A survey of interactions made by the giant protein titin

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SUMMARY

A simple solid-phase binding assay was used to screen for interactions that the giant myofibrillar protein titin makes with other sarcomeric proteins. The titin used in the tests was purified by a modified procedure that results in isolation of ~20 mg relatively undegraded protein in <24 h. In addition to the ~3 MDa polypeptide, bands at ~160 kDa and ~100 kDa were also consistently seen on gels. Binding of titin to myosin, C-protein, X-protein and AMP-deaminase was observed. The interaction with myosin appears to be with the light meromyosin part of the molecule.

Key words: titin, thick filaments, sarcomere, giant proteins

INTRODUCTION

Single molecules of the giant protein titin (also known as connectin, ~3000 kDa; (Maruyama et al., 1984; Kurzban and Wang, 1988) span between the M- and Z-lines in vertebrate striated muscle myofibrils (Furst et al., 1988; Whiting et al., 1989). The I-band region of the molecule forms an elastic connection between the end of the thick filament and the Z-line. These connections centre the A-band between adjacent Z-lines and are the main route of mechanical continuity through relaxed muscle fibres (Magid and Law, 1985). Elastic behaviour in this region of titin is evidenced by the movement of epitopes away from both the M- and Z-lines as sarcomere length is increased (Furst et al., 1988; Ito et al., 1988; Whiting et al., 1989), and by the loss of a central A-band after selective cleavage of titin by protease inhibitors (Nave et al., 1989; Kimura et al., 1992). The following method results in ~20 mg of purified titin in 24 h: myofibrils were prepared from chilled fresh rabbit skeletal muscles by homogenisation in 3 volumes of 50 mM KCl, 5 mM EGTA, 1 mM NaHCO₃, 5 µM E64 (Sigma), pH 7.0, at 4˚C. After the 4th spin the myofibrils were resuspended and extracted on ice for 5 min with stirring in 2 volumes of 0.9 M KCl, 2 mM MgCl₂, 10 mM imidazole, 2 mM EGTA, 1 mM PMSF, 10 µg/ml trypsin inhibitor, 0.5 mM DTT, 5 µM E64, pH 7.0. The extract was clarified at 20,000 g for 30 min, diluted 3× with water (final ionic strength ~0.2) and after 1 h precipitated myosin was removed by spinning for 30 min at 20,000 g. The supernatant was diluted a further 5 times (final ionic strength 0.05), left for 40 min and spun at 11,000 g for 30 min. The crude titin pellet was resuspended in 0.6 M KCl, 30 mM potassium phosphate, pH 7.0, clarified for 30 min at 25,000 g and chromatographed in this buffer on a 90 cm × 1 cm Sepharose CL2B column maintained at 1˚C. On occasion the titin at this stage was also passed through a Q-Sepharose column, as described by Nave et al. (1991), but this was not generally used for the present work. The purification was monitored by SDS-polyacrylamide electrophoresis in gradient slab gels (4% to 15%). Samples for these were dissociated at 56°C for 20 min in SDS/urea, as described by Fritz et al. (1989).

MATERIALS AND METHODS

Purification of titin

Titin was first isolated in undenatured form by a relatively protracted procedure (Trinick et al., 1984) during which time endogenous proteinases caused some degradation. This was evidenced by length heterogeneity of molecules seen by electron microscopy, and by the progressive appearance of lower molecular mass bands on SDS-gels. Recently, procedures have been described that accelerate purification and reduce degradation with protease inhibitors (Nave et al., 1989; Kimura et al., 1992). The following method results in ~20 mg of purified titin in 24 h: myofibrils were prepared from chilled fresh rabbit skeletal muscles by homogenisation in 3 volumes of 50 mM KCl, 5 mM EGTA, 1 mM NaHCO₃, 5 µM E64 (Sigma), pH 7.0, at 4˚C. This was followed by 3 cycles of centrifugation (2000 g) and resuspension in buffer without E64 inhibitor. After the 4th spin the myofibrils were resuspended and extracted on ice for 5 min with stirring in 2 volumes of 0.9 M KCl, 2 mM MgCl₂, 10 mM imidazole, 2 mM EGTA, 1 mM PMSF, 10 µg/ml trypsin inhibitor, 0.5 mM DTT, 5 µM E64, pH 7.0. The extract was clarified at 20,000 g for 30 min, diluted 3× with water (final ionic strength ~0.2) and after 1 h precipitated myosin was removed by spinning for 30 min at 20,000 g. The supernatant was diluted a further 5 times (final ionic strength 0.05), left for 40 min and spun at 11,000 g for 30 min. The crude titin pellet was resuspended in 0.6 M KCl, 30 mM potassium phosphate, pH 7.0, clarified for 30 min at 25,000 g and chromatographed in this buffer on a 90 cm × 1 cm Sepharose CL2B column maintained at 1˚C. On occasion the titin at this stage was also passed through a Q-Sepharose column, as described by Nave et al. (1991), but this was not generally used for the present work. The purification was monitored by SDS-polyacrylamide electrophoresis in gradient slab gels (4% to 15%). Samples for these were dissociated at 56°C for 20 min in SDS/urea, as described by Fritz et al. (1989).
Purification of other proteins

