

Studies of the Interaction between Titin and Myosin

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Abstract. The interaction of titin with myosin has been studied by binding assays and electron microscopy. Electron micrographs of the titin–myosin complex suggest a binding site near the tip of the tail of the myosin molecule. The distance from the myosin head–tail junction to titin indicates binding 20–30 nm from the myosin COOH terminus. Consistent with this, micrographs of titin–light meromyosin (LMM) show binding near the end of the LMM molecule. Plots of myosin- and LMM-attachment positions along the titin molecule show binding predominantly in the region located in the A band in situ, which is consistent with the proposal that titin regulates thick filament assembly. Estimates of the apparent dissociation constant of the titin–LMM complex were ~ 20 nM. Assays of LMM cyanogen bro-

mid fragments also suggested a strong binding site near the COOH terminus. Proteolysis of a COOH-terminal 17.6-kD CNBr fragment isolated from whole myosin resulted in eight peptides of which only one, comprising 17 residues, bound strongly to titin. Two isoforms of this peptide were detected by protein sequencing. Similar binding data were obtained using synthetic versions of both isoforms. The peptide is located immediately COOH-terminal of the fourth “skip” residue in the myosin tail, which is consistent with the electron microscopy. Skip-4 may have a role in determining thick filament structure, by allowing abrupt bending of the myosin tail close to the titin-binding site.

SINGLE molecules of the giant protein titin (also known as connectin, $\sim 3,000 M_r$) (Maruyama et al., 1984; Kurzban and Wang, 1988) span between the M and Z lines in striated muscle myofibrils (Maruyama et al., 1985; Fürst et al., 1988). Based initially on evidence of movement of monoclonal antibodies, titin was proposed to have fundamentally different roles in different parts of the sarcomere: in the I band, titin epitopes move away from both the M and Z lines as sarcomere length increases, which suggested elastic behavior in this part of the molecule (Fürst et al., 1988; Itoh et al., 1988; Whiting et al., 1989; Wang et al., 1993). Subsequently it has become clear that elastic titin connections link thick filaments to the Z line, thereby centering the A band in the sarcomere and transmitting passive tension. These conclusions are also supported by evidence from selective radiation scission (Horowitz et al., 1986) and by studies of titin isoforms in different muscles (Wang et al., 1991; Horowitz, 1992).

In contrast, titin epitopes in the A band remain fixed with respect to the M line as sarcomere length is varied, which suggested that this region of the molecule is an integral part of the thick filament (Fürst et al., 1988; Whiting et al., 1989). Moreover, a few of the titin monoclonal anti-

bodies that bind in the A band do so at multiple sites spaced 43 nm apart (Fürst et al., 1989), which is the repeat distance of the helices that describe the arrangement of myosin molecules in the thick filament. The idea that titin is associated with thick filaments has since been supported by data from sequencing and in vitro binding experiments. The sequence of titin is mainly composed of two motifs similar to type III fibronectin and I-set immunoglobulins (Labeit et al., 1992). Both motifs therefore fold to form separate domains. Throughout much of the A band the motifs are arranged in an 11-domain super repeat that probably spans the 43-nm helical repeat of the thick filament. In addition, both whole titin and titin constructs prepared by expression of cDNAs bind in vitro to myosin, and to the other thick filament components C- and X protein (Labeit et al., 1992; Soteriou et al., 1993). The interaction with myosin appears to be predominantly with the light meromyosin (LMM)¹ portion of the molecule that forms the thick filament backbone. An interaction with the S1 fragment from the head of the molecule has also been reported (Wang et al., 1992). One possible role for titin in the thick filament is to act as what has been termed a protein-ruler to regulate the exact assembly of myosin and the other filament proteins (Wang and Wright, 1988; Whiting et al., 1989). Here we present further studies of the interaction between titin and myosin. The data suggest

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1. *Abbreviation used in this paper:* LMM, light meromyosin.

that the strongest interaction is via a site ~20 nm from the tip of the myosin tail. A preliminary report of some of the results was published previously (Houmeida et al., 1994).

Materials and Methods

Protein and Peptide Preparation

Titin was purified from rabbit back muscle (Soteriou et al., 1993). These preparations contained molecules ~1 μm long, which is slightly shorter than the 1.25- μm full-length molecules, probably because of loss of an NH_2 -terminal peptide by proteolysis (see Nave et al., 1989; Suzuki et al., 1994). Rabbit myosin was purified and digested with chymotrypsin (Sigma Chemical Co., St. Louis, MO) to make LMM as described by Margossian and Lowey (1982). Fragments of LMM were produced by further cleavage by either trypsin or cyanogen bromide. Trypsin digestion used an enzyme to substrate ratio of 1:200 (wt/wt) in 0.1 M KCl, 10 mM EDTA, 20 mM Tris-HCl, pH 8 at room temperature for 40 min (Nyitray et al., 1983) and was stopped with soybean trypsin inhibitor.

CNBr digestion was initially done in 70% formic acid at room temperature for 18 h using a 50-fold molar excess of CNBr (Sigma Chemical Co.) over methionines. Excess formic acid and CNBr were removed by two cycles of dilution with water and lyophilization. In later experiments both LMM and myosin were dissolved at ~10 mg/ml in 6 M GuCl, 0.15N HCl before treatment with CNBr. For a typical cleavage, 100 μl of this solution was mixed with 18 μl CNBr (1 mg/ μl in acetonitrile) and incubated 30 min at 37°C. These mixtures were fractionated directly on an HPLC column (C18; VYDAC, The Seps. Group, Inc., Hesperia, CA) (4.6 \times 250 mm), equilibrated in 0.1% trifluoroacetic acid, and developed with a gradient of acetonitrile. Fractions were characterized by amino-terminal sequencing (G001; Hewlett-Packard Co., Palo Alto, CA) and solid-phase binding assays.

The J' peptide isolated by HPLC of whole myosin (see Results) was further cleaved with 5% wt/wt endoproteinase Lys-C (Promega Corp., Madison, WI) in 0.1 M Tris-Cl for 18 h at 37°C. This digest was fractionated by reverse-phase HPLC and the purified peptides were screened for titin-binding activity by solid-phase assay. The titin-reactive peptides identified (LEARVKELENEVESEQK and LEARVRELEAEVESEQK) were also prepared synthetically (Immune Systems Ltd., Bristol, UK). The synthetic versions were purified by HPLC and verified by mass spectrometry and sequencing.

Proteins and peptides were monitored by SDS-PAGE using 4–15% gradient gels for large polypeptides (Soteriou et al., 1993), and zone gels of 6, 10, and 16% acrylamide for small polypeptides (Schägger and von Jagow, 1987). Gels were stained either with Coomassie brilliant blue or silver. Where peptides were isolated from gels for sequencing or binding assays, half of the gel was first stained to locate the bands. Appropriate slices from the other half were then extracted with 0.1% SDS, 25 mM Tris-base, 190 mM glycine, pH 8.3, using an electroelution apparatus (Bio-Rad Laboratories, Hercules, CA) for 4 h at 8 mA at room temperature. The eluates were extensively dialyzed against 6 M urea to remove SDS and then against PBS (0.1 M KCl, 10 mM K-phosphate, pH 7.0).

