assembled in the Presence of Cellular Titin Forms Large Supramolecular Aggregates**

To determine whether the cellular titin interacts with cellular myosin II, we assembled purified brush border myosin into bipolar filaments in the presence and absence of cellular titin. When cellular titin was present, the myosin assembled into bipolar filaments that over time aggregated into large, highly ordered arrays in which the myosin filaments were associated end-to-end and aligned side-by-side. The striated pattern reflecting the end-to-end alignment of myosin filaments in the coassemblages had a periodicity of ~300 nm (Fig. 3, *c–f*). The arrangement of myosin filaments at the splayed ends of the supramolecular arrays suggest that formation of the coassemblage starts as a reticular network that then condenses into highly ordered arrays. It also appears that the ends of coassemblages can both splay and knit together to yield branched and sometimes fused coassemblages. Under similar solution conditions, myosin alone formed bipolar filaments that remained unassociated with each other (Fig. 3 *b*). Cellular titin alone formed large, cablelike associations (Fig. 3*a*) similar to those found with rabbit psoas muscle titin-II (21, 43).

Immunogold localization with our anti-cellular titin antibody confirmed the presence of cellular titin in the supramolecular assemblies. Following fixation, the coassemblages were incubated in anti-cellular titin polyclonal antibody or rabbit IgG as a control followed by a secondary antibody conjugated to 15-nm gold particles. Gold particles were best detected in understained assemblages (Fig. 4, *a–d*).



**Figure 1.** Immunolocalization of cellular titin and myosin in cultured chick embryo skin fibroblast stress fibers. A cultured chick embryo skin fibroblast double-labeled with an anti-cellular titin rabbit polyclonal antibody that was detected with an FITC-conjugated donkey anti-rabbit IgG antibody (A) and an anti-cellular myo-



**Figure 2.** Purification of brush border cellular titin and myosin II and pectoralis muscle titin and myosin. SDS-PAGE analysis of samples from steps in the purification of cellular titin and brush border myosin: 0.15 M KCl + ATP extract of isolated brush borders (lane B); Sephacryl S-500 fraction containing cellular titin (lane C); purified cellular titin eluted from HAP column (lane D); Sephacryl S-500 column fraction containing brush border myosin II (lane E); and purified brush border myosin II eluted from a HAP column (lane F). SDS-PAGE analysis of samples from steps in the purification of muscle titin and myosin: 0.6 M KCl + ATP extract of chicken pectoralis muscle (lane G); HAP column fraction containing titin (lane H); purified titin obtained from ammonium sulfate precipitation (lane I); and purified muscle myosin (lane J). Lane A is an SDS extract of pectoralis muscle used as a marker for titin and myosin heavy chain migration in the 2.5–20% polyacrylamide gradient gels. T, muscle titin or cellular titin; M, myosin heavy chain; A, actin.

