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Visualization of the Polarity of Isolated Titin Molecules: A Single Globular Head on a Long Thin Rod As the M Band Anchoring Domain?

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Abstract. TII, the extractable form of titin, was purified from myofibrils and separated by high resolution gel permeation chromatography into two fractions (TII_A and TII_B). Novel specimen orientation methods used before metal shadowing and EM result in striking pictures of the two forms. Molecules layered on mica become uniformly oriented when subjected to centrifugation. TII_B comprises a very homogeneous fraction. All molecules reveal a single globular head at one end on a long and very thin rod of uniform diameter. The lengths of the rods have a very narrow distribution (900 ± 50 nm). TII_A molecules seem lateral oligomers of TII_B, attached to each other via the head regions. While dimers are the predominant species, trimers and some higher oligomers can also be discerned. Mild proteolysis destroys the heads and converts TII_A and TII_B into TII_B-like rods. Similar molecules also result from titin purified from myofibrils by certain established purification schemes. Headless titin molecules show in gel electrophoresis only the TII band, while head bearing molecules give rise to two additional polypeptides at 165 and 190 kD. Immunoelectron microscopy of myofibrils identifies both titin-associated proteins as M band constituents. We speculate that in the polar images of TII the globular head region corresponds to the M band end of the titin molecules. This hypothesis is supported by immunoelectron micrographs of TII_B molecules using titin antibodies of known epitope location in the half sarcomere. This proposal complements our previous immunoelectron microscopic data on myofibrils. They showed that epitopes present only on the nonextractable TI species locate to the Z line and its immediately adjacent region (Fürst, D. O., M. Osborn, R. Nave, and K. Weber. 1988. *J. Cell Biol.* 106:1563–1572). Thus, the two distinct ends of the titin molecule attach to Z and M band material respectively.

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THE laboratories of Maruyama and Wang have clearly established that sarcomeres contain in addition to the thin and thick filaments with their various associated proteins a third filament system (for reviews, see Maruyama, 1986; Wang, 1985). An extremely high molecular mass protein is the major component of this elastic system. It is referred to either as titin, to indicate its giant molecular mass (Wang et al., 1979), or as connectin, to stress its bridging function (Maruyama et al., 1977). Direct solubilization of myofibrils by dodecylsulfate provides a titin doublet in gel electrophoresis. While the parent TI molecule cannot be extracted under native conditions, various protocols allow purification of the smaller TII species (Kimura and Maruyama, 1983; Trinick et al., 1984; Wang et al., 1984), which is thought to be a proteolytic derivative (Maruyama, 1986; Wang, 1985).

Micrographs of rotary shadowed TII, although surprisingly heterogeneous in appearance (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984), indicate very long and flexible strings. Consequently, titin molecules have been proposed to link the M band and either the N_2- (Wang, 1982) or the Z line (Maruyama et al., 1985). Using distinct monoclonal antibodies, we have recently mapped more than 10 unique titin epitopes by immunoelectron microscopy of antibody decorated myofibrils. The epitope map describes the titin molecules as polar structures, which start at the Z line and extend into the M band. Interestingly, epitopes present only on the larger TI species are restricted to the Z line and its direct environment. Thus, one physical end of the TI molecule seems firmly attached to the Z line (Fürst et al., 1988; 1989). In an attempt to characterize the other end of the polar titin molecules, we have tried to overcome the heterogeneity of isolated TII molecules seen previously by rotary shadowing (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984). Here we report the separation of two distinct TII fractions by high resolution gel permeation chromatography and describe methods to orient the molecules before shadowing. The resulting uniform images show that TII_A and TII_B molecules bear a small globular head at one end of the long rod. Gel electrophoresis shows two associated proteins, which we identify here as M band constituents (a 165- and 190-kD protein). Since mild proteolysis of

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titin II destroys the head and results in a loss of the two M band proteins, we further propose that the head reflects at least in part the M band anchoring domain of the polar molecules. This hypothesis is supported by immunoelectron micrographs of TII_B molecules using antibodies of known epitope location in the half sarcomere.

Materials and Methods

Purification of Titin TII

The method of Trinick et al. (1984) was modified extensively. Chicken breast muscle removed immediately post mortem was chopped into small pieces. These were homogenized for 20 s with a Polytron homogenizer in ice cold low salt buffer (100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM NaN_3, 10 mM Tris-maleate, pH 6.8) containing 2 mM $Na_2P_2O_7$. The following protease inhibitors were present in LSB and in all subsequent buffers: PMSF up to 1 mM, trypsin inhibitor II (T-9253; Sigma Chemical Co., St. Louis, MO) up to 10 μg/ml and E64 (E-3132; Sigma Chemical Co.) at a final maximal concentration of 5 μM (for variations in protease inhibitors see text). Myofibrils were harvested (15 min, 3,000 g at 4°C), washed three times with LSB and resuspended in extraction solution (0.6 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM 2-mercaptoethanol, 10 mM imidazole-HCl, pH 7) for maximally 30 min (for time dependent extraction effects see Results). The supernatant obtained after centrifugation (60 min, 20,000 g) was extensively dialyzed against buffer T (2 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM NaN_3, 50 mM Tris-HCl, pH 7.9) containing 80 mM KCl, clarified by centrifugation (100,000 g for 45 min), and applied to a DEAE cellulose (DE-52; Whatman Biosystems Ltd., Maidstone, England) column (1.6 × 15 cm for 20 g muscle) equilibrated with buffer T containing 80 mM KCl. After washing with the same buffer, bound protein was stepped off with buffer T containing 150 mM KCl. The titin pool was dialyzed against buffer T containing 500 mM KCl and gelfiltered through Superose 6 FPLC (HR 10/30; Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in the same buffer. An aliquot of TII, which emerges in the void of the S6 column, was subjected to high resolution gel permeation chromatography (GPC). A TSK 6000 PW column (7.5 × 600 mm, LKB Instruments Inc., Bromma, Sweden) equilibrated in buffer T plus 500 mM KCl was developed at 12 ml/h. The two gel filtration columns were run at room temperature. No protease inhibitors were present in the last step. Samples of TII or its two subfractions TII_A and TII_B were stored at 4°C after addition of E64.

Variations in the Purification

(*a*) Titin prepared in the presence of ATP. Solutions for homogenization, washing, extraction (5 min only), and first dialysis were 0.5 mM in ATP. DEAE dialysis buffer, equilibration buffer, and eluting solution were 0.2 mM in ATP. For gel filtration on Superose 6 titin was dialyzed into buffer T plus 0.5 M KCl containing 0.2 mM ATP. The same solvent was used for the GPC-column. (*b*) Titin purified by the procedure of Maruyama's laboratory (Kimura and Maruyama, 1983; Itoh et al., 1986). Homogenization with a Polytron homogenizer in 50 mM KCl, 1 mM $NaHCO_3$, the subsequent seven washing steps in the same buffer, the serial extraction steps and the hydroxyapatite chromatography were as in Itoh et al. (1986). We added as final steps gel filtration on Superose 6 FPLC and TSK 6000 PW as above.

