Monoclonal Antibodies Distinguish Titins from Heart and Skeletal Muscle

Craig Hill and Klaus Weber

Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany

Abstract. Murine monoclonal antibodies specific for titin have been elicited using a chicken heart muscle residue as antigen. The three antibodies T1, T3, and T4 recognize both bands of the titin doublet in immunoblot analysis on polypeptides from chicken breast muscle. In contrast, on chicken cardiac myofibrils two of the antibodies (T1, T4) react only with the upper band of the doublet indicating immunological differences between heart and skeletal muscle titin. This difference is even more pronounced for rat and mouse. Although all three antibodies react with skeletal muscle titin, T1 and T4 did not detect heart titin, whereas T3 reacts with this titin both in immunofluorescence microscopy and in immunoblots. Immunofluorescence microscopy of myofibrils and frozen tissues from a variety of vertebrates extends these results

and shows that the three antibodies recognize different epitopes. All three titin antibodies decorate at the A-I junction of the myofibrils freshly prepared from chicken skeletal muscle and immunoelectron microscopy using native myosin filaments demonstrates that titin is present at the ends of the thick filaments. In chicken heart, however, antibodies T1 and T4 stain within the I-band rather than at the A-I junction. The three antibodies did not react with any of the nonmuscle tissues or permanent cell lines tested and do not decorate smooth muscle. In primary cultures of embryonic chicken skeletal muscle cells titin first appears as longitudinal striations in mononucleated myoblasts and later at the myofibrillar A-I junction of the myotubes.

T ITIN is a protein of extremely high polypeptide molecular weight. Values of one to two million have been discussed in the literature (14, 22, 25, 29). Titin comprises a substantial percentage of the total protein mass of muscle myofibrils. In the presence of sodium dodecyl sulfate (SDS) it migrates as a doublet on low percentage polyacrylamide gels and the lower band is thought to represent a proteolytic breakdown product of the upper band. This protein was first detected in an extract of skeletal muscle consisting of several myofibrillar proteins (12). This mixture of proteins was collectively called "connectin" by Maruyama et al. (12). Wang et al. (29) independently isolated the high molecular weight component of connectin from skeletal muscle and called it titin. Both groups originally purified the protein in a denatured form using harsh treatments such as SDS. More recently it has been possible to extract a native molecule which is thought to correspond to the lower molecular weight component of the doublet (7, 8, 22, 30). Electron micrographs have characterized native titin as a long, elastic string ~3 nm in diameter with a variable length from 100 to 1500 nm (14, 22, 30). Although titin has been identified also in cardiac muscle, almost all detailed studies to date have been performed on titin from skeletal muscle.

A second high molecular weight protein (~500,000-mol-wt) was detected in SDS gels of skeletal muscle and was named nebulin (28). This protein was found to be immunologically distinct from titin and was localized at the N2-line

of skeletal muscle myofibrils by immunofluorescence microscopy.

Titin is believed to be a component of a putative third filament system in striated muscle where it could play an elastic length-limiting role within the myofibril (15, 26). Initial studies with polyclonal antibodies against the SDS denatured protein have localized it by immunofluorescence microscopy primarily at the A-I junction of the myofibril although it was also observed at other more variable positions within the myofibril such as the Z-line and throughout the A-band (29). Other studies using polyclonal antibodies against the native protein have yielded similar results (15). Current hypothesis based on the immunofluorescence data assume that titin stretches throughout the myofibril from the M-line to the Z-line or the N2-line within the I-band. Proteolytic degradation with subsequent redistribution of the molecule within the myofibril could explain the variable images obtained with immunofluorescence microscopy. Recent electron microscopical studies by Trinick et al. (22) have strongly suggested that titin may make up the "end filaments" observed at the ends of native myosin filaments. Despite this progress many aspects of the exact localization and attachment points of the titin molecules within the myofibril are unknown.

In the present study, we report the isolation and characterization of three different monoclonal antibodies against chicken heart titin. Using these antibodies we have found significant immunological differences between cardiac and

skeletal muscle titin among several species. These studies have additionally localized an attachment point for titin more precisely within the myofibril using both immunofluorescence and immunoelectron microscopy. The developmental sequence of titin in primary cultures of embryonic chicken skeletal and heart muscle cells is also reported.

Materials and Methods

Immunization and Monoclonal Antibodies

The residue of chicken heart used for immunization was prepared according to the fascia adherens isolation procedure given by Maher and Singer (11). Two female BALB/c mice 6–8 wk old were immunized with the residue (100–200 μg protein/injection) using Freund's complete adjuvant for the first injection and incomplete adjuvant for the booster injection at day 24. After the booster injection, test sera were screened by immunofluorescence microscopy on sections of chicken heart muscle. A final boosting schedule was used as in Debus et al. (3) except that all injections were intraperitoneal.

Spleen cells were fused with cells from the mouse myeloma line PAI using the procedures previously given (3). After fusion, cells were aliquoted into fifteen 24-well plates in hypoxanthine/aminopterin/thymidine (HAT) medium. Medium was changed twice a week and macroscopic colonies were visible by day 9. Supernatants were assayed by immunofluorescence microscopy and Western blot analysis as described below. Positive colonies were cloned twice by limiting dilution. Ascites fluids were produced in female BALB/c mice that had been injected 7–10 d previously with 0.5 ml Pristane (Janssen Pharmaceuticals, Beerse, Belgium).

Immunofluorescence Microscopy

All tissue sections were from material snap frozen in isopentane at −140°C and stored at −70°C until use. Sections were fixed in acetone for 6 min at 4°C and air dried before staining with antibody.

Myofibrils from chicken breast and heart muscle used in immunofluorescence microscopy were prepared essentially according to Wang and Ramirez-Mitchell (27). Glycerinated myofibrils were prepared according to Knight and Trinick (9). Myofibrils were kept in the myofibril extraction buffer during the antibody staining procedure. For the KI and KCl extraction experiments, fresh myofibrils processed as above were attached to coverslips followed by incubation in the solutions at 21°C for the time periods stated in the Results section. The 0.6 M KCl solution was the same as in Maruyama et al. (15) and the 0.6 M KI solution was the same as in Wang and Ramirez-Mitchell (27).

Embryonic chicken skeletal muscle cultures were obtained according to Bullaro and Brookman (2) using mechanical dissociation of the cells rather than trypsinization. Cells were grown on coverslips in Dulbecco's modified Eagle's medium + 10% horse serum and fixed in ethanol for 6 min at 4°C. After air drying, indirect immunofluorescence microscopy was performed. Primary cultures of chicken embryonic heart cells were obtained by removing hearts from day 8 embryos and incubating in a trypsin solution (Gibco, Karlsruhe, FRG) consisting of 0.05% trypsin, 5 mM EDTA in phosphate-buffered saline at 37°C for 10 min with gentle agitation. Digestion was terminated by addition of an equal volume of F-12 medium. The suspension was filtered through nylon mesh followed by centrifugation and resuspension in fresh F-12 medium. Cells were plated onto glass coverslips and fixed the following day as described above for the embryonic chicken skeletal cells. The human RD cell line derived from a rhabdomyosarcoma has been described (3).