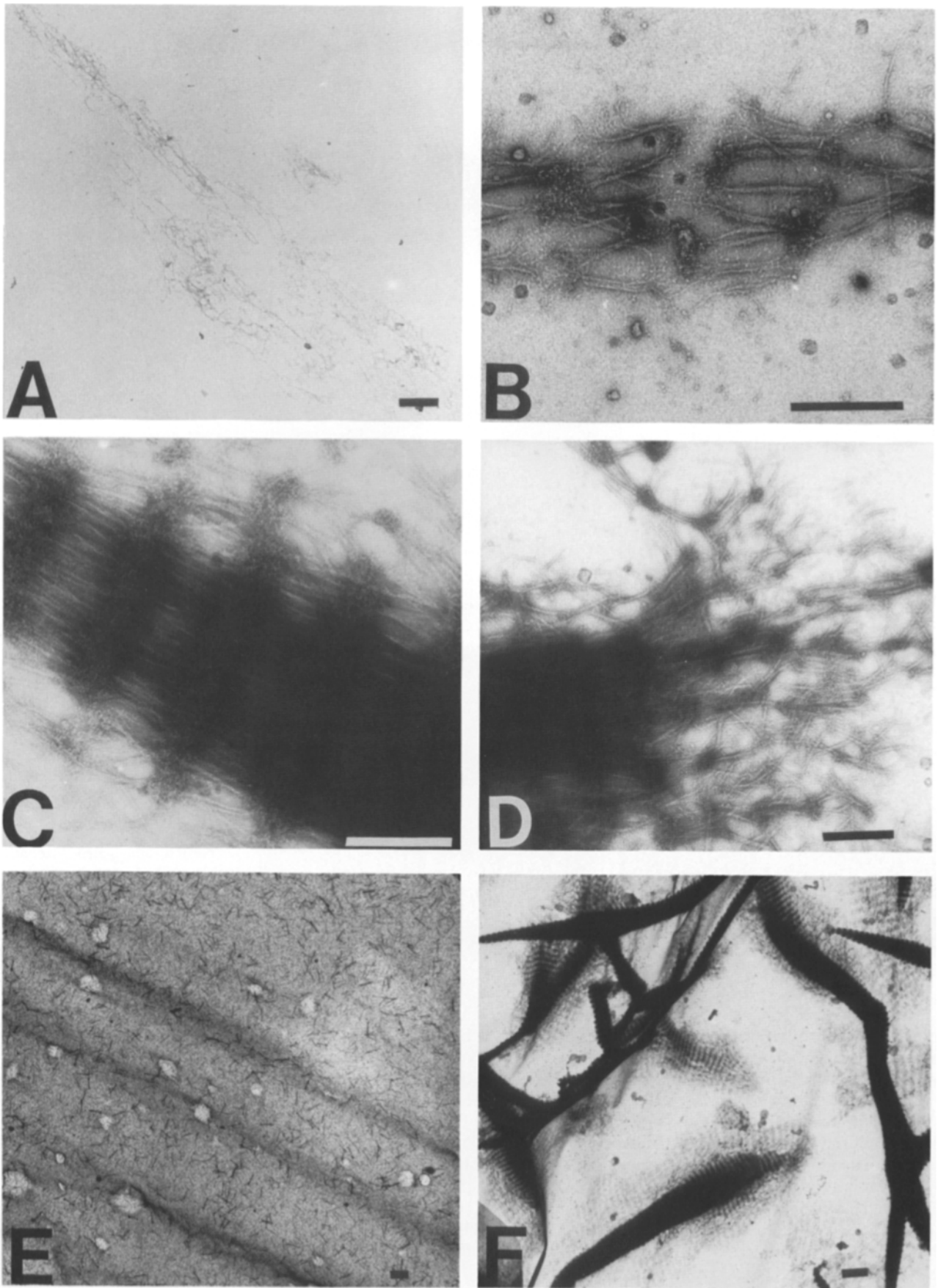
### ***Muscle Titin Forms Less-ordered Aggregates with Sarcomeric Myosin***

Muscle myosin that was assembled in the presence of muscle titin also formed large aggregates (Fig. 5). These aggregates were consistently more reticular and less ordered than the cellular isoform structures. Gold labeling with our anti-cellular titin antibody, which also crossreacts with muscle titin, confirmed titin association with these aggregates.