Gel Electrophoresis and Related Procedures

Gel electrophoresis on gradient slab gels (2-12% acrylamide, 0.5% bisacrylamide) and immunoblotting was as described (Fürst et al., 1988). Monoclonal antibodies to titin (T3, T12, T30, and T33) have been characterized previously (Hill and Weber, 1986; Fürst et al., 1988; 1989). The monospecific rabbit antibody against the 190-kD M line protein was affinity purified via preparative blots from an antiserum raised against native TII according to Olmsted (1981) as modified by Gassner (1986). Monoclonal antibody B4, specific for myomesin (Grove et al., 1984) was kindly provided by Dr. H.M. Eppenberger. Rabbit antibody to skeletal muscle actin was a gift from Dr. U. Gröschel-Stewart.

1. *Abbreviation used in this paper*: GPC, gel permeation chromatography.

Limited Protease Treatment

TII or its subfractions TII_A and TII_B in buffer T plus 250 mM KCl were treated at room temperature with trypsin using an enzyme to substrate ratio of 1:300 wt/wt. After 30 min, trypsin inhibitor was added. In other experiments, chymotrypsin was used for 20 min under the same conditions.

Electron Microscopy

The rotary shadowing procedure used a modification of the layering technique described by Trinick et al. (1984). A drop of the solution (titin at 10 μg/ml in buffer T containing 500 mM KCl and 50% glycerol) was applied to freshly cleaved mica. Excess liquid was removed by drawing one edge of the mica across filter paper. The mica was adhered horizontally by double-sided adhesive tape to a cardboard plate (at a radius of 4 cm for TII_B or 6 cm for TII_A) that was placed on top of the rotor of a centrifuge (12,000 rpm; model No. 3200; Eppendorf Gerätebau, Hamburg, FRG). Centrifugation at top speed was for 15 s. A standard procedure of low angle rotary or unidirectional shadowing with tantalum/tungsten at 5° and carbon at 90° followed. Replicas were floated off on a surface of distilled water and collected on copper grids.

Specimens of titin were also obtained using a mechanical device that is described in detail by Jahn and Strey (1988). A small drop of titin solution (1 μl) was placed on the surface of a formvar (1595E; E. Merck, Darmstadt, FRG) or pioloform (2295; Bio-Rad Laboratories, Cambridge, MA) coated grid held vertically by tweezers mounted to this device. A torsion spring was wound up to generate a spring tension so that the specimen is moved very fast in an arc to the other side of the apparatus. The high acceleration (entrance velocity ~10 m/s, transfer time 15 ms) splashed most of the liquid away leaving a very thin layer on the grid. The grid was dried in vacuo and rotary shadowed with tantalum/tungsten at an angle of 5°. Using this procedure, glycerol could be omitted from the original sample.

Immunoelectron Microscopy on Myofibrils

Sarcomeric epitope location by immunoelectron microscopy was as in Fürst et al. (1988).

Immunoelectron Microscopy of Titin Molecules

Titin (0.2 mg/ml) dialyzed into 150 mM KCl, 20 mM imidazole-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 2 mM EGTA was incubated for 4 h at room temperature with antibodies using a molar ratio of 1:10. Purified IgM (antibody T33) or IgG (antibody T30) were at a concentration of ~1 mg/ml. The mixture was passed through a small column (0.5 × 5 cm) of Sepharcryl S 1000 to separate titin-antibody complexes from unbound antibodies and processed for rotary shadowing as above.

Analytical Ultracentrifugation

Samples of the central portion of GPC peaks were analyzed in a centrifuge (model E; Beckman Instruments, Inc., Palo Alto, CA) with Flossdorf optics and a scanner. Titin at 20-200 μg/ml in buffer T containing 250-500 mM KCl was used. Centrifugation at 20°C was at 28,000 rpm. Sedimentation coefficients were corrected to $s_{20,w}$ values.

Results

Purification of Native Titin TII

Since titin is prone to proteolytic breakdown, our standard procedure made use of very fresh chicken muscle, minimal extraction times and the presence of different protease inhibitors. We also avoided ammonium sulfate fractionation steps since they can lead to some irreversible aggregation of titin. To obtain a high enough concentration of titin by ion exchange chromatography to allow subsequent gelfiltration steps, we found it necessary to step the protein off the DEAE-column (0.15 M KCl) instead of using a linear salt gradient. The resulting material, already 70-80% pure (Fig. 1 *b*), had an optical density of up to 3 (280 nm). It could be directly processed by gel filtration on Superose 6. Use of an FPLC

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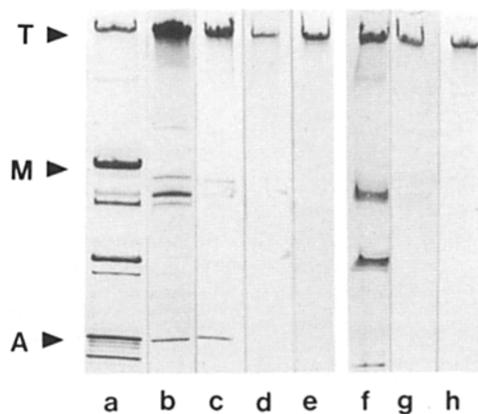

Figure 1. Purification of titin II by the standard procedure (lanes *a–e*). The gels (2–12% linear polyacrylamide gradient gels) show the 0.6 M KCl extract (lane *a*), the crude titin obtained by step elution from the DE52 column (lane *b*) and the titin peak from the Superose 6 column (lane *c*). Lanes *d* and *e* show Titin II_A and II_B obtained by gel permeation chromatography on the TSK 6000 PW column (for elution profile see Figs. 2, *a* and *c*). Lanes *f–h* monitor the purification according to Itoh et al. (1986). Lane *f*, sodium phosphate, pH 6.6 extract; lane *g*, titin II obtained from hydroxyapatite column; lane *h*, titin from the TSK 6000 PW column. The positions of titin (*T*), myosin heavy chain (*M*) and actin (*A*) are indicated.

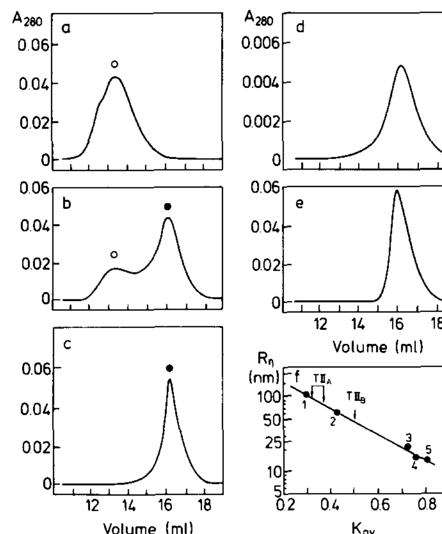

Figure 2. Gel permeation chromatography of different titin II preparations on a TSK 6000 PW column (7.5 × 600 mm). Profiles *a–c* illustrate the influence of protease inhibitors (see Results). In the presence of PMSF (1 mM) and trypsin inhibitor (10 μg/ml) the column profile shows two peaks: TII_A and TII_B (*b*). This profile results from our standard purification scheme. In the absence of inhibitors there is essentially only the TII_B peak (*c*). Addition of E64 (5 μM) and the two other inhibitors provides only a broad TII_A peak (*a*). *d* shows the elution profile of TII_A after mild trypsin treatment (see Materials and Methods). Note that the resulting material elutes at a position corresponding to TII_B. The elution positions of TII_A are indicated with open circles and the positions of TII_B with filled circles. Titin II extracted and purified by the method of Itoh et al. (1986), is essentially in the TII_B form (*e*). Calibration of the TSK 6000 PW column (*f*) according to viscosity radii (Potschka, 1987) used the following standards: 1 = dimer of tobacco mosaic virus (TMV); 2 = TMV; 3 = spectrin tetrameter; 4 = turnip yellow mosaic virus; 5 = spectrin dimer. The elution positions of TII_A and TII_B (*arrows*) correspond to 75–95 nm and 48 nm, respectively.