Standard procedures for indirect immunofluorescence microscopy were used for sections, cells, and myofibrils. Fluorescein isothiocyanate–labeled goat anti-mouse antibodies (Cappel Laboratories, Cochranville, PA) were used at a final concentration of 250 μg/ml.

SDS Gel Electrophoresis and Immunoblot Analysis

SDS gel electrophoresis was based on the 3.2% polyacrylamide gels of Wang (25) except that 1-mm-thick slab gels were used rather than the tube gels described. The 2–15% or 3–15% gradient gels were performed as described (18) except that sucrose was added to 3% and 20% in the lower and higher percentage acrylamide solutions, respectively.

Electrophoretic transfer onto nitrocellulose was done essentially as described (19). After incubation of the nitrocellulose sheets with mouse monoclonal antibody, antibody decoration was visualized by treatment with peroxidase-labeled rabbit anti-mouse antibody (DAKO, Gloustrup, Denmark) followed by incubation with substrate.

Electron Microscopy

Native thick filaments from chicken breast muscle were prepared according to Trinick (21). Antibodies were reacted with a suspension of native thick filaments in a "relaxing buffer" consisting of 100 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 5 mM ATP, 0.1 mM dithiothreitol, and 6 mM potassium phosphate, pH 7, for 1–2 h at room temperature followed by centrifugation at 17,000 g and resuspension in the same buffer. After centrifugation, the pellet was resuspended in 10 nm gold-labeled goat anti-mouse IgG (Janssen Pharmaceuticals) diluted 1:1 with the same buffer and incubated for 1 h. The filaments were washed by centrifugation and resuspension in the relaxing buffer. Filaments were negatively stained by floating a carbon-coated piece of mica on a drop of filament suspension and allowing the mica to sink to the bottom of the droplet. The carbon film was then picked up on a grid and stained with 1% uranyl acetate. Grids were viewed on a Phillips 301 electron microscope using an accelerating voltage of 80 kV.

Other Procedures

Antibody type was determined by Ouchterlony immunodiffusion tests using sera specific for each of the distinct immunoglobulin classes (Litton Bionetics, Kensington, MD). Antibody concentration was determined by the enzyme-linked immunosorbent assay according to the protocol published by Dianova (Hamburg, FRG). Native titin was enriched from chicken breast muscle by the method of Trinick et al. (22) using the crude KCl extract after removal of myosin rather than the column-purified material.

Results

Monoclonal Antibodies against Titin

BALB/c mice were immunized with myofibrillar residues obtained from chicken heart muscle. Immunofluorescence microscopy of chicken heart sections was used to screen immune sera. One serum gave a characteristic myofibrillar staining emphasizing the A-I junction. Therefore, immune spleen cells from this mouse were fused with PAI mouse myeloma cells and grown in culture. Culture supernatants were initially screened using immunofluorescence microscopy on chicken heart muscle sections or myofibrils isolated from chicken breast muscle. Clones that gave myofibrillar banding patterns were further tested on Western blots using chicken breast myofibrillar proteins separated on 3.2% slab gels and transferred electrophoretically to nitrocellulose paper. Hybridoma supernatants from five clones detected two polypeptides of extremely high apparent molecular weight, which corresponded to the position of titin as described by others (14, 29). Of these five clones, two also stained additional bands on the Western blots and were therefore not characterized further. The other three hybridomas were cloned again and then grown as ascites. The resulting three monoclonal antibodies (T1, T3, and T4) stained both bands of the titin doublet band in Western blots of isolated chicken breast myofibrils (Fig. 1 a). All three antibodies also stained partially purified native titin derived from chicken breast muscle which migrates as a single band as previously reported (22) (Fig. 1 b). No other polypeptides in the preparations were labeled when 2–15% gradient gels were used for blotting. Such gels provided a good resolution of the lower molecular weight polypeptides present in addition to titin. Immunodiffusion showed that T3 and T4 are IgG1 antibodies while T1 is of the IgG2a type. Further experiments (see below and Table I) proved that all three antibodies recognize different epitopes.

To better separate the titin doublet, the tracking dye was run off the end of the polyacrylamide gels which had been loaded with myofibrillar polypeptides of chicken breast muscle. Subsequent immunoblots showed that all three antibodies decorated both bands of the titin doublet (Fig. 2 a). Surpris-

ingly this, however, was not the case when similar blots were made with chicken heart myofibrils. Fig. 2b demonstrates that T1 and T4 stain only the upper band of the cardiac titin
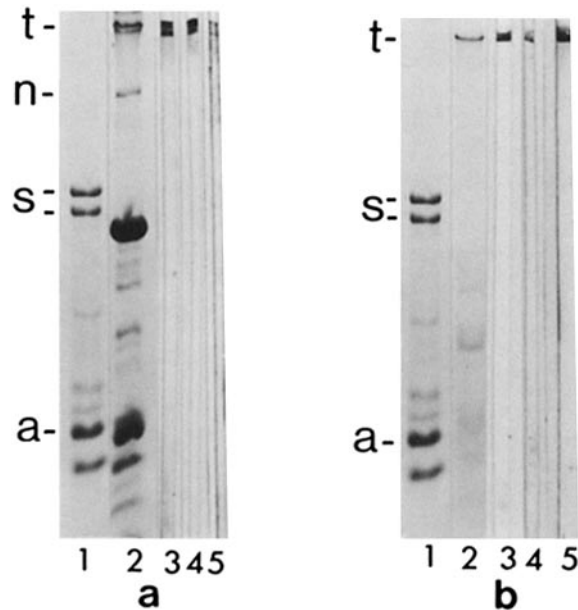
doublet while T3 stains both bands as do all three antibodies on chicken skeletal muscle myofibrils.

Western blots made on rat skeletal and heart myofibrils revealed additional differences between titin from the two sarcomeric muscles. All three antibodies stained the titin doublet in rat skeletal muscle as in chicken skeletal muscle (Fig. 2c). In rat heart, however, only T3 stained both bands of the doublet (Fig. 2d). T4 gave a very weak reaction

Figure 1. Immunoblotting of monoclonal titin antibodies against (*a*) chicken skeletal muscle myofibrils and (*b*) partially purified chicken skeletal muscle titin. (*a*) Lane *1*, Coomassie-stained standards. *s*, spectrin doublet; *a*, actin. Lane *2*, Coomassie-stained chicken breast myofibrils. *t*, titin; *n*, nebulin. Lanes *3-5*, immunoblots with (lane *3*) T3, (lane *4*) T4, and (lane *5*) T1. (*b*) Lane *1*, Coomassie-stained standards as in lane *1* of *a*. Lane *2*, Coomassie-stained partially purified skeletal muscle titin. Lanes *3-5*, immunoblots with (lane *3*) T3, (lane *4*) T4, and (lane *5*) T1. All three antibodies recognize purified titin (*b*) as well as the titin doublet present in myofibrils (*a*).