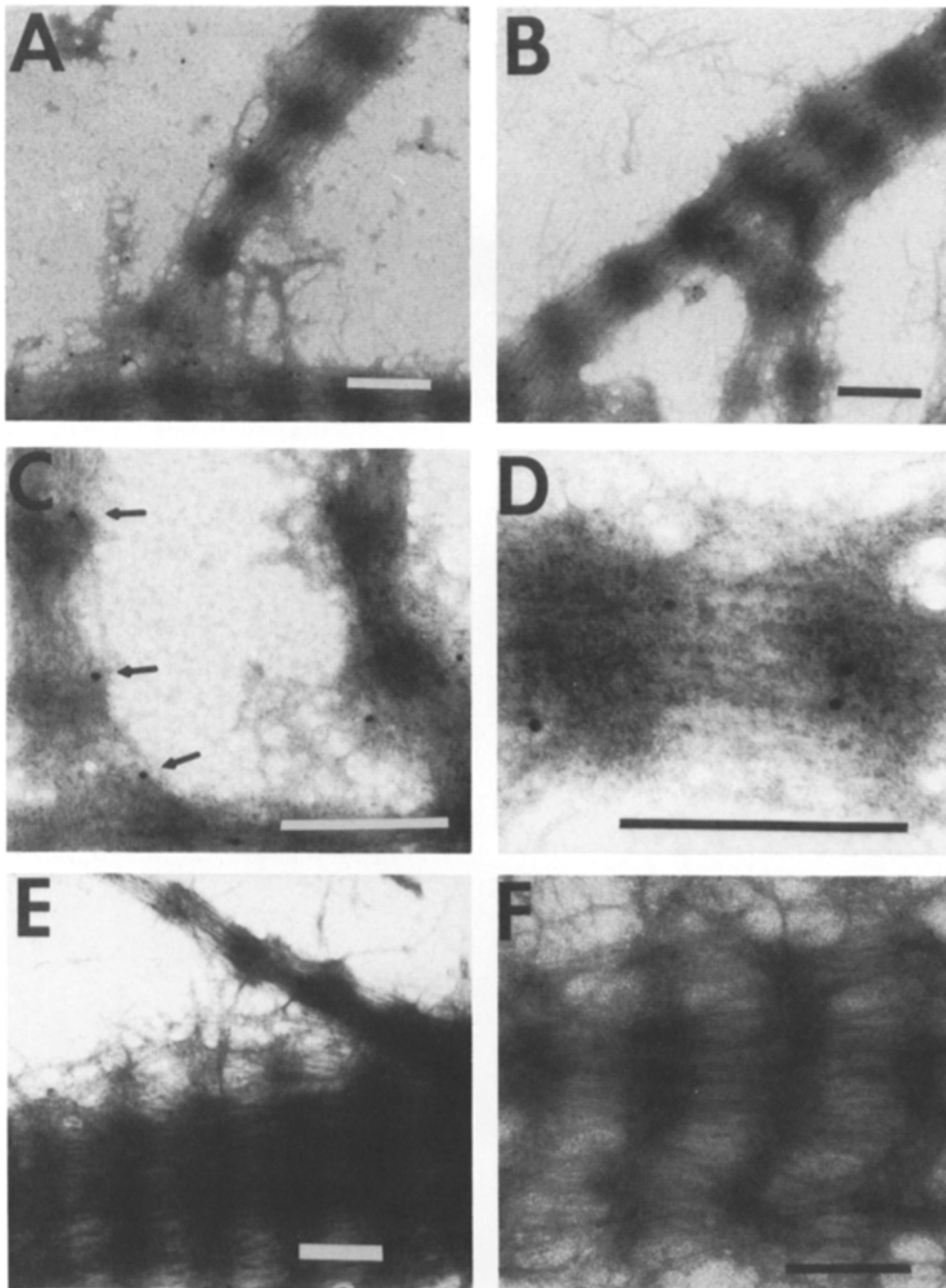
### ***Sucrose Density Gradient Analysis of Coassembly Interactions***

We further characterized the cellular and muscle titin-myosin coassemblies by sucrose density gradient centrifugation. Coassemblies of cellular titin and brush border myosin banded at the interface between the 5–60% sucrose gradient and a pad of 65% sucrose (Fig. 6, lanes 2–4). To determine the ratio of cellular titin to myosin in the coassemblies, we analyzed duplicate coassembly reactions from each of two independent purifications of cellular titin and myosin. Sam-

ple mouse monoclonal antibody that was detected with a TRITC-conjugated donkey anti-mouse IgG antibody (B) displayed colocalization of cellular titin and myosin in stress fibers. Control cells incubated either with nonimmune serum IgG followed by the FITC-conjugated donkey anti-rabbit IgG antibody (C) or only the TRITC-conjugated donkey anti-mouse IgG antibody (D) displayed no staining. Bar, 10  $\mu$ m.



**Figure 3.** Electron micrographs of cellular titin and brush border myosin interaction. Aliquots of purified cellular titin (*A*), brush border myosin (*E*), and a mixture of cellular titin and brush border myosin (*B–D*, and *F*).



**Figure 4.** Immunogold localization of cellular titin in cellular titin-brush border myosin coassemblages. Samples of coassembled cellular titin and brush border myosin on grids were fixed, incubated with an anti-cellular titin polyclonal antibody (A-D) or a nonimmune antibody (E and F) followed by a 15-nm gold particle-conjugated secondary antibody, and stained with 1% uranyl acetate. Arrows indicate gold label associated with myosin filament head domains (C). Bars, 300 nm.