apparatus allowed the isolation of 200 μg of titin within 20 min. Therefore, this step could be performed at room temperature. Superose 6 purified titin was at least 90% pure (Fig. 1 *c*). Since titin eluted in the void of the column a subsequent gel permeation step was indicated. We found that on a TSK 6000 PW HPLC column (7.5 × 600 mm) titin eluted in the included volume. Two distinct peaks were obtained. TII_A, a rather broad peak, eluted ~13–14 ml, while TII_B, a much narrower peak, eluted around 16 ml (Fig. 2). No significant changes in the ratio of TII_A to TII_B of a single preparation were observed, when the KCl concentration was varied in the range of 150 mM to 1 M KCl and the pH changed between 7.0 and 8.5. Unexpected changes in the ratio were, however, observed when different titin preparations were routinely analyzed by GPC.

The major factors governing the ratio of TII_A to TII_B were the type and the relative concentration of the protease inhibitors used in the purification as well as the time of the myofibrillar extraction step. Fig. 2 summarizes only some of the results collected during this study. Titin purified by the original protocol of Trinick et al. (1984), which does not call for protease inhibitors, showed exclusively TII_B. If PMSF (1 mM) and trypsin inhibitor II protein (10 μg/ml) were included in the homogenization step, the resulting titin II revealed in addition to the major TII_B peak a very prominent TII_A peak (Fig. 2 *b*). Additional inclusion of E64 (5 μM) at all early steps provided a titin preparation consisting nearly exclusively of TII_A, which eluted in a rather broad profile (Fig. 2 *a*). Although prolonged extraction times increased the yield of titin, they always resulted in increased amounts of TII_B and noticeable losses of TII_A even when E64 was present in the preparation. For instance, starting with 10 g of muscle tissue under optimal conditions (short extraction time and E64 present) we obtained only 5 mg of titin which was essentially all TII_A. When the same material was processed without protease inhibitors and a long extraction time (30 min) was used, 20 mg of TII_B and no TII_A were obtained (Fig. 2 *c*). Thus, even with high concentrations of protease inhibitors only relatively small amounts of TII_A could be purified. Since TII_B is particularly suitable for EM (see below) we arrived at a compromise purification protocol (see Materials and Methods). It leads to useful amounts of TII_B and also provides TII_A for additional experiments. Since titin has also been prepared by Maruyama's laboratory (Itoh et al., 1986) under quite different conditions, again without protease inhibitors, we also followed this isolation in detail (see Materials and Methods and Fig. 1, *f–h*). Gel permeation chromatography on TSK 6000 PW showed that such titin preparations consist exclusively of TII_B-like molecules (Fig. 2 *e*) even when protease inhibitors are present during the extraction.

Calibration of the TSK 6000 PW column with known standards yielded hydrodynamic information for both titin species. Following the general approach of Potschka (1987) the viscosity radius of TII_A was in the range of 75–95 nm, while TII_B gave a value of 48 ± 2 nm (Fig. 2 *f*). Because of this strong difference, we performed analytical sedimentation studies on material present in the centers of the two peaks. TII_B gave rise to a sharp boundary characterized by a $s_{20,w}$ value of 12.5S, which was not influenced by increased ionic

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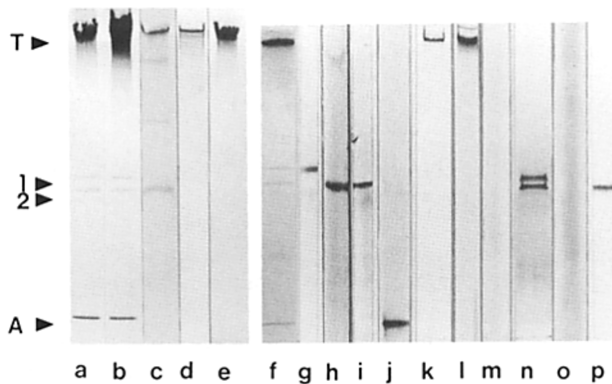

Figure 3. Identification of two proteins associated with titin. Highly overloaded SDS gels of titin TII_A (*a*) and TII_B (*b*) show three minor polypeptides at 190, 165, and 43 kD (marked with *1*, *2*, and *A*, respectively). TII_B-like material obtained from a preparation that included ATP in all steps (see Materials and Methods) does not contain the 43-kD band (*c*). TII_B rechromatographed after mild trypsin treatment (*d*) and TII_B-like material purified by the procedure of Maruyama's laboratory (*e*) only reveal the titin band and lack all associated proteins. Lanes *f–p* show the identification of the three proteins by immunoblotting. In a blot of titin TII_B (for Ponceau red stain, see lane *f*) the 190-kD polypeptide was specifically recognized by an antigen-affinity-purified polyclonal rabbit antibody (lane *g*). Similar blots treated with the myomesin specific monoclonal antibodies B4 (lane *h*) and U256 (lane *i*) show exclusive decoration of the 165-kD band (myomesin). The 43-kD band was identified as actin by an immunoblot with antibodies specific for muscle actin (*j*). Gelfiltration of Superose purified titin II on TSK 6000 PW in 4 M guanidine-HCl separates the pure TII polypeptide (peak 1) from the mixture of myomesin plus 190 kD (peak 2; elution profile not shown). Lane *k* shows a Ponceau red–stained blot of the titin fraction (peak 1). It is recognized by titin antibody T3 (lane l) and not by myomesin antibody U256 (lane *m*). Lane *n* shows the Ponceau red–stained blot of the second fraction (peak 2). It is recognized by myomesin antibody U256 (lane *p*) and not by titin antibody T3 (lane *o*).

strength. TII_A, however, yielded a diffuse boundary. While the main species sedimented at ~27S, additional species at 40S and 57S were observed. Using a different TII preparation, which in our hands is greatly reduced in TII_A, Trinick et al. (1984) reported a major species at 13S and a minor species at 40S.

Are M Band Proteins (165/190 kD) Titin-associated Proteins?

Gel electrophoresis showed that TII_A and TII_B seemed essentially pure, since only the TII band was detected by dye staining (Fig. 1). However, overloaded gels as well as silver stained gels revealed three additional polypeptides at 190, 165, and 43 kD in both TII_A and TII_B (Fig. 3). While the 190- and 165-kD polypeptides seemed always present in approximately equal amounts, the 43-kD polypeptide varied in quantity in different preparations. The 43-kD band comigrated with actin and immunoblotting with a rabbit antibody specific for skeletal muscle actin confirmed this assignment (Fig. 3 *j*). Since titin purification proceeds in the absence of ATP (see Materials and Methods), it seemed that this minor contaminant could represent denatured actin. Therefore, titin was purified with ATP present in all steps (see Materials and Methods). In such preparations crude titin obtained after the DE-52 column was heavily contaminated by myosin. Subsequent analytical gel permeation chromatography in 0.5 M KCl provided myosin-free TII_A and TII_B. Such preparations clearly lacked the actin contamination in SDS gels (Fig. 3 *c*). Again, however, both titin species revealed the 190/165-kD doublet. The interaction of these proteins with TII was resistant to high salt (up to 1 M KCl was used) but could be broken by gelfiltration on TSK 6000 PW in 4 M guanidine-HCl, which separates a peak containing only denatured TII from the mixture of the two associated proteins (Fig. 3, *k–p*).