Interaction of Electrophoretically Isolated Fragments

LMM fragments were resolved by SDS-PAGE and transferred to Immobilon paper (Millipore Corp., Bedford, MA) in 25 mM Tris-base, 192 mM glycine, 20% methanol at a constant current of 250 mA for 60 min. The paper was washed and blocked for 2 h at room temperature with 0.5 M KCl, 50 mM Tris, 1 mM EDTA, 0.3 mM DTT, pH 7.9, containing 3% gelatin, 0.1% Tween-20, and then washed five times with PBS containing 0.1% Tween (PBS-Tween). It was then reacted overnight with 50 $\mu\text{g}/\text{ml}$ titin in 0.18 M KCl, 50 mM Tris, 1 mM EDTA, 0.3 mM DTT, pH 7.9, (buffer A) containing 3% gelatin, 0.1% Tween at 4°C. After washing with PBS-Tween it was incubated for 1 h with mAb CH 11 (Whiting et al., 1989) diluted 1:1 in buffer A, followed by addition of anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co.). After five further washes in PBS-Tween and one wash in PBS alone, titin-reactive bands were visualized by addition of alkaline phosphatase substrate.

Fluorescence Polarization Measurements

Interaction of titin and LMM was monitored in solution by fluorescence polarization using dansylated LMM. This was prepared by reaction with a 15 M excess of 1,5-IAEDANS (Sigma Chemical Co.) in 0.5 M KCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.6, at room temperature for 1 h in the dark.

The reaction was stopped by adding β -mercaptoethanol to 0.2 M. Free dye was removed on a G-25 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated with the same buffer. Fluorescence polarization was measured in buffer A with a spectrofluorimeter (Perkin-Elmer Corp., Norwalk, CT) equipped with polarization accessory. Titrations were performed at excitation/emission wavelengths of 340 and 480 nm, respectively, by adding increasing amounts of titin to a solution of labeled LMM at room temperature. After 30 s to allow for equilibration, fluorescence polarization was monitored for a further 60 s. The average value was taken for determination of the apparent binding constant.

Solid-Phase Binding Assays

Solid-phase assays on ELISA (Engval, 1980) plates (Falcon; Becton Dickinson, Oxnard, CA) were used to monitor the interaction between titin and LMM and its fragments. Triplicate samples were tested using a constant amount of protein per plate well. The amounts of LMM, LMM peptides, or synthetic peptides applied to each well were 0.4, 1, or 5 μg , respectively. These were diluted in 50 mM carbonate, pH 9.6, and immobilized overnight at 4°C. Unbound protein was removed and the plates were washed six times with PBS-Tween. (Control assays were treated similarly but contained no coating protein.) The wells were then blocked with 3% gelatin in PBS-Tween for 1 h at room temperature and again washed. (All incubations were accompanied by gentle swirling.) Titin (in 3% gelatin in PBS-Tween containing additional KCl to a total concentration of 0.18 M) was then added and the plate incubated for 1 h at room temperature. After a further six washes, bound titin was reacted for 1 h at room temperature with antibody CH 11 diluted 1:1 in gelatin-PBS-Tween. After a further wash, the plate was reacted with anti-mouse IgG coupled to peroxidase for 1 h, followed by the hydrogen peroxidase substrate. Amounts of titin bound were estimated at 450 nm in a microtiter plate reader (Multiskan MCC/340; Labsystems, Helsinki, Finland). Results were plotted after correction for nonspecific adsorption to the ELISA wells in the absence of LMM. All values quoted are the average of at least two independent experiments. These assays were dependent on the assumption that the extensive washing procedure used with the ELISA plates did not reduce significantly the amounts of titin bound. This assumption is valid for solid-phase assays of complexes with apparent association constants $>10^8 \text{ M}^{-1}$. For such high affinities, six washes result in $<20\%$ of the complexed protein being dissociated (see e.g., Fig. 8.3 in Tijssen, 1985).

Competition experiments were carried out as previously described (Mejean et al., 1992). LMM was coated on the ELISA plate as above, and then reacted with a constant amount of titin that had previously been incubated with increasing concentrations of the J peptide (see Results) at room temperature. After 2 h, bound titin was measured as described above. Inhibition was expressed as the decrease of absorbance when the J peptide was incubated with the titin, compared with the absorbance produced by the titin alone.

Apparent Dissociation Constants

By definition

$$K_d = [T_{\text{free}}] [L_{\text{vacant}}] / [TL_{\text{complex}}] \quad (1)$$

where $[T_{\text{free}}]$, $[L_{\text{vacant}}]$, and $[TL_{\text{complex}}]$ are the concentrations of free titin, free LMM, and the complex, and K_d is the apparent binding constant. In solution binding experiments using fluorescence, this equation can be expressed as

$$P = P_{\text{max}} / (1 + K_d / [T_{\text{added}}]) \quad (2)$$

where P is the fluorescence polarization and P_{max} the maximum value. K_d was obtained by fitting the data using a nonlinear least squares plot. Eqs. 1 and 2 do not depend on stoichiometry, but assume multiple sites are similar and independent. Eq. 2 also assumes $[T_{\text{added}}] \gg [TL_{\text{complex}}]$. K_d was 15 nM (see Results) and since the number of sites is >25 (see Discussion) the molecular-binding constant is ~400 nM; therefore this condition was satisfied. For solid-phase assays the apparent dissociation constant was determined by measuring the titin concentration necessary for half-saturation (Van Heyningen et al., 1983).