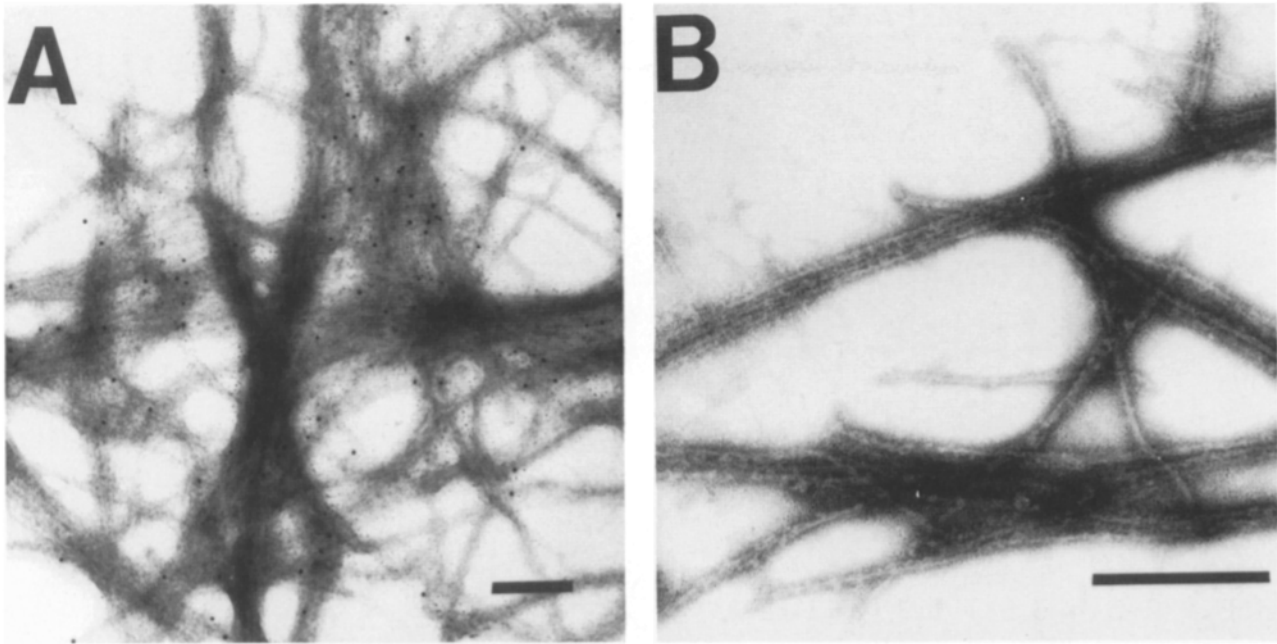
ples of three interface fractions from each of the gradients were analyzed by scanning densitometry of Coomassie blue-stained SDS gels. The mean optical density ratio for myosin to cellular titin in the 12 interface fractions analyzed was  $3.29 \pm 0.07$  (SE). Using an approximate molecular weight of  $3 \times 10^6$  D for cellular titin and  $4 \times 10^5$  D for two myosin heavy chains in each myosin molecule, we calculated the myosin/cellular titin ratio in the coassemblies to be  $\sim 25:1$ .

In experiments where brush border myosin was in excess of this ratio, all of the cellular titin migrated to the interface (as in Fig. 6 a). The excess myosin remained higher in the gradient and typically separated into two peaks (Fig. 6, a, lanes 8, 9, and 12). The faster sedimenting major peak appeared to be assembled but unaggregated myosin bipolar filaments (Fig. 6 a, lanes 8 and 9). The slower sedimenting

minor peak appeared to be unassembled myosin (Fig. 6 a, lane 12). In experiments where cellular titin was in excess of the 1:25 molar ratio to myosin, the coassemblies spread throughout the gradient, and the cellular titin/myosin ratio in various fractions was greater than 1:25 (data not shown). This is consistent with the ability of cellular titin to aggregate in the absence of myosin. When present, the unidentified 100-kD peptide that contaminated certain preparations of purified cellular titin was found in the coassembly fractions (Fig. 6 a, lanes 2-4, arrow). The variable presence of the 100-kD protein, however, makes it an unlikely requirement for cellular titin-myosin coassembly.