Myosin and its subfragments were prepared as described by Margossian and Lowey (1982). C-, H- and X-protein were prepared as described by Starr and Offer (1983). AMP-deaminase was prepared as described by Smiley et al. (1967). A monoclonal antibody to \( \alpha \)-actinin was a generous gift from J. M. Wilkinson. Other proteins were purchased from Sigma Chemical Co.

Electron microscopy

Titin molecules were visualised in the electron microscope by rotary metal shadowing with platinum after drying in vacuo, as described by Trinick et al. (1984) and modified by Nave et al. (1989). This procedure involves flow straightening of the molecules by centrifuging off buffer prior to drying.

Solid-phase binding assays

Dilutions of candidate proteins to interact with titin were spotted onto nitro-cellulose paper in 0.5 M KCl, 50 mM Tris, 1 mM EDTA, 0.3 mM DTT, pH 7.9, at 4°C. After blocking for 2 h at room temperature in this buffer containing 0.25% BSA, 0.25% gelatin and 1% TWEEN-20, the paper was reacted overnight in the cold with 50 \( \mu \)g/ml titin (~20 nM) in 0.25% BSA, 0.25% gelatin in 1% TWEEN, 0.2 M KCl, 50 mM Tris, 1 mM EDTA, 0.3 mM DTT, pH 7.9, at 4°C. The paper was then given three, 5 min washes in PBS-TWEEN and bound titin detected by reaction with titin monoclonal antibody AB5 cell supernatant (Whiting et al., 1989) diluted 1:1 in this buffer. The monoclonal antibody was detected by addition of anti-mouse whole IgG followed by mouse monoclonal peroxidase/anti-peroxidase complex diluted 3000 \( \times \) and 1500 \( \times \), respectively, in PBS-TWEEN. Estimates of the approximate apparent dissociation constants for the interactions found were made with the same assay but applying the proteins to microtitre plates rather than nitro-cellulose paper. The amounts of titin bound were estimated with a microtitre plate reader.

RESULTS

Using the modified purification procedure described ~20 mg titin can be purified in < 24 h. Fig. 1 shows SDS-polyacrylamide gel analysis at different stages during the purification. The main component after gel filtration chromatography is titin, which migrates as a single band at approximately 3 MDa. In addition to this, these preparations also show a faint doublet with mobilities corresponding to approximately 160 kDa. There was also consistently a weak band with a similar mobility to \( \alpha \)-actinin (~100 kDa). In order to test whether this latter protein is \( \alpha \)-actinin, the protein pattern after proteolysis (Cleveland et al., 1977) was compared to that of \( \alpha \)-actinin, and western blots were performed with an anti-\( \alpha \)-actinin antibody. The patterns of peptides on SDS-polyacrylamide gels produced by digestion of the 100 kDa band and \( \alpha \)-actinin (chicken) after digestion with V-8 protease were not similar. On western blots a monoclonal antibody to human platelet \( \alpha \)-actinin that reacted with rabbit \( \alpha \)-actinin did not react with the 100 kDa band on the same blots (data not shown). Thus in neither experiment was the \( \alpha \)-actinin identity confirmed.

Fig. 2 shows an electron micrograph of a rotary-shaded titin preparation in which the molecules had been straightened by centrifuging off buffer after application of the preparation to a mica substratum. The molecules were approximately 900 nm long and usually had a head at one end. Two out of the three examples in Fig. 2 are monomers in which the tail is ~4 nm wide. The third appears to be a dimer, although one of the tails may not extend the full 900 nm. This molecule also has a projection extending from the head. Headless molecules were also observed in these preparations.