At half saturation

Therefore (1) becomes

And since

Then if

Then

$$[L_{\text{vacant}}] = [TL_{\text{complex}}]$$

$$K_d = [T_{\text{free}}]$$

$$[T_{\text{free}}] = [T_{\text{added}}] - [TL_{\text{complex}}]$$

$$[T_{\text{free}}] \gg [TL_{\text{complex}}]$$

$$K_d \sim [T_{\text{added}}]$$

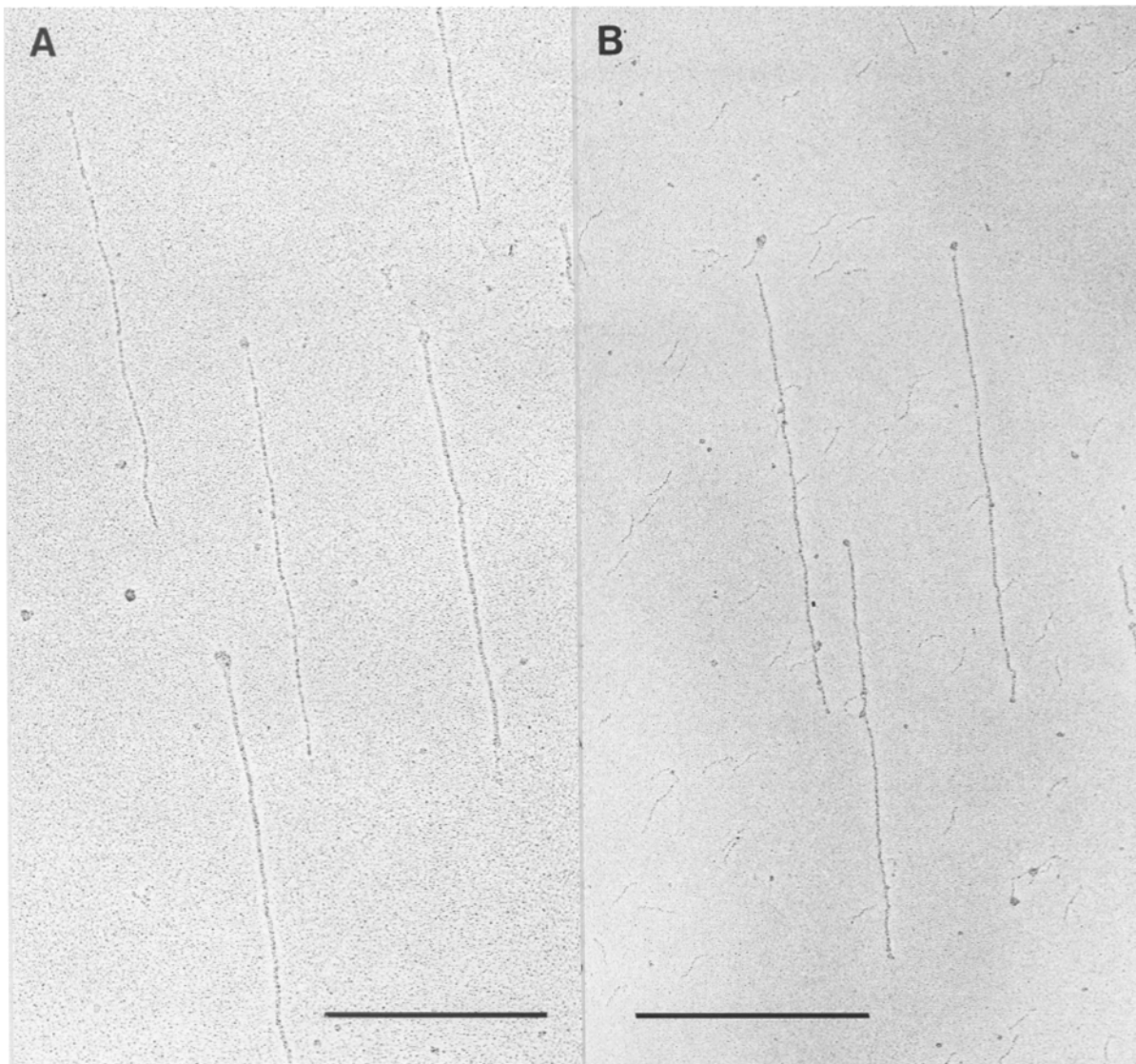


Figure 1. Electron microscopy of titin and titin-LMM complexes. (A) Titin alone (10 $\mu\text{m}/\text{ml}$), rotary shadowed after layering onto mica in 0.5 M KCl, 1 mM NaN_3 , 1 mM DDT, 10 mM imidazole, pH 7.0. (B) Titin and LMM at concentrations of 10 $\mu\text{m}/\text{ml}$ and 0.5 $\mu\text{m}/\text{ml}$, respectively, in 50% vol/vol glycerol, 0.2 M KCl, 1 mM NaN_3 , 1 mM DDT, 10 mM imidazole, pH 7.0. Note that the titin and LMM have different preferential orientations and that only $\sim 20\%$ of the LMM appears attached. Bar, 0.5 μm .

The concentration of titin added to produce half saturation is therefore a measure of the affinity, provided $[T_{\text{free}}] \gg [TL_{\text{complex}}]$. The maximum amount of LMM in the solid-phase assays was ~ 100 ng/well (determined using iodinated LMM, data not shown); thus this condition was satisfied.

Affinity Chromatography

Titin was coupled to CNBr-activated Sepharose 4-B (Pharmacia LKB Biotechnology Inc.) following the instructions of the manufacturer. 4 mg titin were added to 5 ml preswollen CNBr-Sepharose in 0.5 M NaCl, 0.1 M NaHCO_3 , pH 8.3, for 2 h at room temperature. Residual active groups were saturated with 0.2 M glycine, pH 8. LMM-CNBr digest, diluted in buffer A, was loaded onto the column equilibrated with buffer A. After washing, bound protein was eluted in buffer A plus 0.9 M KCl. The eluate was desalted on a small G-25 column equilibrated with buffer A and lyophilized before sequencing.

Electron Microscopy

Titin mixtures with myosin or LMM were dialyzed against 0.15 M KCl, 1 mM NaN_3 , 1 mM DDT, 10 mM imidazole, pH 7.0, at titin concentrations in the range 50–60 $\mu\text{g}/\text{ml}$ and using molar ratios of titin to myosin or LMM

of between 1:1 and 1:12. Immediately before shadowing samples were diluted with 50% vol/vol glycerol, 0.2 M KCl, 1 mM NaN_3 , 1 mM DDT, 10 mM imidazole, pH 7.0, to give a final concentration of 2–10 $\mu\text{g}/\text{ml}$. Shadowing with platinum was carried out essentially as described by Trinick et al. (1984) and modified by Nave et al. (1989). This involves layering the sample onto mica in the presence of 50% glycerol followed by centrifugal removal of bulk liquid. (Tests with the above solid-phase assay indicated that inclusion of 50% glycerol decreased the affinity of the complexes by $\sim 30\%$.) After drying in vacuo, the mica was rotary shadowed at an angle of 5° and coated with carbon. Replicas were floated off on water, collected on grids, and dried. Grids were examined in a microscope (EM400T; Philips Electronic Instrument Co., Mahwah, NJ) operating at 80 kV.

Results

Electron Microscopy of Titin-Myosin and Titin-LMM Complexes

Titin complexes with LMM and myosin were examined by electron microscopy after rotary shadowing with platinum.