Cellular titin assembled in the absence of myosin failed to reach the sucrose gradient-pad interface and was spread throughout several fractions higher in the gradient (Fig. 6 b,





**Figure 5.** Immunogold localization of titin in coassemblies of muscle titin and muscle myosin. Samples of coassembled titin and muscle myosin on grids were fixed, incubated with an anti-cellular titin polyclonal antibody that crossreacts with muscle titin (A) or a nonimmune antibody (B) followed by a 15-nm gold particle-conjugated secondary antibody, and stained with 1% uranyl acetate. Bars, 300 nm.

lanes 5–10). Myosin assembled in the absence of cellular titin also remained higher in the gradient and separated into two peaks similar to those found in samples containing myosin in excess of the coassembly ratio to cellular titin (Fig. 6 c, lanes 7–12):

Under similar assay conditions, coassemblies of muscle titin and myosin also migrated to the sucrose gradient-pad interface (Fig. 7 a, lanes 2–4). We determined the myosin:titin molar ratio in these coassemblies to be  $\sim 38.1$  (mean optical density ratio of  $5.04 \pm 0.15$ ,  $n = 11$  fractions analyzed). Muscle titin dialyzed in the absence of myosin (Fig. 7 b) or muscle myosin assembled without muscle titin (Fig. 7 c) both failed to reach the interface and were spread throughout the gradient with distributions similar to those found with the cellular isoforms under similar conditions (Fig. 6, b and c).

#### ***Isoform Specificity of Titin-Myosin Interaction***

To investigate the specificity of titin-myosin interaction, we assembled muscle myosin in the presence of cellular titin (Fig. 8 a) and brush border myosin in the presence of muscle titin (Fig. 8 b). Sucrose density gradient centrifugation yielded no protein at the gradient-pad interface in either case. Although the presence of myosin and titin in various fractions overlapped, the sedimentation pattern for each protein was characteristic of pure protein, indicating no interaction between cellular titin and muscle myosin or between muscle titin and brush border myosin. Electron microscopy of samples taken prior to sedimentation analysis confirmed lack of interaction between mixed cellular and muscle titin and myosin isoforms.

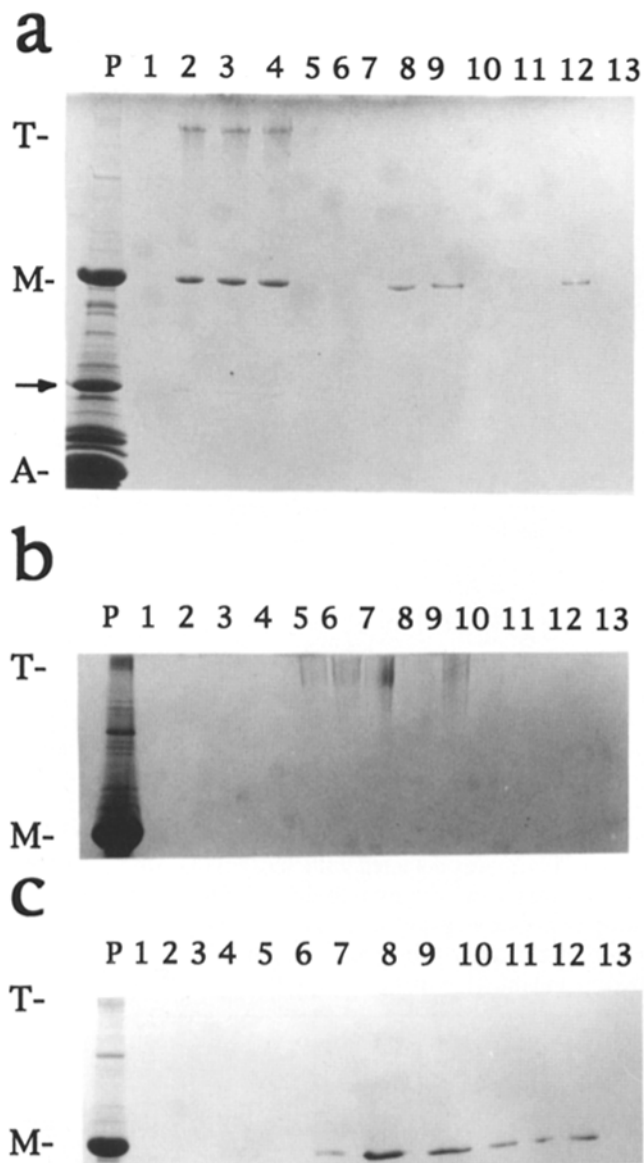
A solid phase binding assay also demonstrated isoform-specific interactions between cellular and muscle titins and myosins. Aliquots of muscle and brush border myosin were blotted onto a strip of nitrocellulose and overlaid with either

cellular titin or muscle titin (Fig. 9). Binding of cellular titin or muscle titin was detected with anti-cellular titin antibody, which crossreacts with muscle titin, followed by an alkaline phosphatase-conjugated secondary antibody. The results indicate that cellular titin bound specifically to brush border myosin and muscle titin bound specifically to muscle myosin.