Table I. Characterization of Titin Monoclonal Antibodies T1, T3, and T4 by Immunofluorescence Microscopy

|	T1	T3	T4
Antibody type	IgG2A	IgG1	IgG1
Human skeletal muscle	+	+	+
Uterus	−	−	−
Monkey skeletal muscle (*Callithrix*)	+	+	+
Heart muscle	+	+	+
Rat skeletal muscle	+	+	+
Heart muscle	−	+	−
Mouse skeletal muscle	+	+	+
Heart muscle	−	+	−
Chicken skeletal muscle	+	+	+
Heart muscle	+	+	+
Embryo fibroblasts	−	−	−
Embryonic skeletal muscle cells	+	+	+
Aorta	−	−	−
Intestine	−	−	−
Gizzard	−	−	−
Alligator skeletal muscle (*Alligator mississippiensis*)	+	+	+
Toad skeletal muscle (*Bufo marinus*)	+	~	−
Heart muscle	+	~	−
Trout skeletal muscle (*Salmo trotta*)	−	+	−
Heart muscle	−	+	−
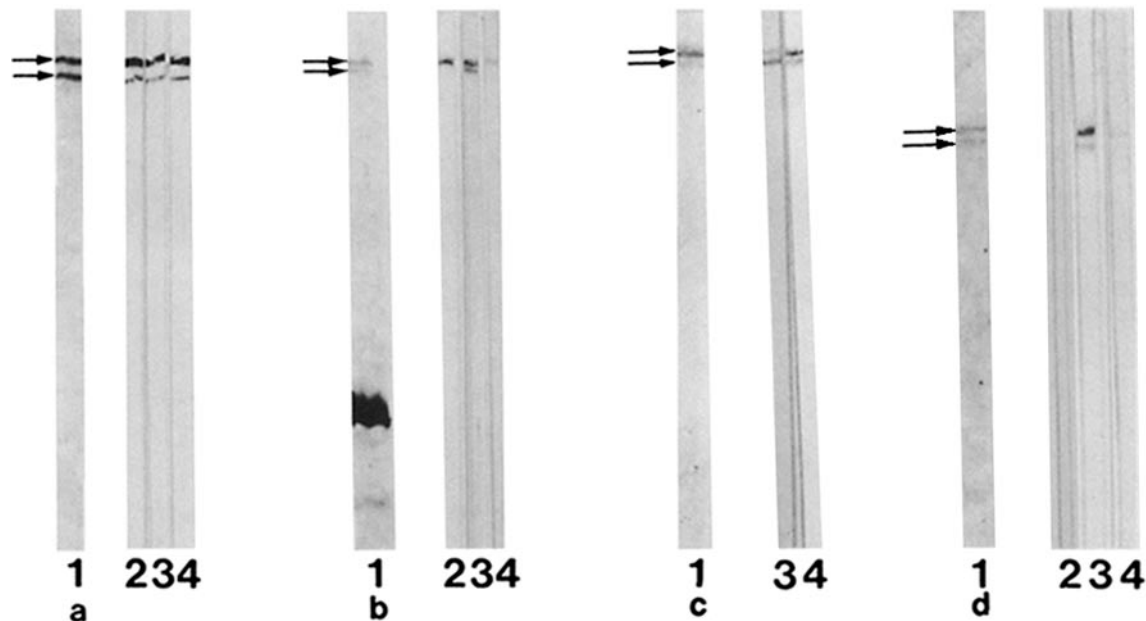
Figure 2. Immunoblots of monoclonal antibodies on (*a*) chicken skeletal muscle myofibrils, (*b*) chicken heart myofibrils, (*c*) rat skeletal myofibrils, and (*d*) rat heart myofibrils. In each group of figures, lane *1* is a nitrocellulose strip stained with Amido black. Lanes *2-4* are adjacent strips stained with antibodies (lane *2*, T1; lane *3*, T3; and lane *4*, T4). Although all three antibodies recognize the titin doublet (arrows) in chicken skeletal muscle (*a*) and rat skeletal muscle (*c*), antibodies T1 and T4 detect only the upper band of chicken cardiac titin (*b*) while only T3 reacts with both bands. In rat heart titin (*d*), T3 again stains both components of the doublet, while T1 gives no reaction and T4 a very weak decoration of the upper band. The thick lower molecular weight band in *b* is myosin heavy chain which was electrophoresed off the other gels in Fig. 2, *a*, *c*, and *d*.

involving only the upper band of the doublet while T1 did not stain either of the two bands of rat cardiac titin even when blots were overdeveloped.

Immunofluorescence Microscopy on Frozen Tissue Sections

Skeletal and cardiac muscle from a variety of vertebrates were frozen, sectioned, and stained with antibodies to titin. The summary in Table I shows that the titin antibodies reacted with sarcomeric muscles from a wide variety of species ranging from human and monkey to toad and trout although there is

not one particular antibody which covers all species and muscles. In many of the positive cases, the label appeared at the A-I junction of the myofibrils as reported for polyclonal antibodies on chicken and rabbit skeletal muscle myofibrils (13, 15, 29). However, the exact labeling pattern was not always deduced with certainty due to the limits of resolution of the immunofluorescence technique on frozen sections.

In certain species, major differences were observed for the differential reactivity of the antibodies on heart and skeletal muscle of the same animal. For example, Fig. 3 shows that antibody T3 stained both skeletal and cardiac muscle of rat
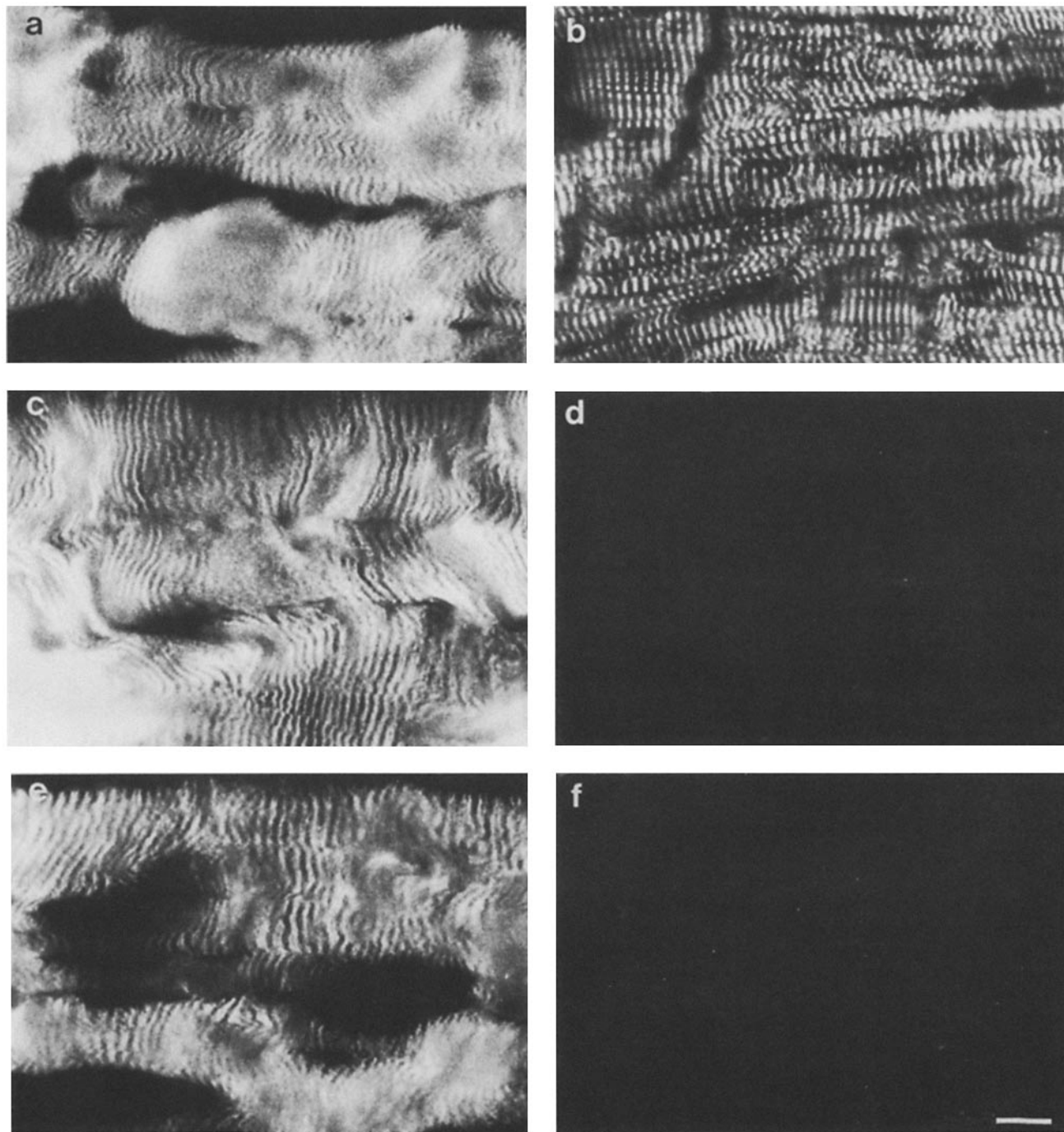