None of our 15 distinct monoclonal antibodies to titin (Fürst et al., 1988; 1989) reacted with the 190/165-kD doublet in immunoblotting. Thus, a proteolytic derivation of the

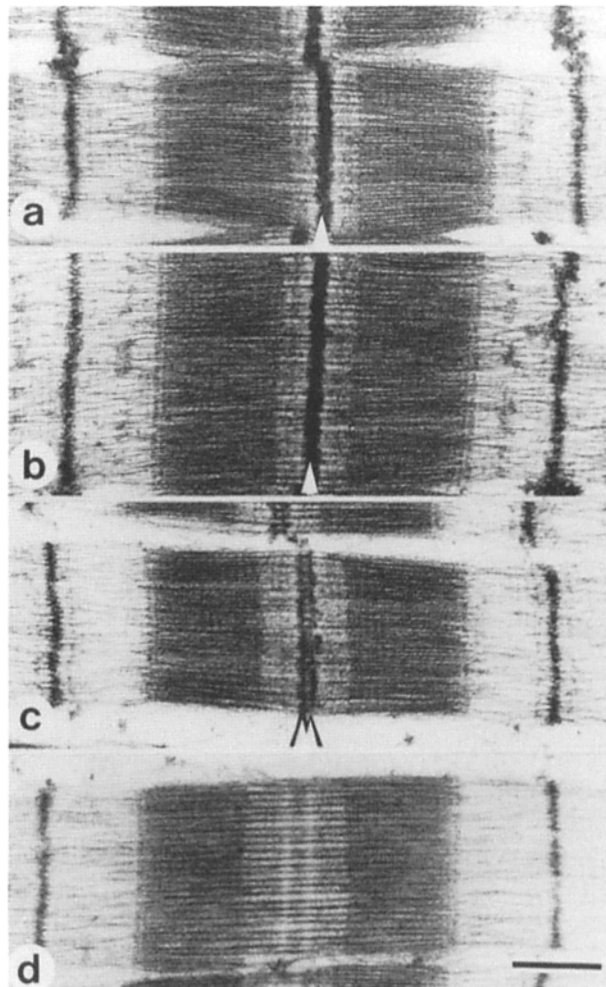

Figure 4. Immunoelectron microscopical localization of putative titin-associated proteins in chicken pectoralis major muscle. The following antibodies were used: (*a*) monoclonal antibody B4 specific for myomesin (see Grove et al., 1984); (*b*) monoclonal antibody U256; (*c*) monospecific rabbit polyclonal antibody to the 190-kD protein; (*d*) control muscle without first antibody. The labeling positions are indicated by arrowheads. Note that the myomesin antibody B4 (*a*) decorates almost the entire M band as does our antibody U256 (*b*). Antibodies to the 190-kD protein provide a strong decoration at a position that could correspond to the M6 and M6′ lines (*b*). Bar, 500 nm.

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doublet from titin itself seemed unlikely. Interestingly, however, one of our monoclonal antibodies (U256), which was originally raised against native titin II, did not detect in immunoblots of a myofibrillar extract the titin doublet and reacted instead only with a band at 165 kD. Fig. 3 *i* shows that U256 detects the 165-kD band of the 190/165-kD doublet present in TII_A and TII_B. Immunoelectron microscopy of myofibrils was used to locate the position of the U256 epitope along the sarcomere. Prominent and exclusive decoration of the entire M band was observed (Fig. 4 *b*). As such a pattern has been reported for antibodies to the M band specific protein myomesin, which seems to have an apparent molecular mass of ~185 kD (Grove et al., 1984), we obtained the corresponding antibody from Dr. H.M. Eppenberger (Swiss Federal Institute of Technology, Zürich, Switzerland). Fig. 3 *h* shows that the bona fide myomesin antibody B4 as well as our U256 antibody recognize the 165-kD polypeptide present in TII_A and TII_B preparations. Fig. 4 *a* shows that antibody B4, like U256, decorates the entire M band of myofibrils. Currently, we don't know whether the difference in apparent molecular masses of 185 kD (Grove et al., 1984) and 165 kD is because of our gel system or potential proteolysis.

Polyclonal rabbit antibodies to TII reacted on myofibrillar extracts with TI, TII, the 165-kD protein, and a 190-kD polypeptide. In purified titin, the reaction with TII and the two associated proteins was retained. Thus, rabbit antibodies specific for the 190-kD proteins could be isolated from preparative blots (Fig. 3 *g*). When these antigen-affinity purified antibodies and myomesin antibodies were used in immunofluorescence microscopy on isolated myofibrils identical images were obtained (data not shown). The assignment of the 190-kD protein to the M band was directly confirmed by immunoelectron microscopy. Here the antibodies to the 190-kD protein decorated exclusively at a position ~35–40 nm distant from the M1 line (Fig. 4 *c*).

Titin II prepared according to Maruyama's laboratory lacked the 165/190-kD polypeptides even in heavily overloaded gels (Fig. 3 *e*), and no reaction with the corresponding antibodies was seen upon blotting (not shown). Interestingly, the same observation was made when we treated TII_A or TII_B mildly with trypsin. Such material chromatographs on the TSK 6000 PW column in a TII_B-like position (Fig. 2 *d*) and lacks the 165/190-kD doublet (Fig. 3 *d*).

TII_B Molecules are Thin Rods with a Single Protease Sensitive Globular Head

In spite of the narrow peak width of TII_B in GPC (Fig. 2), standard glycerol spraying followed by rotary shadowing gave disappointing results, although the stringlike appearance described for titin II (Maruyama et al., 1984; Trinick et al., 1984: Want et al., 1984) was clearly apparent (Fig. 5 *c*). To avoid shearing forces, we further explored the layering technique of Trinick et al. (1984) including their attempts to orient the molecules on the mica by centrifugation. In contrast to their images, we found that molecules could be easily and reproducibly oriented, provided appropriate centrifugation conditions were used and washing steps with volatile salts were rigorously excluded. Optimal conditions were provided, when the mica was adhered 4 cm from the center of a cardboard plate attached to the rotor of a centrifuge (model 3200; Eppendorf Gerätebau), which was run at top speed for 15 s (see Materials and Methods). Impressively uniform images of oriented molecules were obtained (Fig. 5, *a* and *b*). All molecules were fully stretched and surprisingly revealed a single globular head. Attachment to the mica via this head is probably responsible for the unidirectional orientation induced by the centrifugation. The linear molecules lack bends and knicks invariably encountered in experiments lacking centrifugation steps. The diameter of the rod was constant over the entire length. A comparison with the thickness of the rod of the myosin molecule (2 nm, according to Elliott and Offer, 1978) yielded a value of ~3–4 nm. Histograms of the rod length point to a rather narrow distribution with the pronounced peak centered ~850–950 nm (Fig. 5 *d*). Indistinguishable images were obtained for titin, which had been prepared in the presence of ATP (see Materials and Methods).