## ***Discussion***

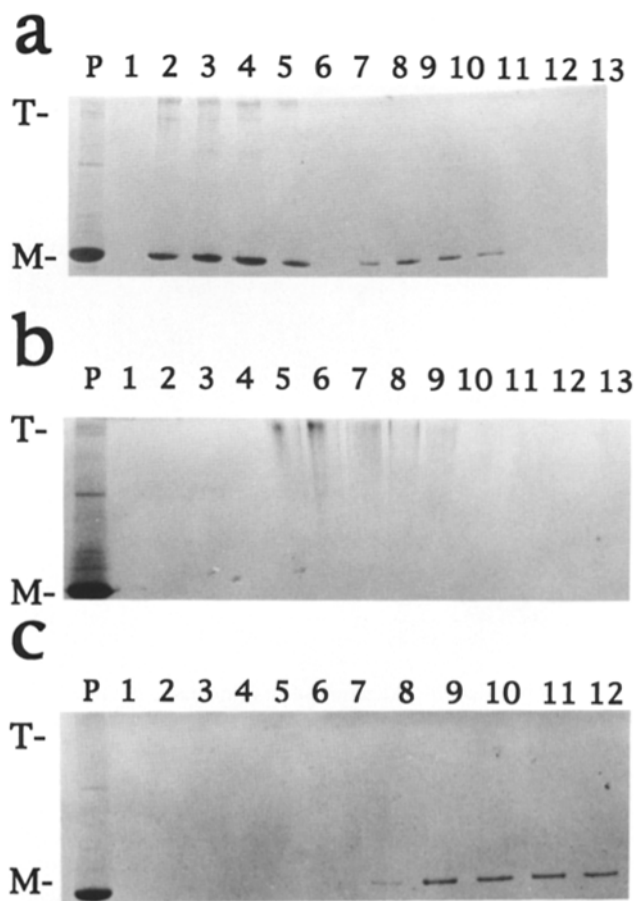
### ***Cellular Titin-Myosin Interaction In Vivo and In Vitro***

Our initial discovery of a cellular isoform of titin (T-protein) colocalized with myosin II in the terminal web domain of intestinal brush borders (7) and demonstration here that cellular titin also is colocalized with myosin in fibroblast stress fibers supports the possibility that cellular titin mediates interactions between cellular myosin filaments and a variety of cytoskeletal structures. Additional characterization of interaction between brush border myosin and cellular titin in vitro reinforces this possibility. Our results reveal that cellular titin can align myosin bipolar filaments side-by-side and end-to-end into highly ordered arrays. Moreover, the highly ordered distribution pattern of myosin bipolar filaments that we find in cellular titin-myosin coassemblies in vitro bears a remarkable resemblance to in vivo myosin filament distributions patterns found in the circumferential ring of intestinal epithelial cell brush borders (6), at the periphery of fibroblasts (40), and in stress fibers (24). Although actin filaments could aid in myosin II localization in these cytoskeletal structures in vivo, the degree of myosin filament organization in in vitro cellular titin-myosin coassemblies that lack actin suggests that the myosin filament localization in several cytoskeletal structures depends more on interactions with cellular titin than with actin.



**Figure 6.** Sucrose density gradient centrifugation analyses of cellular titin, assembled brush border myosin, and coassemblies of cellular titin and brush border myosin. Aliquots of purified cellular titin and brush border myosin were dialyzed separately or together into an assembly buffer and subjected to sucrose density gradient centrifugation. SDS-PAGE analysis of fractions from: (a) coassembly of cellular titin and brush border myosin; (b) dialysis of cellular titin alone; and (c) assembly of brush border myosin alone. Lane 1 in each gel corresponds to the fraction from the bottom of the gradient. Lanes 2–4 are fractions from the 5–60% sucrose gradient 65% sucrose pad interface. Lanes 5–13 are from the bottom to the top of the 5–60% sucrose gradient. *P*, SDS-extract of chicken pectoralis muscle; *T*, titin or cellular titin; *M*, myosin heavy chain; *A*, actin. Arrow, 100-kD protein.