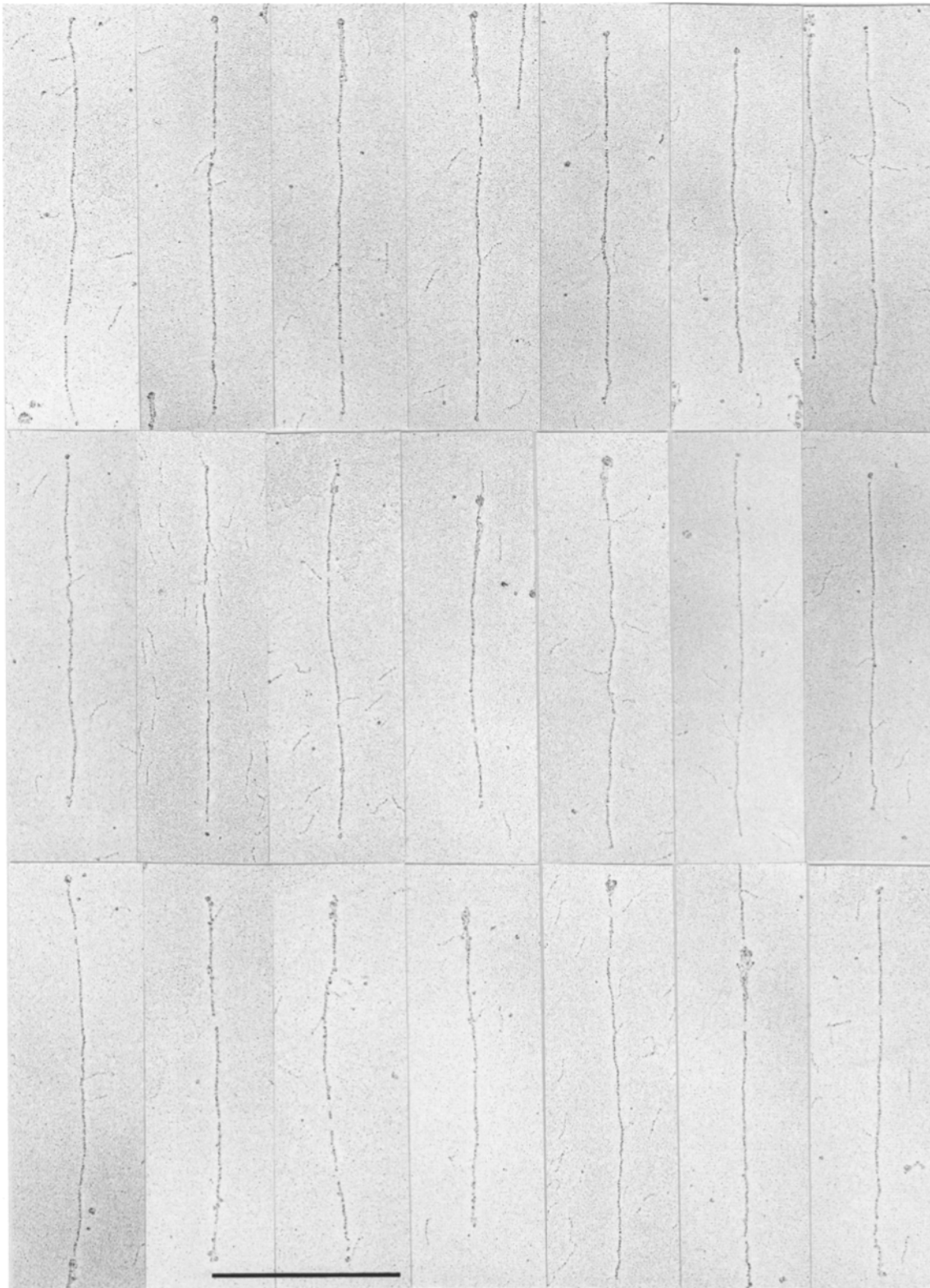


Figure 2. Montage of titin-LMM complexes. The LMM is most often seen attached to the titin through one end of the molecule. Bar, 0.5 μm .

Purified titin molecules are relatively compact in solution but can be straightened by a flow of liquid after attachment to a substrate (Nave et al., 1989). Fig. 1 *A* shows a field of molecules layered onto mica at high (~ 0.5) ionic

strength and is similar to data described previously (Nave et al., 1989; Soteriou et al., 1993). The molecule consists of a tail $\sim 1 \mu\text{m}$ long and 4 nm wide. At one end is a head that is located in the M line in situ (Nave et al., 1989) and con-



Figure 3. Montage of titin–myosin complexes. The characteristic appearance of the myosin molecules is easily recognized, although both heads are not usually resolved. Myosin is most often seen attached to the titin via a site near the tip of the myosin tail. Bar, 0.5 μm .

tains the COOH terminus (Labeit et al., 1992). In addition to monomers, dimers and trimers are found, bound through the head.

When preparing titin specimens with myosin or LMM,

the KCl concentration was reduced to 0.2 M (in 50% glycerol). Tests showed that this was the best compromise consistent with complex formation, straightening of titin, and minimization of aggregation. At 0.5 M KCl, most titin mol-

ecules were straight, but at 0.2 M KCl, many were compact. Straightening was observed only near where the meniscus retracted during drying (the centers of these regions were identified by nonvolatile residues). Here straight molecules pointed towards the centers of dried droplets. Straightening therefore appears to require the raised salt concentration produced during drying.

Fig. 1 *B* shows a mixture of titin and LMM molecules near a dried droplet. The rodlike LMM molecules are similar to those described previously and are ~ 80 nm long and 2 nm wide (Lowey et al., 1969; Stewart and Edwards, 1984). Only $\sim 20\%$ of titin molecules were found closely associated with LMM, and in these cases the most common appearance was of LMM touching titin with one of its ends (Fig. 2). Behavior consistent with Figs. 1 *B* and 2 was seen in mixtures of titin and myosin (Fig. 3). The usual appearance where myosin and titin were closely associated was of the myosin molecule joined by the tip of its tail. The length from the myosin head-tail junction to the point of attachment on titin (Fig. 4 *A*) was $121.6 \text{ nm} \pm 29.3 \text{ nm}$ (SD, $n = 106$). This was significantly shorter than the tail length in unattached myosin (Fig. 4 *B*) which was 158.0 nm

$\pm 9.5 \text{ nm}$ (SD, $n = 105$) and is very similar to the value of 156 nm previously obtained (Elliott and Offer, 1978). The reduced observable tail length in attached myosin suggests that binding is not at the tip of the myosin tail. The fact that the remaining ~ 30 nm of the myosin tail was not seen projecting on the other side of the titin suggests it may have turned to run parallel to the titin.

To discover whether the binding sites for myosin (or LMM) were located in a particular region of the titin molecule, attachment positions were plotted as a function of distance from the titin head (Fig. 5 *A*). Attachment was mainly in the region 0–800 nm from the titin head, which is the part of titin located in the A band in situ. Shown in Fig. 5 *B* is the length distribution of the titin molecules (mean = $1,002 \text{ nm} \pm 77 \text{ nm}$, $n = 82$).