To exclude the possibility that the head structure was generated by the high glycerol content of the original samples, we also explored a different approach. The mechanical device described in detail by Jahn and Strey (1988) allows the very rapid movement of a formvar- or pioloform-coated grid, which causes excess liquid to be removed by centrifugal force. This device can be used to obtain rotary shadowed molecules of titin without the need to introduce glycerol in the sample. As an example, Fig. 6 *a* shows again well-oriented TII_B molecules, which all carry a single head. Thus, the movement exerted by the device leads to a uniform orientation. These results show that the globular head is a normal feature of the TII_B molecule.

Titin TII_A and TII_B mildly treated with trypsin or chymotrypsin (see Materials and Methods) eluted in GPC around the position of TII_B (Fig. 2 *d*). Electron micrographs of shadowed molecules after centrifugal orientation revealed headless rods as shown in Fig. 7 *a* for TII_B mildly treated with trypsin. Titin isolated by the procedure of Maruyama's laboratory and finally purified as TII_B-like material (see Materials and Methods) was also analyzed (Fig. 2 *e*; see above). As in the trypsin-treated material, the resulting molecules lacked heads (Fig. 7 *b*). Electron micrographs generally revealed a more heterogeneous appearance of this material. Individual molecules were less well aligned and often not fully stretched. Preliminary measurements of the contour length revealed a broader distribution than seen with TII_B. The majority of the identifiable molecules measured ~700–900 nm. These headless molecules were sometimes less well oriented and showed a tendency for end to end or side by side alignment (see Fig. 7 *b*), which is currently not clearly understood. A possible explanation lies in the assumption that in the case of normal TII_B molecules the head domain provides the major attachment to the substratum (mica surface). This would subsequently allow orientation of the individual molecules upon centrifugation. Without the attachment at one end provided by the head, the liquid stream introduced could lead to partial aggregation of less firmly attached molecules.

TII_A Molecules Seem Oligomers of TII_B

Using the same centrifugation method for specimen preparation before metal shadowing only a minority of TII_A molecules revealed the same uniform image as TII_B. Instead many molecules showed a strong tendency to form side by side or end to end aggregates. This behavior seemed not to

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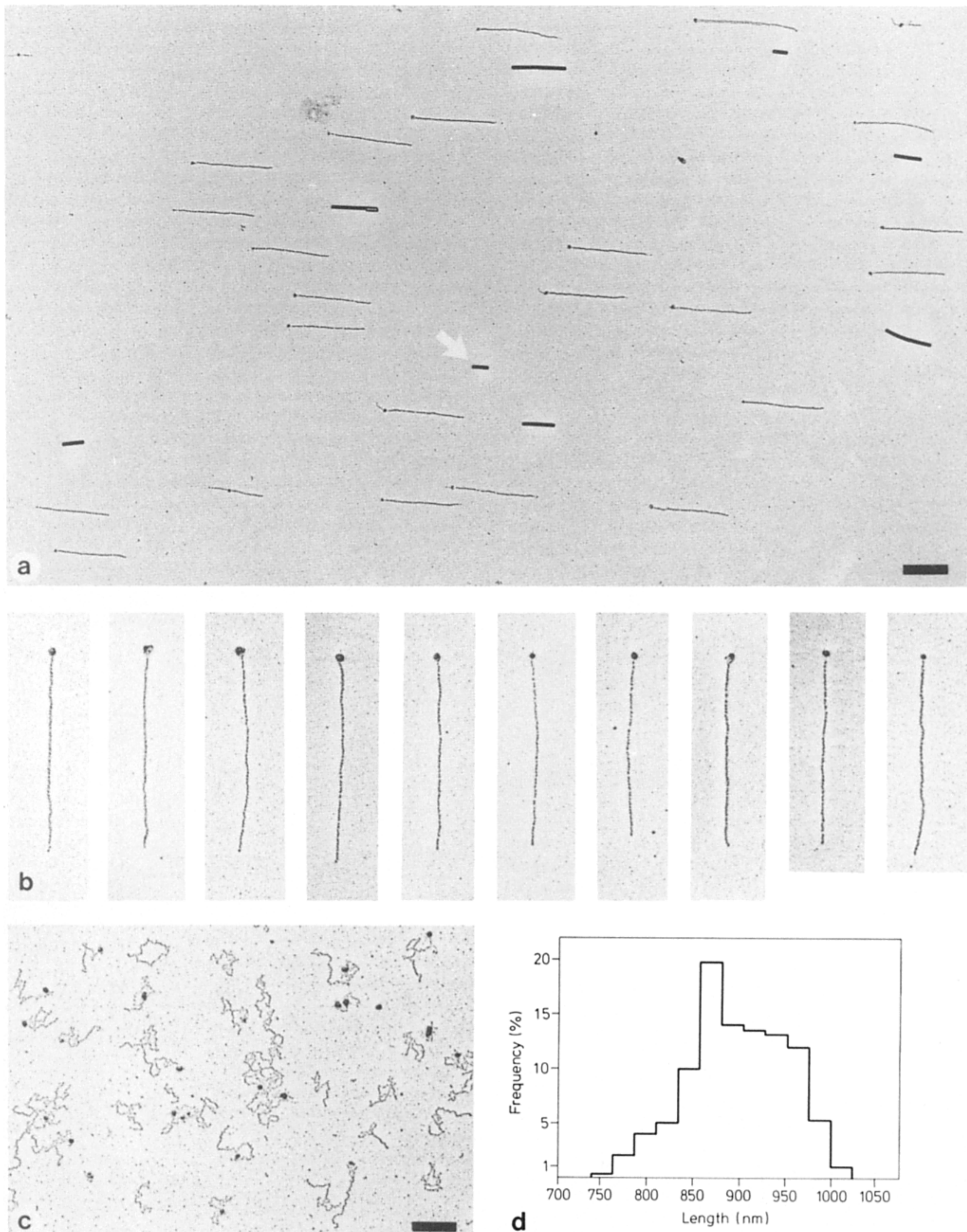

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Figure 5. Electron microscopic images of TII_B after rotary shadowing reveal a single globular head. (*a*) Molecules layered on mica in 50% glycerol were oriented by centrifugation for 15 s at 12,000 rpm (at 4-cm distance from the center of rotation; see Materials and Methods). Tobacco mosaic virus particles (*arrow*) were added as internal standard. Note the uniform orientation of the molecules (glycerol front moved towards the right) and the presence of a globular head present at the left side of nearly all molecules in the field. A gallery of individual TII_B molecules is given in *b* at higher magnification. (*c*) Appearance of TII_B molecules after spraying from 50% glycerol solution without a centrifugation step. *d* gives a histogram of the contour length of the rod of stretched molecules obtained from 300 molecules in a field similar to that shown in *a*. The histogram yields an average length of the rod of 900 ± 50 nm. Bars, 500 nm.