Figure 3. Immunofluorescence micrographs of frozen sections of rat skeletal muscle (*a, c,* and *e*) and heart tissue (*b, d,* and *f*), stained with antibodies against titin. (*a*) Rat skeletal, T3; (*b*) rat heart, T3; (*c*) rat skeletal, T4; (*d*) rat heart, T4; (*e*) rat skeletal, T1; (*f*) rat heart, T1. Note the lack of reactivity of rat cardiac muscle with antibodies T4 and T1 in *d* and *f*. Bar, 10 μm.

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with equal intensity (Fig. 3, a and b) while antibodies T4 and T1 stained only rat skeletal muscle (Fig. 3, c and e) and not rat heart muscle (Fig. 3, d and f). These data correlate well with the results obtained by the Western blots of the rat muscle preparations (Fig. 2, c and d). In immunofluorescence microscopy, mouse sarcomeric muscles behaved in their decoration pattern like the corresponding muscles of rat.

We have tested only three cold-blooded vertebrates in more detail (Table I). T4 antibodies were negative on heart and skeletal muscle of both the trout and the toad while the other two antibodies showed differential species but not tissue reactivity. Both heart and skeletal muscle were stained by T1 in the toad but not in the trout. T3 showed the opposite reactivity. It was positive on the trout muscles but not on the toad muscles. The combined results (see Table I) emphasize that the three antibodies recognize different epitopes. In the case of alligator we had only skeletal muscle available. It stained positively with all three antibodies.

Immunofluorescence Microscopy of Isolated Myofibrils

Myofibrils were isolated from chicken breast muscle and reacted with the mouse monoclonal antibodies followed by fluorescein-labeled goat anti-mouse antibodies. All three titin antibodies clearly stained the A-I junction of the skeletal muscle myofibrils. The fluorescent transverse bands in freshly prepared myofibrils were centered at the A-I junction and overlapped into both the A and the I-bands (Fig. 4a). The fluorescent bands seemed to remain at the A-I junction in both stretched and contracted myofibrils (Fig. 4b). There were, however, differences in the staining pattern obtained with antibodies T1 and T3 depending on the age of the myofibril preparation. Freshly prepared myofibrils stained on the same day with T1 or T3 gave the normal A-I junction pattern. When myofibrils were glycerinated according to standard procedures (9) and stored for several weeks in 50% glycerol at −20°C, subsequent immunofluorescence revealed in many of the myofibrils fluorescent images in addition to the normal A-I band staining pattern (Fig. 5a). These variations in banding patterns were observed between different myofibrils within the same preparation with no obvious correlation to the length of the myofibril. In T3 antibody-stained glycerinated myofibrils, most of the variant staining patterns consisted of M-line staining although also many instances of diffuse staining within the entire A-band were seen. Glycerinated myofibrils stained with T1 antibodies often had diffuse staining in the I-band with occasional discrete bands at the Z-line (data not shown). T4 antibodies stained only at the A-I junction in both glycerinated and nonglycerinated, freshly isolated myofibrils.

When freshly isolated skeletal muscle myofibrils were extracted for 10 min with a 0.6-M KI solution to remove most of the actin and myosin, the titin antibodies were localized at the remaining phase-dense bands of the extracted myofibril (Fig. 5b). These phase-dense areas represent the Z-line of the myofibril (5, 27) as confirmed by labeling with antibodies to α-actinin (data not shown). Similar results were also obtained with myofibrils which had been extracted with 0.6 M KCl for 10 min (Fig. 5c). This treatment extracts primarily myosin as well as some of the titin and leaves the I-Z-I "brushes." The titin antibodies again only labeled at the Z-line. When, however, myofibrils were extracted with 0.6 M KCl for only 20 s,
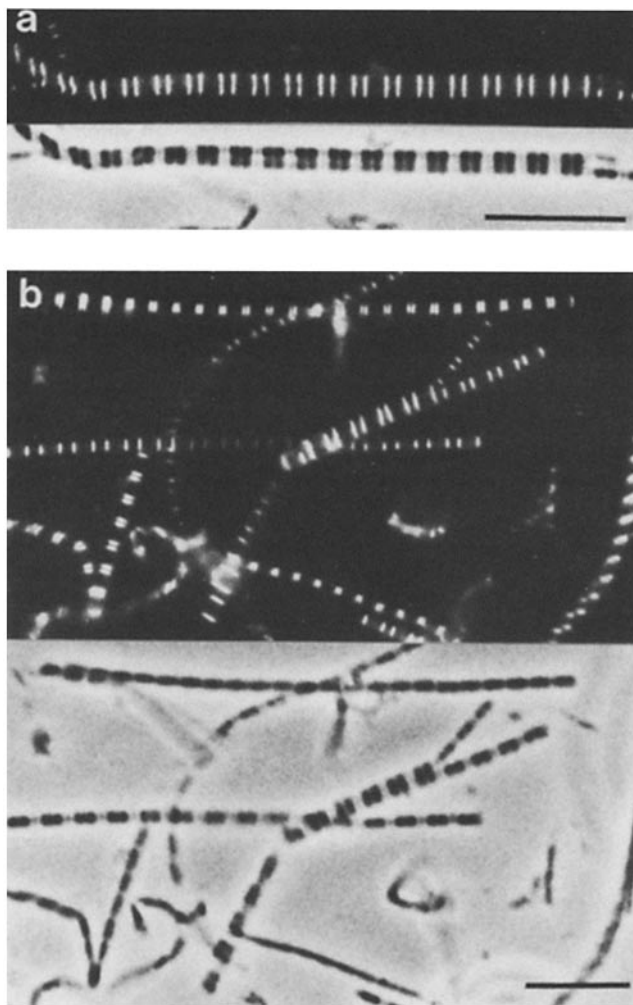