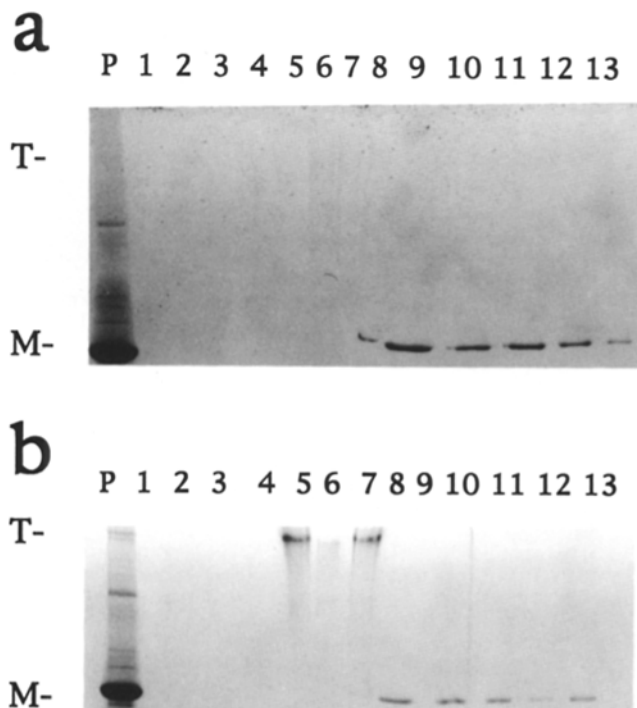
Undoubtedly, similar *in vitro* interactions between cellular titins and myosins have been observed before, indicating that cellular titin may be a widespread cytoskeletal component. For example, *Acanthamoeba* and platelet myosin bipolar filaments were found to align following assembly (32, 35). Nevertheless, the mechanism by which cellular titin organizes myosin filaments *in vitro* remains to be elucidated.



**Figure 7.** Sucrose density gradient centrifugation analyses of muscle titin, assembled muscle myosin, and coassemblies of muscle titin and myosin. Aliquots of purified titin and muscle myosin were dialyzed separately or together into an assembly buffer and subjected to sucrose density gradient centrifugation. SDS-PAGE analysis of fractions from: (a) coassembly of titin and muscle myosin; (b) dialysis of titin alone; and (c) assembly of muscle myosin alone. Lane 1 in each gel corresponds to the fraction from the bottom of the gradient. Lanes 2–4 are fractions from the 5–60% sucrose gradient 65% sucrose pad interface. Lanes 5–13 are from the bottom to the top of the 5–60% sucrose gradient. *P*, SDS-extract of chicken pectoralis muscle; *T*, muscle titin or cellular titin; *M*, myosin heavy chain.

Brush border myosin filaments coassembled with cellular titin appear to be similar in size to platelet myosin filaments that were estimated to contain 28 myosin molecules (32). Our determination of a 25:1 myosin to cellular titin molar ratio in the coassemblies, therefore, is most consistent with one cellular titin molecule per myosin bipolar filament. This apparent 1:1 cellular titin:myosin filament ratio does not preclude the possibility that a single titin molecule interacts with multiple myosin filaments. We previously have observed cellular titin molecules with conformations ranging from 900 to 1700 nm in length (7). If extended along the length of the coassembly, one cellular titin molecule could span multiple, possibly three to five, bipolar filament regions. Fibrous material is evident between the head regions of adjacent myosin bipolar filaments and appears to be oriented along the coassembly (see for example Fig. 3 *d*). This fibrous mate-



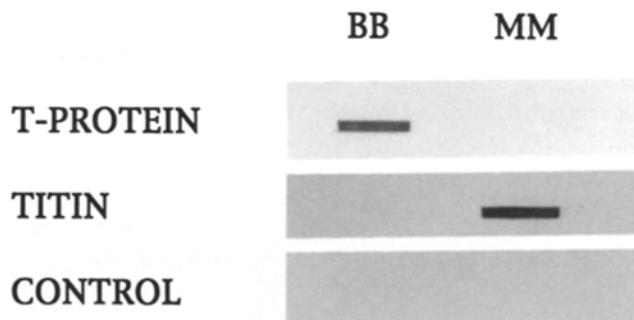


**Figure 8.** Sucrose density gradient centrifugation analyses demonstrating the specificity of titin and myosin isoform interactions. SDS-PAGE analysis of fractions from: (a) coassembly of cellular titin and muscle myosin, and (b) coassembly of muscle titin and brush border myosin. Lane 1 in each gel corresponds to the fraction from the bottom of the gradient. Lanes 2–4 are fractions from the 5–60% sucrose gradient 65% sucrose pad interface. Lanes 5–12 are from the bottom to the top of the 5–60% sucrose gradient. P, SDS-extract of chicken pectoralis muscle; T, muscle titin or cellular titin; M, myosin heavy chain.

rial is likely to be titin molecules spanning the gap and tethering myosin filaments end-to-end.