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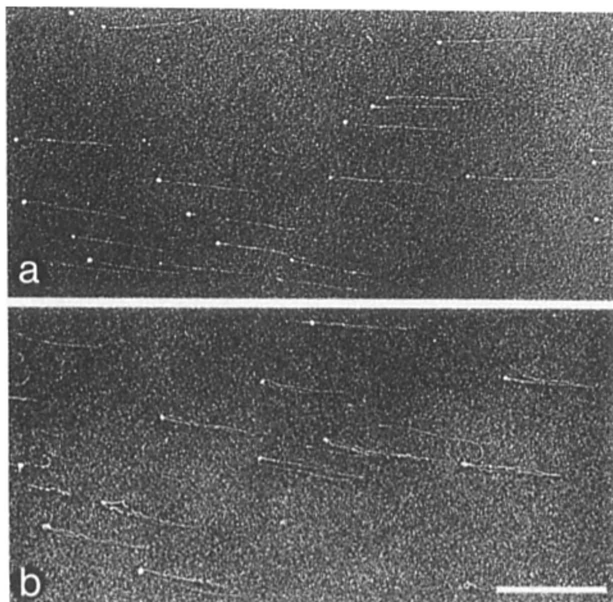

Figure 6. Electron microscope appearance of native TII_B (*a*) and TII_A (*b*) molecules in the absence of glycerol. Samples were layered on pioloform-coated grids without addition of glycerol and excess liquid was catapulted away using the mechanical device described by Jahn and Strey (1988) (see Materials and Methods). The specimens were rotary shadowed and the micrographs printed in reverse contrast. Molecules are again well aligned and reveal a single globular head. TII_A molecules (*b*) seem to be lateral dimers of the TII_B form. Bar, 1 μm.

be influenced by salt or glycerol content of the sample. To obtain well oriented and stretched molecules of TII_A a higher relative centrifugal force was necessary (see Materials and Methods). Acceptable images were obtained once the mica was placed 6 cm rather than 4 cm from the center of rotation. The resulting molecules also showed single heads and long rods as described for TII_B (Fig. 8 *a*). While the length of the rods was similar to TII_B for the majority of the molecules, ~10% of them exhibited a contour length of up to 1.2 μm (e.g., the first three molecules in the gallery shown in Fig. 8 *b*).

TII_A molecules were ~1-nm thicker than TII_B forms. The reason for this difference in diameter was more clearly revealed in suboptimally stretched preparations. A number of molecules in Fig. 8 *b*, for instance, gives the impression of dimers of TII_B-like molecules somehow connected at the head region. While the majority of the analyzable molecules exhibits this dimeric character, one occasionally encounters also trimers (two examples are given in Fig. 8 *b*) or higher oligomers (e.g., the last molecule in Fig. 8 *b*). Independent proof of the predominantly dimeric character of TII_A comes from microscopy experiments in which glycerol was omitted during sample preparation and the molecules were oriented via mechanical movement (see Materials and Methods). Fig. 6 *b* shows several molecules with two parallel rods, that are connected only at the head region.

Decoration of TII_B Molecules with Antibodies of Established Epitope Location in the Half Sarcomere

To obtain an independent assessment of our interpretation

Figure 7. Electron microscopic images of mildly proteolyzed TII_B and of titin prepared by the procedure of Itoh et al. (1986). *a* shows TII_B previously mildly treated with trypsin. *b* shows the morphology of TII purified according to Itoh et al. (1986). In both cases the material was passed through the GPC column and oriented by centrifugation (see Materials and Methods and Fig. 5). Note that both preparations appear as headless rods with a more heterogeneous length distribution. In addition, such molecules are less well oriented than TII_B (see Fig. 5). Bar, 500 nm.

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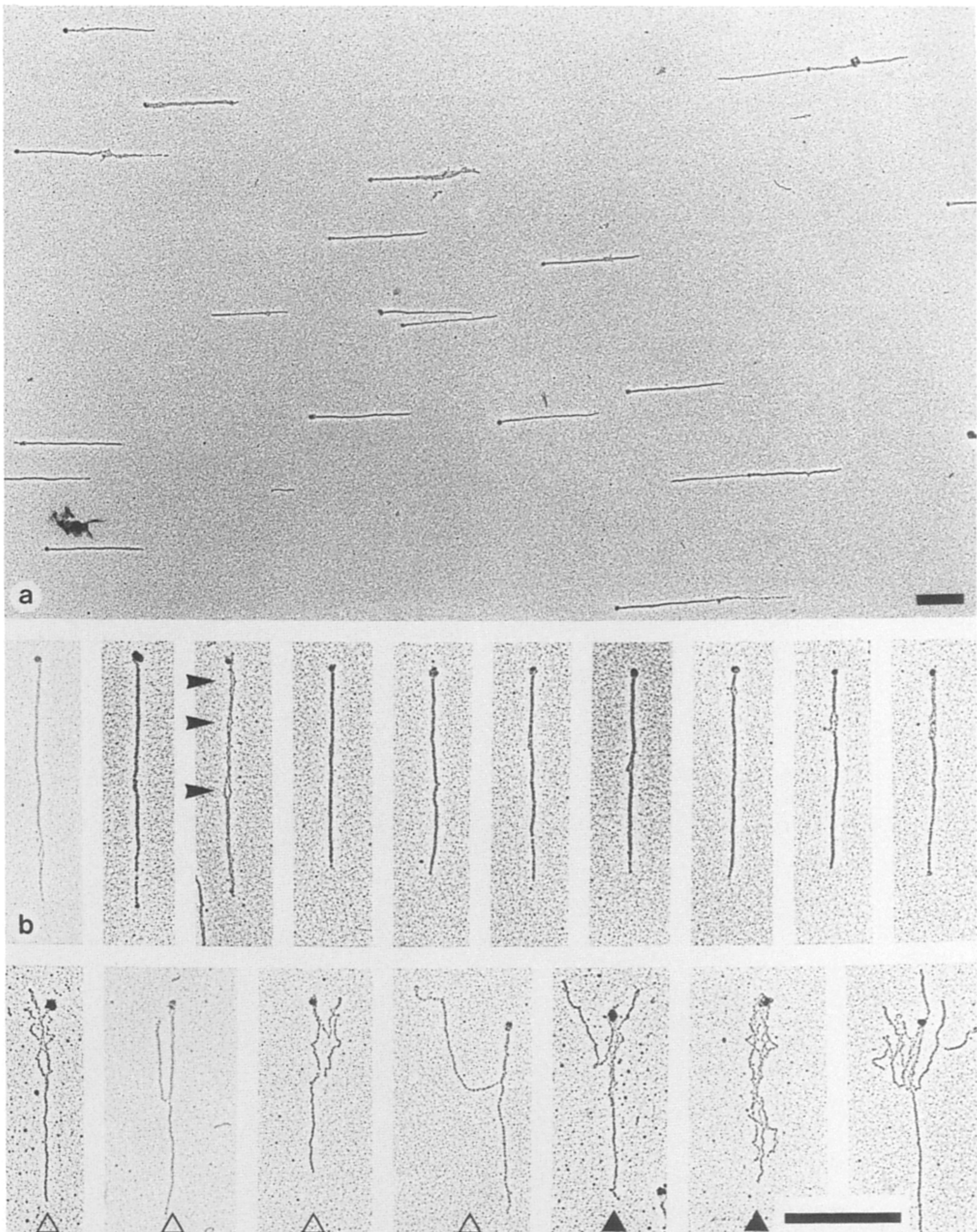

Figure 8. Electron microscopic images of TII_A after rotary shadowing. (*a*) Molecules were oriented by centrifugation for 15 s at 12,000 rpm (see Materials and Methods). Note that the overall morphology is very similar to TII_B (see Fig. 5). *b* shows a gallery of individual TII_A molecules at higher magnification. While most molecules have a length similar to TII_B, a few seem longer (see the first three molecules in the gallery). The higher magnification images show in many cases unraveling of the rod at different positions (e.g., *arrowheads*), revealing the underlying oligomeric character. The primarily dimeric state demonstrated in Fig. 6 is also seen at bottom (*molecules marked with open triangles*). Occasionally trimers (*molecules marked with filled triangles*) and higher oligomers (e.g., the last molecule in the gallery) can be observed. Bars, 500 nm.