Figure 4. Immunofluorescence and phase-contrast micrographs of freshly prepared chicken skeletal muscle myofibrils stained with T3 antibody. (a) Fluorescence and corresponding phase-contrast micrograph of a single myofibril stained with T3 antibody. (b) Fluorescence and corresponding phase-contrast micrograph of chicken skeletal myofibrils at different sarcomeric lengths stained with T3 antibody. Fluorescent label was observed only at the A-I junction regardless of the myofibril length. Bars, 10 μm.

a completely different staining pattern was obtained (Fig. 5d). The titin antibodies were localized not only at the Z-line but also as a doublet in the area previously occupied by the A-band. Each band of this doublet is located at the edge of a slightly phase-dense area that apparently represents a shortened A-band (arrows). All three titin antibodies gave the same immunofluorescence staining pattern in the extracted myofibrils. Given the report that contractile proteins such as myosin and tropomyosin can translocate to the Z-lines in salt-extracted myofibrils (5) and the electron micrographs of similar material (27), the immunofluorescence data on titin in these specimens do not allow a decision as to its true disposition in normal myofibrils.

Using the same procedure as with skeletal muscle, myofibrils were freshly isolated from chicken heart and stained for titin. Antibody T3 gave the same staining pattern as in chicken skeletal muscle with the fluorescent bands centered at the A-I junction and overlapped into both the A- and the I-bands
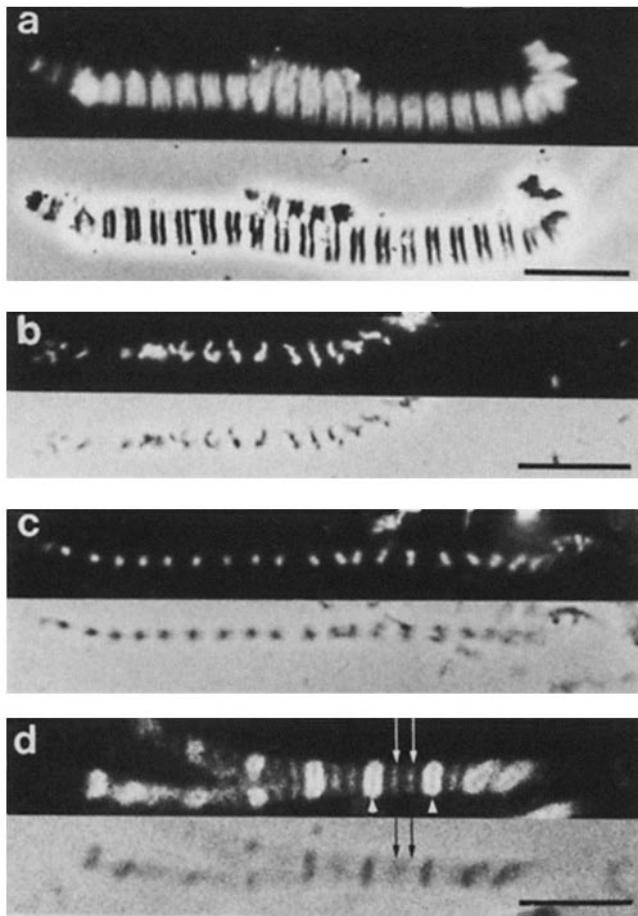
Figure 5. (a) Immunofluorescence and corresponding phase-contrast micrographs of chicken skeletal myofibrils which had been stored at −20°C in 50% glycerol. Fluorescent label is observed at the M-line and throughout the A-band in addition to the A-I junction stain. b–d are immunofluorescence and corresponding phase-contrast micrographs of chicken skeletal myofibrils extracted with (b) 0.6 M KI for 10 min, (c) 0.6 M KCl for 10 min, and (d) 0.6 M KCl for 20 s followed by immunofluorescence staining with T1 antibodies. Fluorescent bands are observed at the A-I junction (arrows in d), as well as at the Z-lines (arrowheads in d). Bars, (a–c) 10 μm; (d) 5 μm.
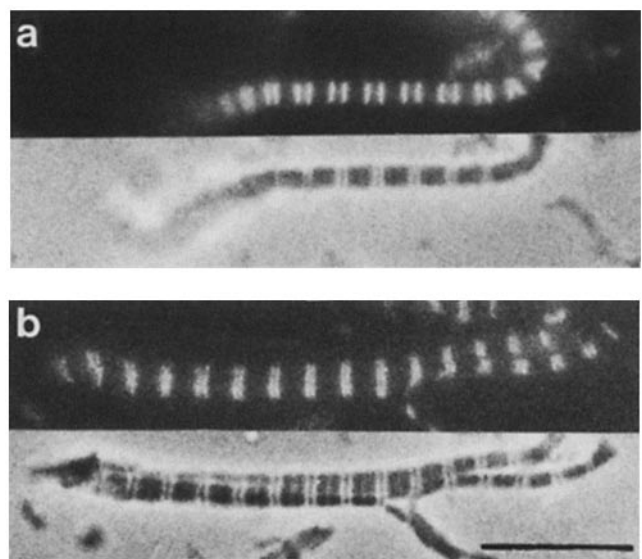

Figure 6. Immunofluorescence and corresponding phase-contrast micrographs of chicken heart myofibrils stained with (a) T3 and (b) T1 antibodies. The myofibrils are of the same sarcomere length and the micrographs are the same magnification. Note that antibody T1 stains within the I-band region while antibody T3 stains at the A-I junction. Bar, 10 μm.

(Fig. 6a). Antibodies T1 and T4, however, demonstrated different patterns (for T1 see Fig. 6b). Both antibodies gave fluorescent bands which were localized entirely within the I-bands of the myofibrils rather than at the A-I junctions. No staining was observed overlapping into the A-bands as was seen with the T3 antibody in heart and all three antibodies in skeletal muscle. These observations seem to correlate with the immunoblot results which also indicated differences between chicken skeletal and heart titin (Fig. 2, a and b). For instance, antibody T3 stains both bands of the cardiac titin doublet whereas T1 and T4 stain only the upper band of the doublet in immunoblotting. In skeletal muscle however, all three antibodies recognize both components of the doublet.

Titin Expression in Cultured Embryonic Chicken Skeletal and Heart Muscle Cells

Primary cultures of embryonic chicken skeletal muscle cells were prepared by mechanical dissociation of the leg muscles of day 12 chicken embryos. Immunofluorescence microscopy was performed with the monoclonal antibodies. Titin was first observed in mononucleated myoblasts where it occurred in a longitudinal pattern (Fig. 7, a and b). These longitudinal fibers sometimes had a striated appearance. No staining was observed in the nonmuscle cells surrounding the myoblasts. After fusion of myoblasts into multinucleated myotubes and the formation of myofibrils, titin fluorescence was located at the A-I junction as in adult skeletal muscle (Fig. 7, c and d). Often titin was located at the A-I junctions in the middle of the myofibril and occurred in longitudinal fibers at the ends of the myofibril near the plasma membrane. Distinct staining patterns were also obtained with primary cultures of embryonic myocardial cells isolated from the hearts of day 8 chicken embryos (Fig. 7, e and f).