It is difficult to envisage how threads aligned only along the length of the coassembly could account for the side-by-side arrangement of the myosin filaments, however. This aspect of the alignment suggests that there are specific interactions between adjacent myosin filaments or more likely between cellular titin molecules. Although brush border myosin filaments remain unaggregated in the absence of cellular titin, both cellular titin (Fig. 3 a) and muscle titin (43) molecules form loose aggregates in the absence of myosin. We have detected no regular distribution pattern of immunogold labeling in bundles of pure cellular titin indicative of a specific alignment (data not shown), suggesting that the myosin filaments may impart a specific alignment to self-associating cellular titin molecules in the coassembly.

In addition to a role in higher order organization of myosin, cellular titin also might provide a template for bipolar filament assembly. One possible template role would be to stabilize cellular myosin bipolar filaments in the presence of ATP. ATP normally causes filament disassembly unless the myosin regulatory light chains are phosphorylated (4, 20). This sensitivity to ATP has led to the postulate that light chain phosphorylation regulates myosin filament formation in vivo. We have found that the aggregated myosin bipolar filaments associated with cellular titin likewise disassemble



**Figure 9.** Solid phase assay of cellular and muscle titin and myosin isoform interactions. Brush border myosin and muscle myosin were blotted onto nitrocellulose. The blots were incubated with brush border cellular titin or muscle titin. The binding of cellular titin and muscle titin was detected with an anti-cellular titin antibody that reacts with both, followed by an alkaline phosphatase-conjugated secondary antibody.

in the presence of ATP (Eilertsen, K., and T. Keller, unpublished observations), thereby negating the possibility that cellular titin provides a stabilizing template. Another possible template role could be to specify the size of bipolar filaments; but we have detected no significant difference in size between bipolar filaments assembled with and without cellular titin. Nevertheless, the myosin heads in coassembly filaments appear to be more tightly clustered at each end than in free myosin filaments, indicating that cellular titin may have at least a subtle effect on the conformation of myosin filaments, even though it plays no apparent role as a template for assembly.

### Muscle Titin–Myosin Interaction

The interaction of cellular titin with cellular myosin differs significantly from that of muscle titin and myosin under similar in vitro conditions. We find that titin associates with the large bipolar filaments of muscle myosin in loosely ordered arrays. The molar ratio of 1:38 for titin to myosin in these in vitro coassemblies is most consistent with an association of eight titin molecules with each sarcomeric thick filament (~294 myosin molecules; reference 45) in vivo. This ratio falls within the range of 6–12 titins per thick filament previously estimated from the mass ratio of titin and myosin in muscle (45). Other investigations have demonstrated in vitro interactions between muscle titin and other proteins including myosin (31). In solid phase binding assays, for example, titin has been shown to interact with C-protein, AMP deaminase, and X-protein, as well as with the tail domain of muscle myosin (12, 21, 22, 37). Our results indicate that titin and myosin require no other protein to coassemble, at least in vitro. Nevertheless, other proteins could be important in organizing or stabilizing titin-myosin interaction in the sarcomere.

### Isoform Specificity

Consistent with the general differences in interactions between cellular titin and myosin and muscle titin and myosin, we have found a discrete isoform specificity for titin-myosin interaction. Cellular titin interacts specifically with cellular myosin, whereas muscle titin interacts specifically with mus-

cle myosin. Most cells express one or the other isoform of myosin, not both. During muscle differentiation, however, stress fibers containing myosin coexist in the same cytoplasm with the developing array of muscle sarcomeres containing muscle myosin (for examples see references 9 and 36). Our *in vitro* results indicate that the specificity of interactions between titin and myosin isoforms could contribute to the segregation of the cytoskeletal and sarcomeric structures in the cytoplasm of developing muscle *in vivo*.

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