Titin, LMM, and myosin all tended to be aligned in parallel with the other molecules of the same type. Unexpectedly, however, the orientations of the different molecule types were often different in the same field (Fig. 1 *B*). Moreover, the angle between these orientations varied in different regions of specimens taken from the same piece of mica. These different angles suggest that titin molecules

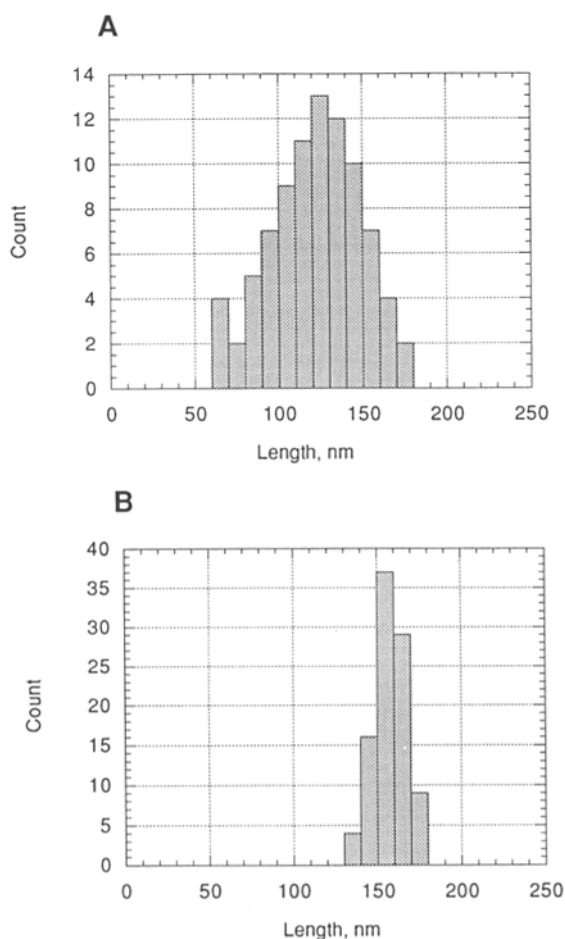


Figure 4. Length of the observable myosin tail in molecules closely associated with titin. The distance from the head-tail junction in myosin molecules found closely associated with titin molecules (*A*) was significantly less than tail length in free molecules (*B*). The data in *A* and *B* were also analyzed by *t* test and the means were confirmed different with a *P* value of 0.001.

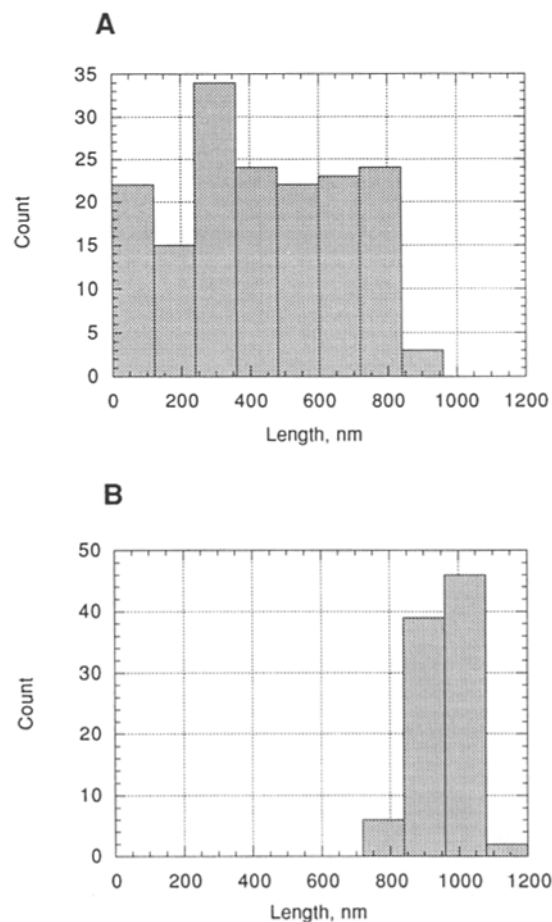


Figure 5. (*A*) Histogram of attachment positions as a function of distance from the head of the titin molecule. The combined data from LMM and myosin are plotted here, but similar histograms were obtained separately. (*B*) Length distribution of the titin molecules. (That binding along the titin was not random was also demonstrated by χ^2 tests and was confirmed at the 0.1% significance level.)

were aligned at a different time from the myosin or LMM (see Discussion).

Quantitation of the Interaction between Titin and LMM

The affinity of titin for LMM was assessed by both solution and solid-phase binding assays. Fig. 6 *A* shows a solution-binding curve obtained by fluorescence depolarization using dansylated LMM. The apparent binding constant calculated from curve fitting was 15 nM (average of 5 determinations). Affinity was also estimated by coating ELISA plate wells with LMM, which were then reacted with titin. Fig. 6 *B* shows titin binding estimated by antibodies and has a typical sigmoid curve. The apparent dissociation constant given by the titin concentration at half-maximal saturation was 12 nM ($n = 5$). Similar values were obtained using plates coated with 2, 0.9, and 0.4 $\mu\text{g/ml}$ LMM.

Reactivity of CNBr Fragments of LMM with Titin

To localize the interaction site(s) in LMM more accurately, CNBr fragments were prepared and their binding to titin assayed. Fig. 7 shows solid-phase assays of titin reacted with two peptides eluted from an SDS-polyacryl-

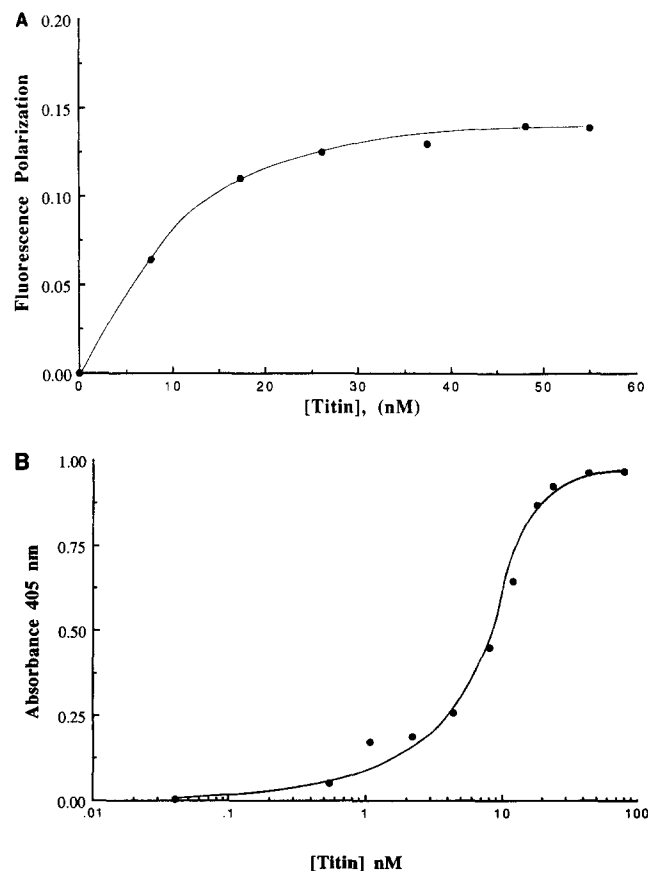


Figure 6. Quantitation of the interaction between titin and LMM. (A) Fluorescence polarization of IEADANS labeled LMM as a function of titin concentration. The curve was generated by nonlinear least-squares fitting and gave a K_d of 15 nM. The amounts of LMM used in these determinations were in the range 8–12 nM. (B) Interaction of titin with immobilized LMM. ELISA plates were coated with LMM and reacted with titin that was detected by antibodies.