The analysis of embryonic chicken skeletal and heart muscle cells indicated that titin is a marker of sarcomeric muscle differentiation. This is in line with labeling experiments using titin antibodies on a variety of nonmuscle tissues and cell lines of rodent and human origin as well as cultured chick embryo fibroblasts. Although titin-like proteins have been reported occasionally in some nonmuscle cell types (4, 6), our results with T1, T3, and T4 antibodies were uniformly negative (Table I). The sole permanent cell line which showed titin is the RD line derived from a human rhabdomyosarcoma (see reference 3). However, even here positive staining was restricted to the occasional differentiated cell which had a myotube-like morphology (Fig. 8). Such cells account for only a small percentage of the total cell number in the culture. None of the three antibodies stained smooth muscle tissue such as human uterus, chicken aorta, or chicken gizzard although titin-like molecules were postulated for the latter tissue (10).

Immunoelectron Microscopy of Native Thick Filaments

Preliminary immunoelectron microscopic experiments were performed with isolated native thick filaments reacted with T3 or T4 antibodies followed by gold-labeled second antibody
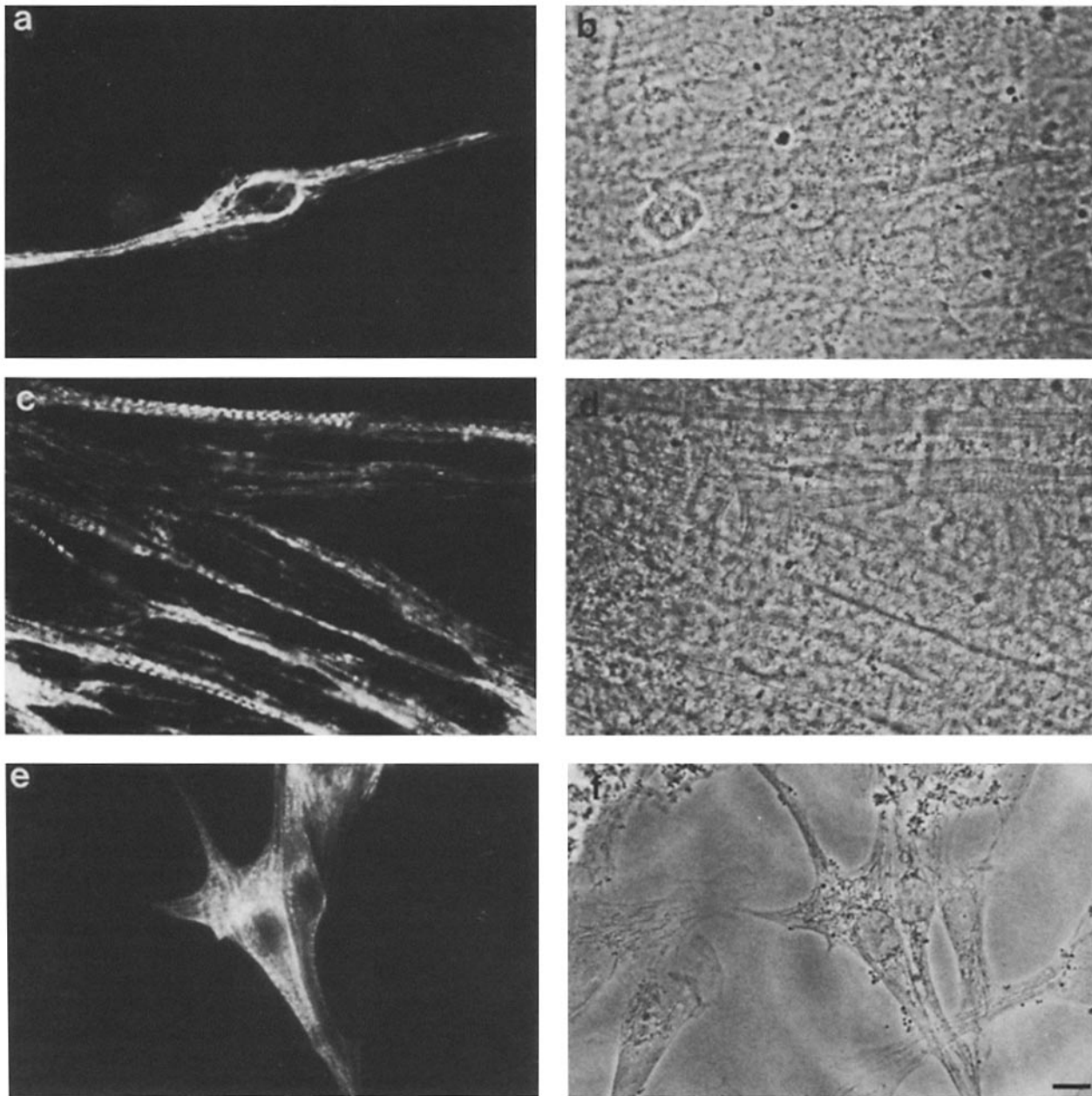
Figure 7. Immunofluorescence micrographs of primary cultures of chicken skeletal and heart muscle cells stained with T1 antibodies. (*a*) Mononucleated skeletal muscle myoblast. (*b*) Corresponding phase-contrast micrograph of *a*. (*c*) Multinucleated skeletal muscle myotubes. (*d*) Corresponding phase-contrast micrograph of *c*. (*e*) Chicken heart myocardial cells. (*f*) Corresponding phase-contrast micrograph of *e*. Note the absence of titin staining in all the cells surrounding the muscle cells. Bar, 10 μm.

and then visualized in the electron microscope by negative staining with uranyl acetate.

Native thick filaments were prepared from chicken skeletal muscle according to Trinick (21). When such a preparation was solubilized in SDS sample buffer and analyzed on 3.2% gels, large amounts of titin were observed along with myosin heavy chain and actin (data not shown). Native thick filament preparations stained with uranyl acetate and visualized in the electron microscope demonstrated numerous myosin thick filaments (15 nm) along with contaminating actin filaments.