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that the globular head of TII could reflect the M band anchorage of titin molecules, we tried to locate the binding sites of at least a few of our antibodies on the intact TII_B molecule (see Materials and Methods). In our bank of monoclonal antibodies, T33 was particularly useful because it is an IgM. In immunoelectron microscopy on muscle fibers it decorates a single line per half sarcomere, which is situated ~55 nm away from the center of the M band (Fürst et al., 1989). The TII_B molecules in Fig. 9 *b* show that antibody T33 labels at the head-rod junction. Because of the decoration and the presence of a head domain, the antibody-decorated titin molecules appear double headed. Another useful antibody was T30. This IgG_1 has been shown to stain on myofibrils a unique set of repetitive epitopes with a 42-nm spacing, which seem to coincide with some of the known C-stripes in the interior of the A band (Fürst et al., 1989). Fig. 9 *c* shows multiple IgG molecules on TII_B. At least three strongly stained epitopes could be visualized per molecule and their spacing correlates with the reported myofibrillar decoration pattern.

Discussion

Our molecular characterization of the native TII molecule has been greatly helped by the use of GPC and by novel methods of specimen preparation before metal shadowing and EM. Probably because of the large size of the stringlike molecules, GPC was previously not exploited in titin characterization. Although TII is excluded from commonly used matrices such as Superose 6, we found that it can readily be fractionated on the silica based TSK 6000 PW column. The elution profiles of standard titin preparations document the presence of at least two distinct molecular species. The narrow TII_B peak, which emerges later from the column, comprises a very homogeneous population of molecules when analyzed by improved metal shadowing procedures. The broad TII_A peak reflects oligomers of TII_B molecules, primarily dimers (see below). Led by the results of GPC and EM, we have developed a purification scheme which provides a good yield for TII_B and sufficient amounts of TII_A (for elution profile see Fig. 2 *b*). In addition, we have defined conditions to control the relative yield of the two titin forms by differential use of protease inhibitors.

Encouraged by the GPC results, we tried systematically to improve the specimen preparation before metal shadowing. We have now defined the experimental conditions that allow titin molecules on a mica surface to be oriented by centrifugal force. This procedure resulted in remarkably uniform images of TII_B. All molecules in the field were oriented in the same direction and unexpectedly all of them revealed a single globular head at one end. As the rods of all molecules pointed in the direction of the front of the retracting glycerol drops (Fig. 5 *a*), we wondered whether the head region was artificially induced by the high glycerol concentration. Using a special mechanical device, a catapult movement removes the excess liquid from the pioloform grid. This procedure allows subsequent rotary shadowing without the necessity of introducing glycerol in the original sample. The resulting

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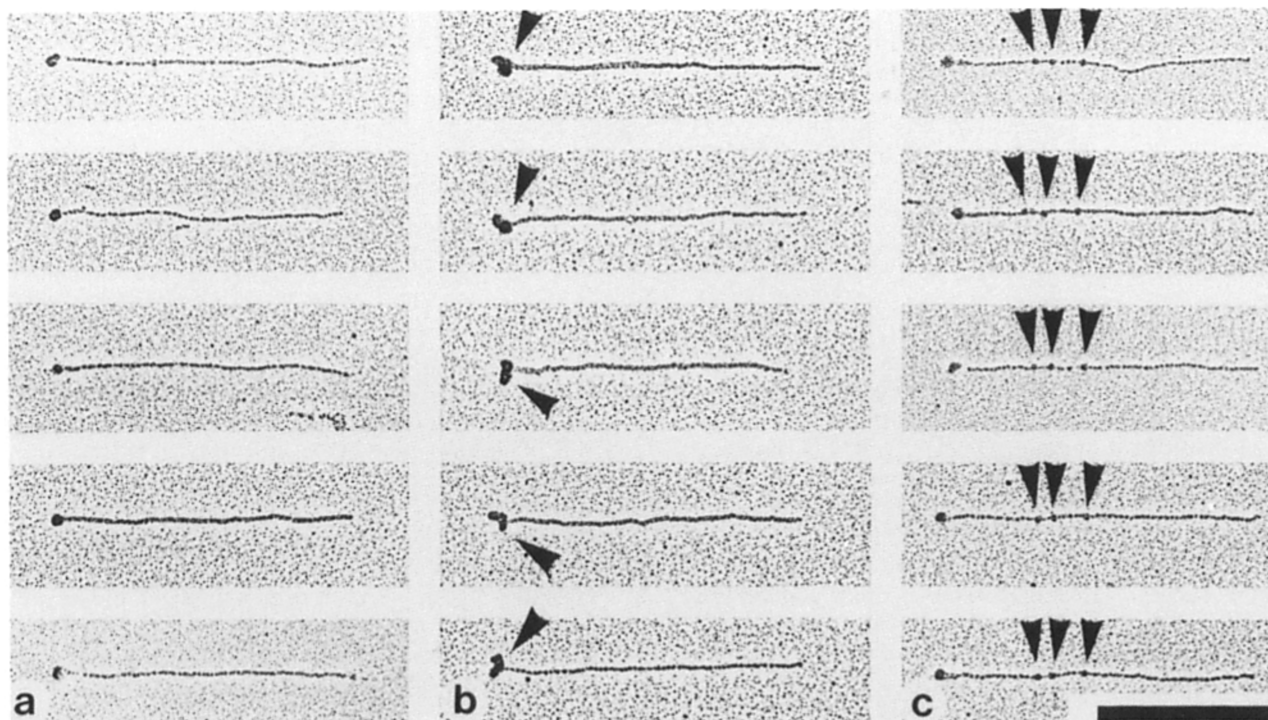

Figure 9. Identification of antibody binding sites on native TII_B molecules. Titin and monoclonal antibodies were mixed, incubated, and unbound antibodies removed by gel filtration. After addition of glycerol the samples were layered on mica, centrifuged, and rotary shadowed (for details see Materials and Methods). Representative molecules are shown. *a* gives unlabeled TII_B molecules as control. The following monoclonal antibodies were used: (*b*) T33, and (*c*) T30. The labeling positions are marked with arrowheads. Note that the decoration occurs specifically in an antibody dependent distance from the head. T33, an IgM, detects an M band epitope on myofibrils (Fürst et al., 1989) and labels the neck region of TII_B molecules (*b*). T30, an IgG_1, labels on myofibrils repetitive epitopes in the C-zone of the A band (Fürst et al., 1989). At least three IgG molecules/TII_B are seen in *c*. Bar, 500 nm.