amide gel. These peptides were selected by an overlay assay shown on the same figure. The gel was blotted onto nitrocellulose paper and reacted with titin, which was then detected by antibodies. The gel of the CNBr digest showed six main components of molecular mass >10 kD (lane 1). Of these, one with an estimated chain mass of 15 kD reacted directly with titin on the blot (lane 2). There was also a weaker reaction with a peptide of ~ 30 kD. This is shown in lane 3 after first concentrating the peptide. Based on the titin concentration at half-maximal saturation, the apparent dissociation constant of the 15-kD peptide was ~ 20 nM. Since the binding curve for the 30-kD peptide did not saturate under the conditions used, the titin concentration at half maximum could not be measured. However, when the data were replotted on a double reciprocal graph (data not shown) it was clear that the affinity was $\sim 100\times$ less than the 15-kD peptide. The 15- and 30-kD fragments isolated by electroelution were also sequenced and in two separate determinations began EQTVKLD and IDVERSA, respectively. These peptides begin at positions 528 and 171, respectively, in LMM (Fig. 8).

Titin-binding peptides from LMM-CNBr digests were also isolated by affinity chromatography and by screening peaks after HPLC. For affinity chromatography, the CNBr digest was passed through a column to which titin was coupled. The fraction retained on the column was analyzed by reverse phase HPLC and only one major peak was detected (Fig. 9). This had the NH_2 -terminal sequence EQTVKLD and a molecular mass ~ 15 kD (estimated from electrophoresis). When the whole LMM digest was fractionated by HPLC, 11 main peaks were identified, and these were labeled alphabetically (Fig. 10). The tenth and eleventh peaks, named the J and K peptides, had sequences beginning EQTVKLD and IDVERSA, respectively. The binding of the J peptide to titin was tested with the solid-phase assay and the apparent dissociation constant was estimated to be 30 nM. Thus the same titin-reactive LMM peptide was identified by the gel overlay, affinity chromatography, and by screening HPLC peaks.

Competition between LMM and J Peptide Binding to Titin

To confirm the importance of the J peptide, experiments were carried out in which it was reacted with titin in the presence of LMM. For this purpose, the wells of an ELISA plate coated with LMM were reacted with aliquots of titin that had previously been incubated with varying amounts of J. Taking the amount of titin bound to LMM in the absence of J as 100%, the maximum inhibition of binding was $\sim 60\%$ (Fig. 11). The fact that inclusion of large amounts of J with titin did not completely inhibit binding may have been due to interaction via site(s) in the K peptide, or could have been due to J interacting directly with LMM.

Fractionation of Myosin-CNBr Digests by HPLC

A similar but longer version of the J peptide was isolated from whole myosin. This was done since there was reason to believe that J from LMM did not extend to include the myosin COOH terminus: myosin COOH-terminal peptides were not detected in J digests, and there is evidence

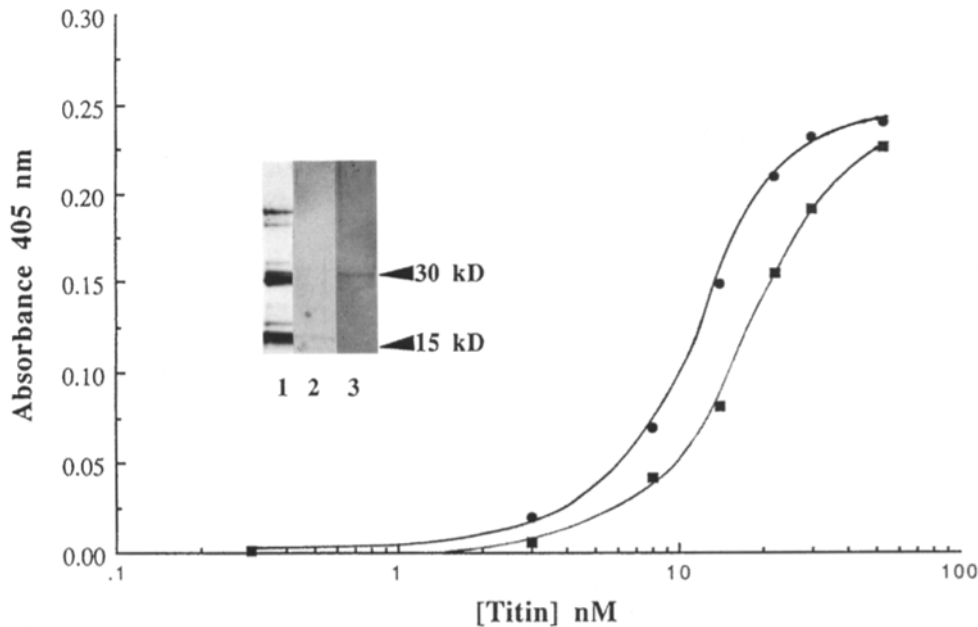


Figure 7. Reaction of LMM CNBr peptides with titin. The graphs show the curves from solid-phase assays demonstrating binding of titin to two peptides eluted from an SDS-polyacrylamide gel of LMM digested by CNBr. These peptides, ~15 kD (left curve) and ~30 kD (right curve), were chosen by an overlay assay shown inset. In the overlay assay the LMM CNBr fragments were separated on a gel and blotted onto nitrocellulose paper. The paper was then reacted with titin, which was detected by antibodies. (Lane 1) Stained paper showing CNBr fragments. (Lane 2) Reaction of titin with a fragment of ~15 kD. (Lane 3) Reaction of titin with a fragment of ~30 kD. (To improve the visibility of this reaction, the 30-kD band from across a whole gel was excised and then concentrated by lyophilization. The concentrated peptide was then reelectrophoresed, blotted, and reacted with titin.)

that ~60 terminal residues are easily lost during chymotryptic preparation of LMM (Maita et al., 1991). It therefore seemed possible that the titin binding to myosin seen by EM might be through a site not be present in J, and that there might be further strong titin-binding site(s) very near the intact tip of the myosin tail. The larger version of

J was termed J' and included the intact COOH terminus. It was prepared by passage of whole myosin CNBr digests through a C18 HPLC column. J' was 17,200 kD, determined by mass spectrometry, which is within 100 D of the predicted value from the sequence beginning EQTVKLD to the myosin COOH terminus. In addition, sequencing of proteolytic fragments of J' showed myosin COOH-terminal peptides. The affinity of J' for titin was indistinguishable from J in the solid-phase assay, which suggests that there are no stronger titin-binding sites in the difference region between the COOH termini of LMM and myosin.