The thick filament preparations were reacted with mouse T3 or T4 antibodies followed by gold-labeled anti-mouse antibodies. When such preparations were negatively stained and visualized in the electron microscope, gold label occurred exclusively at the ends of the thick filaments (Fig. 9, *a* and *b*). Some filaments were labeled only at one end of the filament rather than at both ends. This was usually only found in filaments shorter than 1.5 μm. The lack of labeling was probably due to some of the filaments being partially broken during preparation and thus having only one titin-containing end available for antibody staining. Structures similar to the "end filaments" described by Trinick et al. (20, 22) were sometimes observed at the ends of the myosin filaments and appeared to label with antibody (see Fig. 9*a*, arrows). These filaments were slightly smaller in diameter than the actin filaments in the same area of the grid and had a different substructure. No label was observed at the sides of the thick filaments.
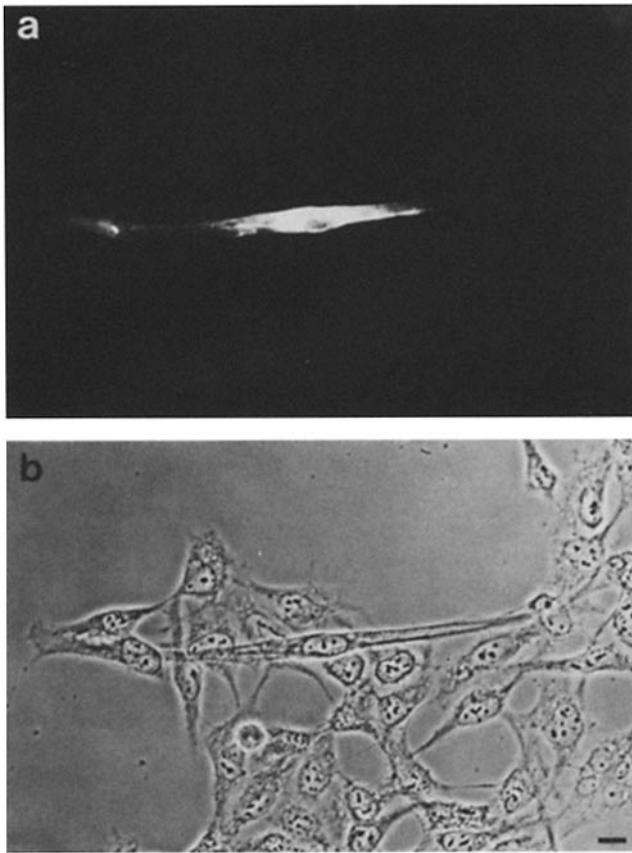
Figure 8. Immunofluorescence (*a*) and corresponding phase-contrast (*b*) micrographs of cultured RD cells, a line derived from a human rhabdomyosarcoma stained with T3 antibodies. Note the single titin-positive cell in the field (*a*). This cell differs strongly in morphology (*b*) from all the other cells which do not display titin. Bar, 10 μm.

Discussion

In this study, we have raised monoclonal antibodies to chicken heart muscle which had been exhaustively extracted with both high and low ionic strength buffers. During the immunofluorescence screening of the sera from immunized mice, we detected a strong myofibril banding pattern which seemed related to some of the patterns reported for titin with polyclonal antibodies (13, 24, 29). Three monoclonal antibodies were subsequently isolated which reacted exclusively with the titin doublet on immune blots of chicken skeletal myofibrils.

The three antibodies T1, T3, and T4 react with different epitopes as demonstrated by their differential tissue reactivity on cardiac and skeletal muscle in rat and mouse and their differential species reactivity observed on two cold-blooded vertebrates (Table I). The different reactivities between heart and skeletal muscle are most pronounced in the rat and mouse. This differential reactivity indicates that skeletal and heart muscle titin of the same animal are immunologically distinct molecules. Using immunofluorescence microscopy, we have demonstrated that in rat and mouse all three antibodies react on skeletal muscle but only antibody T3 decorates cardiac myofibrils. Confirming evidence comes from immunoblots on rat myofibrillar proteins. Whereas all three antibodies decorate skeletal muscle titin strongly, T1 fails to react with heart titin and T4 gives only an extremely weak reaction. An immunological difference between heart and skeletal muscle titin is also indicated for chicken. On immunoblots of

chicken muscle proteins, all three antibodies recognize the titin doublet of skeletal muscle while on heart muscle T1 and T4 recognize exclusively the upper titin band. Immunofluorescence microscopy of isolated heart myofibrils demonstrates that T1 and T4 have a different decoration pattern than that observed on isolated skeletal myofibrils. In addition, chicken heart and skeletal muscle titins show differences in fragmentation patterns when treated with either trypsin or chymotrypsin (our unpublished results). Many myofibrillar proteins are known to be coded for by different genes in the case of heart and skeletal muscle (for review see reference 1). This includes even actin as heart and skeletal muscle actin differ by four amino acid replacements in chicken and cow (23). Thus the differences between skeletal muscle and heart muscle titin documented here indicate that titin most likely also belongs to the developmentally controlled muscle proteins which differ on the gene level for the two sarcomeric muscle types. In contrast to this situation for true myofibrillar proteins, desmin, the myogenic intermediate filament protein, is coded for by only one gene (16).

The results on primary cultures of embryonic chicken skeletal muscle cultures indicate that titin first appears in single myoblasts in longitudinal filaments. After fusion of the myoblasts to myotubes, titin seems to be localized in the mature location at the A-I junction of the myofibrils. It is particularly interesting that titin is localized in longitudinal fibers in the myoblasts before mature myofibrils are observed. This suggests that titin may possibly serve as a scaffold for the assembly of the other sarcomeric proteins of the myofibril. These longitudinal fibers are also observed at the ends of the mature myofibrils in the myotubes, an area which may be the point where sarcomeric proteins are assembled during elongation of the growing myofibril.

From our immunofluorescence microscopical results on frozen tissue sections and on permanent cell lines, it seems that titin is a marker of sarcomeric muscles. Immunologically identical molecules seem absent in smooth muscles and the various nonmuscle tissues of the body. This conclusion is strengthened by two further observations. During muscle differentiation, titin was first observed in mononucleated myoblasts and was never seen by immunofluorescence microscopy in the numerous surrounding cells of the culture. Only one of the cell lines tested revealed titin staining and this was the human line RD, known to be derived from a rhabdomyosarcoma—a tumor of skeletal muscle origin (3). These results differ from a few reports suggesting that titin may be present in smooth muscle like chicken gizzard (10) and even in human lymphocytes (6). Although these proposals are based primarily on immunofluorescence microscopical results with polyclonal antibodies, we could accomodate them only by assuming that smooth muscle and nonmuscle cells, which are not reactive with any of our monoclonal antibodies, would express titins distinct from those found in sarcomeric muscles. As titin can now be purified in native form (7, 22, 30), a reinvestigation of the putative titin-like proteins present outside sarcomeric muscles seems indicated.