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micrographs again visualized oriented TII_B molecules with a single head (Fig. 6). In addition, this procedure was very useful in documenting the dimeric character of the TII_A molecules. They seem to reflect parallel TII_B-like molecules attached only at their head regions. Since the centrifugal orientation is experimentally easier and provides better quality images than standard glycerol spraying, we have used it as a routine assay. Varying the glycerol concentration between 30 and 60% did not change the images. Optimal results were obtained at a final KCl concentration of 0.5 M. Subsequent washing steps are not necessary and invariably lead to poorer images. Particularly detrimental is the use of volatile salts such as ammonium formiate in the samples or in the washes. Preliminary attempts to follow the stretching process by using lower centrifugation forces or shorter times have not been successful. Probably under such conditions too much liquid remaining on the mica does not allow a good shadowing in the subsequent step.

The diameter of the rod (3-4 nm) of TII_B molecules is constant over the entire length, which measures ~900 nm. At present, it is unclear whether the residual length variability of the rod (850-950 nm) is influenced by differential proteolysis or by different degrees of stretch exerted on elastic molecules, which seem attached to the support primarily by their head domain. Additional physical parameters are available from hydrodynamic results. Calibration of the TSK 6000 PW column provided a viscosity radius of 48 nm for TII_B. Together with the s value of 12.5 S the approach of Potschka (1987) leads to a molecular weight of ~2 million. Given the length measurements (see above), a mass per unit length of 2.2 kD/nm is calculated for the rod. These independent estimates are in fair agreement with scanning transmission electron microscopy values recently reported by Hainfeld et al. (1988). On less homogeneous TII preparations, which were not stretched, they measured 2.4 $\pm$ 0.5 million and 2.7 $\pm$ 0.9 kD/nm. The TII_B rods seem to contain only one polypeptide chain, since equilibrium centrifugation of titin in 6 M guanidine-HCl gives a value of 2.4-2.6 million (Kurzban and Wang, 1988). Our estimate of 2 million is necessarily somewhat lower since a possible contribution from the head is not included in the calculation.

Exploring other purification schemes, we found that the procedure of Itoh et al. (1986) provides only the TII_B-like molecules, which clearly lacked the head domain. Such headless molecules can still be oriented by centrifugation but somewhat less impressively than our TII_B molecules. Headless molecules are also obtained when TII_A or TII_B are mildly treated with trypsin or chymotrypsin. Such material elutes on the TSK 6000 PW column in a TII_B-like position and shows in gel electrophoresis the TII band. Heavily overloaded gels or silver stained gels clearly revealed a molecular difference between headless titin preparations and TII_A or TII_B. Only the latter molecules show a pair of titin associated proteins at 165 and 190 kD (Fig. 3, *a* and *b*). Although the interaction of the two proteins with titin is resistant to high salt they are readily destroyed by mild proteolysis. Both associated proteins are clearly identified by immunoelectron microscopy as M band constituents but their identity still poses some problems. The 165-kD protein reacts with a bona fide myomesin antibody, although an apparent molecular mass of 185 kD was reported for this M band protein (Grove et al., 1984). A possible explanation would be the difference in the gel systems used. The 190-kD component is not recognized by the myomesin antibody and could be a novel M band protein since the so-called "M protein" is thought to have an apparent molecular mass of only 165-170 kD (Masaki and Takaiti, 1974; Trinick and Lowey, 1977). Therefore, we suggest that the globular head of TII_A and TII_B molecules marks the M band anchoring domain of the titin molecules. This view is supported by micrographs of TII_B molecules decorated by titin antibodies with known epitope location along the half sarcomere. Thus T33, which labels a single position 55 nm before the center of the M band (Fürst et al., 1989) decorates TII_B molecules directly adjacent to the globular head (Fig. 9 *b*). T30 detects five repetitive epitopes with a 42-nm spacing in C-zone of the inner part of the A band (Fürst et al., 1989). On isolated TII_B several antibody molecules of similar spacing are seen in the region of the rod preceding the head (Fig. 9 *c*). We note specifically that our results cannot yet distinguish between two obvious models. The globular head may arise nearly entirely from the two M band proteins, or it reflects an extension of the rod to which these proteins are bound. Current experiments aim at a molecular understanding of the associated proteins.

Polarity of the titin molecule was previously predicted by our immunoelectron microscopical analysis of the location of more than 10 distinct epitopes along the half-sarcomere (Fürst et al., 1988; 1989). There we found that two epitopes, located at the Z line (T20) and at a position 0.04 μm before it (T21) were found in immunoblotting of muscle extracts on the TI band only. Thus the TI specific end of the titin molecules seems anchored at the Z line. Although the precise positions of the proteolytic cleavage site or sites converting the nonextractable TI of the myofibril into extractable TII are not yet known, a major site seems to occur around the N1 line. Purified TII_A and TII_B strongly react with seven monoclonal antibodies, which on myofibrils describe single epitopes located between the N2 line and the M band (Fürst et al., 1988). In contrast, the reaction with antibody T12, which marks a single position 10 nm before the Z line, is weak and often lost upon storage of the protein (data not shown). Thus, TII_A and TII_B extend to the T12 position with their free rod end, while the head is part of the M band. Further continuation of the titin molecules to the Z line can only be analyzed once conditions are found to prepare TI or the TI-specific end of titin molecules under native conditions.

The ultrastructural appearance of TII_A molecules clearly points to oligomers, usually dimers, of the TII_B conformation. TII_A molecules seem firmly associated at the head domain. In optimal specimens, the two parallel rods can be observed without entanglement. Current models of the thick filaments discuss 6 or 12 titin molecules arranged parallel to the myosin filament (Whiting et al., 1989). Our observation of dimers and sometimes even trimers in the TII_A fraction raises the possibility that titin molecules form an oligomeric organization in the myofibril. In line with this view, we found that E64, a potent inhibitor of the calcium activated proteases (calpain) (Barrett et al., 1982), leads to TII preparations that are almost entirely in the TII_A form. We note that TII_A molecules must not necessarily reflect two TII_B species from the same half sarcomere. Our results would also allow that the two TII_B halves are originally situated in the two halves of the same sarcomere. In the latter case, the orientation forces used before metal shadowing would have to align

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the two rods in parallel. Although we have no direct evidence for two modes to release TII_A molecules from the sarcomere this possibility would offer some explanation for the rather broad TII_A peak in gel permeation chromatography.

Given the length of the titin rod domain, a structural organization of the polypeptide in form of consecutive quasi repeats can be expected. Indeed occasional micrographs of negatively stained titin molecules indicate a string of beads with a repeat distance ∼4.3 nm (Trinick et al., 1984; Hainfeld et al., 1988). It is possible that the molecules observed in our study did not reveal this feature as they were straightened by either centrifugation or a very rapid movement before being subjected to metal shadowing. Using a new set of titin monoclonal antibodies in immunoelectron microscopy of myofibrils, we have recently found a quasi repeat of 42 nm in the more interior part of the A band, which is based on repetitive titin epitopes (Fürst et al., 1989). An understanding of these two repeat types in molecular terms should be possible once the cDNA cloning of titin is achieved.

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