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1  KTKEEEHQRL INELSAQKAR LHTESGEFSR QLDEKDAMVS QLSRGGQAPT
51  OQIEGLKRQL EETKAKSAL AHALQSSRRD CDLLREQYEE EQEAKAELQR
101  AMSKANSEVS QWRKTCETA IQRTEELEEA KKKLAQRLQD AEEHVEAVNS
151  KCASLEKTKQ RLQNEAEDLM LDVERSNATC ARMDKKQRNF DKVLAEWKHK
201  YEETQAELEA SQKESRSLST EVFKVKNAYE ESLDHLETLK RENKNLQOEI
251  SDLTEQIAES AKHIHELEKV KKQIDQEKSE LQAALAEAG SLEHEEGKIL
301  RIQLELNQVK SEIDRKTAPK DPEIDQLKRN HLRVVESMQS TLDAEIRSRN
351  DALRIKPKME GDLNEMEIQL NHANRQAAEA IKNLRNTQGI LKDTQLHLDD
401  AVRGQDDHKE QLAMVERRAN LMQAEIEELR ASLEQTERS R VADQDLLDA
451  SERVQLLHTQ NTLINTKKK LETDISIQG EMEDIVQEAR NAEKAKKAI
501  TDAAMMABEL KKEQDTSABL ERMKNMEQT VKDLQORLDE AEQLALKGGK
551  KQIQKLEARV R A KELENEVESE QRRNVEAVKG LRKHERRVKE LTYQTEEDRK
601  NVLRLQDLVD KLQSKVKAYK RQAEAEQES NINLSKFRKL QHELEEAER
651  ADIAESQVNK LRVKSRDVHS KVISEE

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Figure 8. Positions of titin-binding peptides in the sequence of rabbit LMM. The final 676 residues of the rabbit myosin heavy chain sequence are shown (MYSS_RABIT from the SWISS-PROT database). Single underlining shows the extent of the K and J peptides (see Results). As expected, these peptides were the result of cleavage at methionine residues. The COOH-terminal ends are marked by dotted lines because they were not precisely determined. Double underlining shows the position of the reactive peptide isolated from J, within which two isoforms were detected. The asterisk marks the position of skip residue 4.

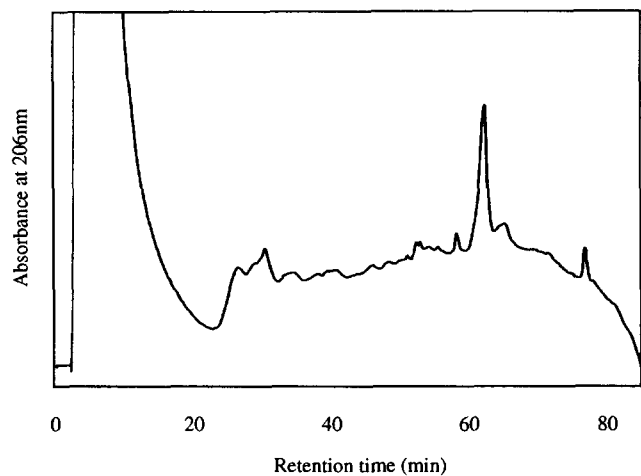


Figure 9. HPLC of bound fraction eluted after passage of LMM digest through titin affinity column. Note there was only one main peak; this had the NH₂-terminal sequence beginning EQTVKLD.

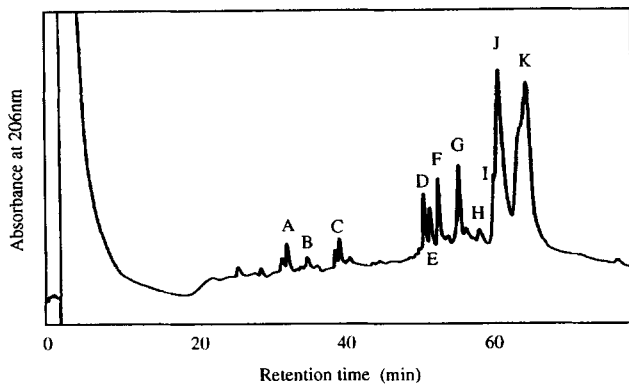


Figure 10. Reverse-phase HPLC of LMM-CNBr digest. Peaks K and J had NH₂-terminal sequences beginning EQTVKLD and IDVERSA, respectively.

Localization of a Titin-binding Site within J'

To localize binding further, J' was digested proteolytically with a lysine proteinase. The resulting peptides were purified by HPLC (Fig. 12) and screened for titin binding. Peak 8 was the only fraction that bound strongly to titin, which is compatible with there being only one tight binding site in LMM. The peak 8 peptide had the 17-residue sequence LEARVRELEAEVESEQK. This sequence begins at residue 556 in the LMM sequence (Fig. 8), and is therefore present in both J and J' peptides. The sequence was different at two positions from the database sequence of rabbit LMM in Fig. 8, which is LEARVKELENEVESEQK. One of these substitutions, alanine for an asparagine, is nonconservative. Rabbit myosin isoforms differing at these positions have been previously noted in myosin CNBr peptides (Elzinga and Trus, 1980). Both versions of the 17-residue peptide were prepared by peptide synthesis, and Fig. 13 shows binding curves with these.

Discussion

Titin and myosin lie approximately parallel in the thick filament. In principle, therefore, many binding sites between

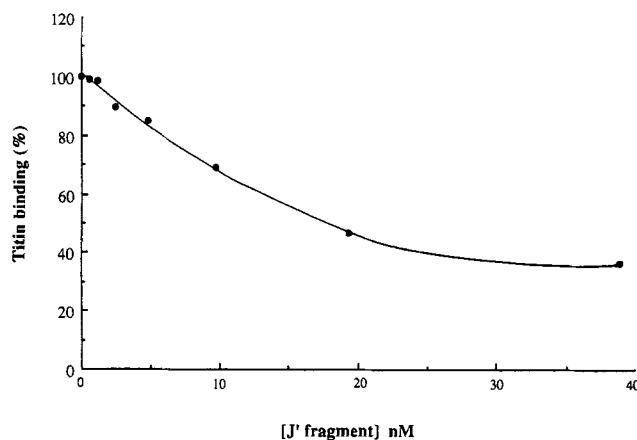


Figure 11. Competition assay. The graph shows a plot of LMM binding to titin (solid-phase assay) in the presence of increasing amounts of the J' peptide.

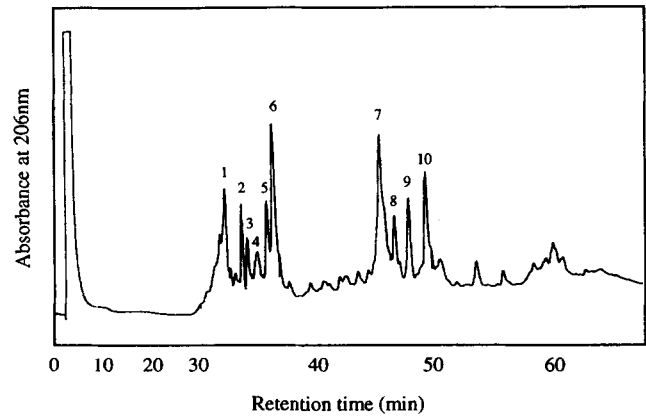


Figure 12. Reverse-phase HPLC of proteolytic digest of J'. 10 major peaks were detected, of which only the eighth (with a sequence beginning LEARV . . .) showed strong reactivity with titin in binding assays.

them could be envisaged. In the most extreme case, since the titin molecule consists mainly of a linear array of immunoglobulin- and fibronectin-like domains, it could be imagined that all the domains in the A-band region interact with myosin, which would give rise to binding sites approximately every 4 nm. In practice, the interaction appears to be simpler. Although not conclusive, both the electron microscope and biochemical data here are consistent with a small number of titin-binding sites in myosin.