In our initial experiments using immunofluorescence microscopy on skeletal muscle myofibrils, we found within the same preparation many variable labeling patterns by titin antibodies T1 and T3 in addition to the A-I junction pattern. This was surprising at the time because monoclonal antibodies
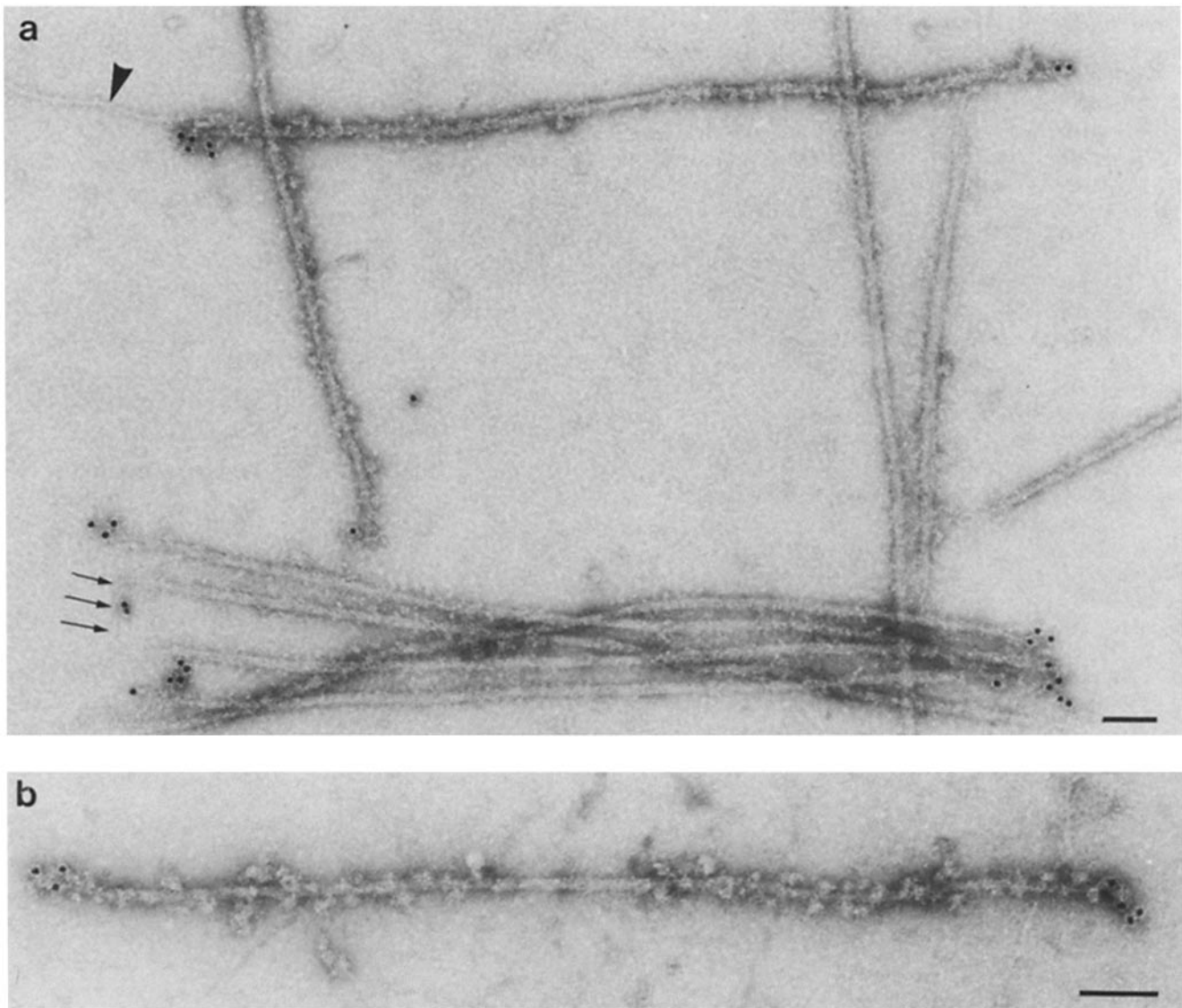
Figure 9. (*a* and *b*) Electron micrographs of negatively stained native myosin filaments decorated first with monoclonal mouse T3 antibodies followed by gold-labeled goat anti-mouse antibodies. Periodic, filamentous structures similar in morphology to end filaments are observed at the end of some of the myosin filaments (arrows) and appear to label with antibody. Arrowhead, actin filament. Bars, 0.1 μm.

specific for only one epitope would be expected to stain at distinct localities rather than diffusely throughout the myofibril if titin would not contain repeating sequences. However, in subsequent experiments, we found that these variant staining patterns occurred only in glycerinated, aged muscle myofibrils. In freshly prepared skeletal muscle myofibrils, we found staining only at the A-I junction with all three antibodies. One possible explanation for the variable staining patterns would be that proteolytic events occurring in the glycerinated myofibrils result in a subsequent redistribution of proteolytic titin fragments throughout the myofibril (see also the discussion in reference 22). This is especially likely as titin is very prone to proteolysis during storage (17, 25).

It has been previously suggested that the intact titin molecule is part of a third filament system in the myofibril which links successive Z-lines. According to this hypothesis, titin molecules are linked to specific loci within the myofibril such as the N-2 line and the ends of the myosin filaments (26). During tissue processing, a proteolytic nick in the titin strand

may occur. The resulting two pieces of the elastic titin strand could then retract to the N2 line and the myosin filaments where they are connected. The latter attachment point is in line with published electron micrographs (20, 22) and our immunofluorescence studies on freshly isolated skeletal myofibrils as well as the immunoelectron microscopical results (Fig. 9) on isolated native thick filaments from skeletal muscle. Our data on the skeletal myofibril do not allow us to identify any other attachment points of titin although they are expected for a putative third filament component which provides elasticity (15, 22, 26). However, in the case of cardiac myofibrils we can identify two possible attachment points of titin within the myofibril. Antibodies T1 and T4 recognize a portion of the cardiac titin present within the I band, possibly at the N2 line, while antibody T3 recognizes a portion of the molecule at the A-I junction, thus suggesting that these are attachment points of titin within the cardiac myofibril. This different staining pattern is in line with a different location of the epitopes for T1 and T4 versus T3 seen in immunoblotting.

When isolated skeletal myofibrils are run on SDS-polyacrylamide gels, titin is recognized as a characteristic doublet where the lower band differs by ~200,000 (30) from the upper band which is thought to have a molecular weight of around one or two million depending on the interpretation of the cross-linked standards used for gel electrophoresis (14). Recent preparations of native titin from such myofibrils seem to correspond to the lower band (7, 22, 30). It is therefore thought that this material is a proteolytic degradation product of the unproteolyzed upper band which remains non-extractable due to a strong attachment site in the myofibril. Electron micrographs of native titin clearly indicate elastic strings although the molecular lengths seem rather variable (from 100 to 1500 nm) and some observations raise the possibility of repeating structural domains (see the discussion in reference 22). If the proteolysis model is indeed correct our immunoblots and immunofluorescence micrographs raise two interesting points about cardiac titin. First, as the epitopes for T1 and T4 antibodies are restricted to the upper band of cardiac titin they would not involve simple repeating domains arising from identical amino acid sequences. Secondly, these two epitopes map closely to the same or to opposite ends of the intact polypeptide represented by the upper band on SDS gels while the epitope for T3 antibody is expected in a more interior position within the molecule as it is retained in the lower cardiac titin band. If we assume that the intact titin polypeptide is a simple strand with one end attached to a locus within the I-band and the other to a locus in the A-band of cardiac myofibrils, we can speculate that the T1 and T4 epitopes map to the same end of the molecule at a position in the I-band while the T3 epitope is localized at the A-I junction. Since recent electron micrographs (22) have suggested that titin may be present in the A-band between thick filaments, our tentative epitope map would place all three antibodies at one end of the titin molecule leaving the second half of the string unaccounted for. These results encourage the view that a more extended set of monoclonal antibodies together with an epitope map along the titin string could help in localizing the molecule more precisely within the myofibril.

Note added in proof: Two other reports on titin localization have just appeared. S. M. Wang and M. L. Greaser (*J. Muscle Res. Cell Motil.* 6:293-312) used a monoclonal antibody to bovine cardiac titin and obtained by immunofluorescence microscopy similar results to ours on normal and extracted skeletal myofibrils. K. Maruyama, T. Yoshioka, H. Higuchi, K. Ohashi, S. Kimura, and R. Natori (J. Cell Biol. *101*:2167-2172) using polyclonal antibodies found in immunoelectron micrographs localization in several discrete transverse bands from the Z-line to a region ~0.15 μm from the center of the A-band.

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