EM of titin complexed with myosin or LMM was complicated by several factors: the protein concentration feasible (~ 10 nM titin) is similar to the affinity of the complexes, consequently complexes were partly dissociated. Additionally, interpretable images of titin are obtained only after molecules are straightened. Titin, myosin, and LMM molecules all showed preferred orientations in micrographs, but surprisingly these were usually different for the two molecule types. This suggests that orientation may have occurred at a different time for titin than for myosin or LMM. It also suggests that complexes did not form until after deposition on mica. Flow orientation could have occurred initially during flinging off of liquid by centrifuga-

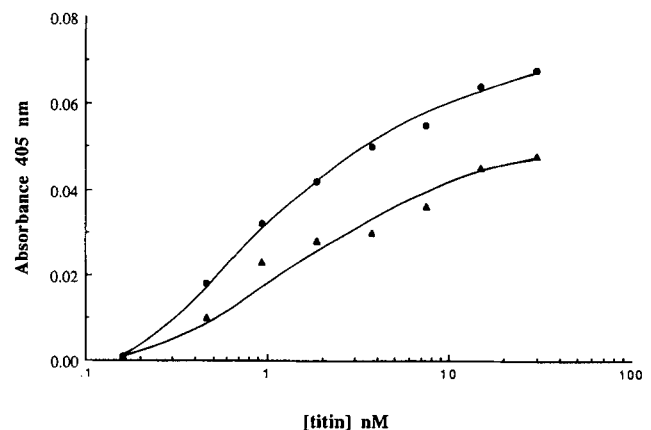


Figure 13. Affinity of synthetic myosin peptides for titin (solid-phase assay). Left curve, LEARVRELEAEVESEQK. Right curve, LEARVKELENEVESEQK.

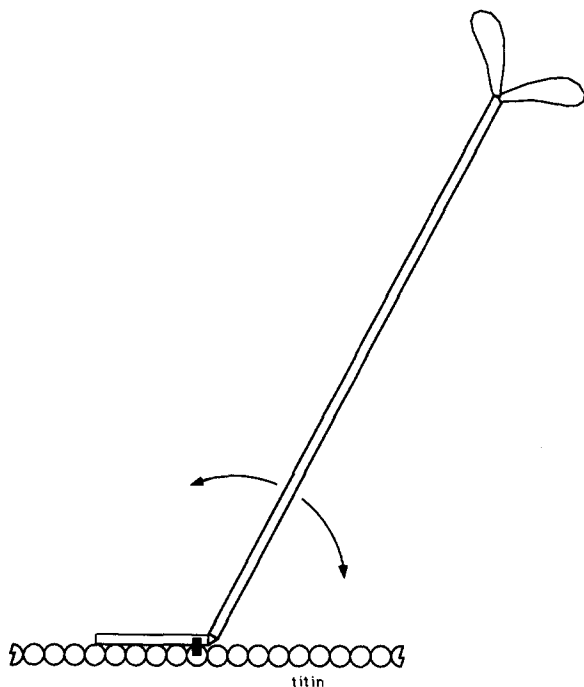


Figure 14. Diagram showing the proposed interaction of titin and myosin. The filled block denotes the binding site in the J peptide. (This diagram is intended to illustrate only the bending of the myosin tail at skip residue 4 and not the detailed packing of the thick filament.)

tion, or subsequently when the meniscus retracted during drying. Drying did not take place until the mica was put under vacuum before shadowing, which was 3–5 min after centrifugation. The myosin tail and LMM are both relatively stiff structures and their orientation may have been fixed by a small number of attachment sites during centrifugation. Titin is more flexible and appears to attach mainly through the molecule head, leaving tail relatively free (see also Nave et al., 1989). Final orientation of titin may therefore not have occurred until it passed through the meniscus on drying.

Although mixtures of titin with LMM or myosin were largely dissociated, it was easy to find instances where they appeared bound. Contact was usually near the tip of myosin tail or at an end of LMM. It could be argued that this was merely overlap, rather than stereospecific binding. The fact that both myosin or LMM were observed joined to titin via their ends far more frequently than through their middle regions suggests stereospecific binding. Measurements of binding positions within titin and myosin are also consistent with this: the distance from the myosin head–tail junction to titin was shorter by ~30 nm than tail length in free molecules. The remaining part of the myosin tail was not seen, although a 30-nm section protruding on the other side of titin would be easily visible. This suggests that the COOH-terminal 30 nm of the myosin tail bent to lie along titin. The data also suggest that the molecules attached to mica were still sufficiently mobile to allow stereospecific attachment before drying.

Similarly, myosin contact positions within titin were mainly in the region that attaches to the thick filament in situ, which is also consistent with stereospecific binding.

This result is important because it is the first evidence that I band titin does not bind myosin. Such a prediction is implicit in the proposal that titin acts as a protein-ruler to specify thick filament assembly (Wang and Wright, 1988; Whiting et al., 1989).

We have also carried out tests where the ratio of LMM to titin was relatively high to determine the number of sites on titin (data not shown). These experiments involved the use of OD at 340 nm as a measure of light scattering and determined the stoichiometry either directly at saturating LMM, or graphically using a modified Scatchard equation (taken from Gutfreund, 1972 p. 71). The ratio of titin/LMM obtained varied between 25 and 50, which is similar to the value of 38:1 recently obtained for myosin/titin using a cosedimentation assay (Eilertsen et al., 1994). A ratio of 49:1 was previously calculated to be present in vivo, assuming there are six titin molecules per thick filament (Whiting et al., 1989). However, it may be noted that the value of 6 does not fit obviously Z-line symmetry which is approximately square.

Within experimental error, the location of binding site in myosin inferred from EM is consistent with the position of the binding peptide determined biochemically. The 17-residue sequence identified is ~17 nm from the myosin COOH terminus (assuming a completely α -helical tail and a translation of 0.15 nm/residue). This site is immediately COOH-terminal of the fourth skip residue in the myosin tail. Skip residues interrupt the regular pattern of hydrophobic residues that stabilize the tail coiled-coil dimer, resulting in enhanced local flexibility or “hinging” (Offer, 1990). The role(s) of striated muscle myosin hinge regions are not known, but skip residues are highly conserved indicating their importance. The proximity of skip-4 to the titin-binding site suggests that in situ it may allow the myosin tail to turn abruptly near where it attaches to titin (Fig. 14). This in turn suggests that the function of other striated muscle skip residues may be to allow sharp bending to facilitate thick filament packing.

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