Fig. 3 illustrates the binding of titin to candidate interacting proteins immobilised on nitro-cellulose paper, as detected by a monoclonal antibody and the peroxidase reaction. A strong interaction was seen between titin and myosin. Evidence as to which part of the myosin molecule was binding the titin was gained by reactions with the light polypeptide chains and with various myosin proteolytic fragments. No reaction was seen against the light chains or heavy meromyosin (HMM); however, a reaction was detected with rod (whole myosin tail) and light meromyosin (insoluble C-terminal portion of the tail). Since HMM
includes the soluble part of the tail, the data are consistent with titin binding to the LMM part of the myosin molecule, which forms the shaft of the thick filament.

Binding to two other thick filament components, C- and X-protein (also known as slow C-protein), was also observed. These are both single polypeptide chain proteins of molecular mass ~140 kDa (Offer et al., 1973; Starr and Offer, 1983) that are bound to thick filaments at intervals of about 43 nm (Bennett et al., 1986), which is repeat distance of the helix that describes the myosin head lattice in the filament. Both are composed of sequence motifs similar to type III fibronectin and C-2 immunoglobulins (Einheber and Fischman, 1990; Vaughan et al., 1992), which are also the motifs that make up the approximately one third of the titin molecule that has so far been sequenced (Labeit et al., 1992). H-protein is another thick filament component which binds on the 43 nm lattice and has no known function (Bennett et al., 1986). It was also tested, but no reaction was observed. No binding was detected to \( \alpha \)-actinin.

Lesser but consistent binding was also seen to actin, although the significance of this result is unclear (see Discussion below). Other proteins tested as controls, which as expected did not react, were cytochrome c, BSA and keyhole limpet haemocyanin.

Approximate estimates of the apparent binding constants for the interactions observed were made by quantitating the binding assay, using microtitre plates rather than nitro-cellulose. The apparent dissociation constants for the interactions with myosin, rod, LMM, C-protein, X-protein and AMP-deaminase were all \( \sim 10^{-7} \) M.

**DISCUSSION**

The modified procedure used to purify the titin used in this study is similar to others recently described (Nave et al., 1989; Kimura et al., 1992) and results in a preparation that is less degraded than that from earlier methods. Its advantages are that it is convenient and produces substantial (~20 mg) quantities of titin in <24 h. Reduced degradation is evidenced by the single band seen at ~3 MDa on SDS-polyacrylamide gels, which is in contrast to the doublet observed in this position when proteolysis is allowed to occur. Electron microscopy of the preparations shows molecules that are generally ~900 nm long and have a head at one end. Degraded preparations are heterogeneous in length and do not usually show heads. Similar titin appearances were also described by Nave et al. (1989) and Sonoda et al. (1990), both of which studies also reported mixtures of monomers, dimers and trimers. Further fractionation to homogeneous oligomeric fractions was not attempted in the present work.

The presence of components other than the ~3 MDa polypeptide in SDS-polyacrylamide gels of the purified titin was also described by Nave et al. (1989) and by Kimura et al. (1992) The two bands at ~160 kDa were reported by Nave et al. (1989), who used antibodies to show that these proteins are unrelated immunologically and are associated with the head of the titin molecule, which they demonstrated to be located in the M-line *in situ*. A band with mobility corresponding to ~100 kDa was also reported by Kimura et al. (1992) on SDS-polyacrylamide gels of titin purified in the presence of urea and it was suggested that this protein might be \( \alpha \)-actinin. Peptide fingerprinting and western blotting did not support this idea, but we cannot completely rule it out.

Previous *in vitro* tests of the interactions made by titin using purified proteins have been done in solution, generally using myosin filaments. Maruyama et al. (1989) showed that titin caused aggregation of synthetic thick filaments, which was seen in the electron microscope to be

![Fig. 2. Electron microscopy of rotary-shadowed titin straightened by solvent flow. Two of the molecules in the field are monomers. The third appears thicker and may be a dimer. There is a projection on the head of this molecule. Similar substructure can be observed in molecules in Figs 5 and 8 of Nave et al. (1989). ×53,000.](image1)

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![Fig. 3. Interaction of titin with other muscle proteins. 5 µl droplets of dilutions containing 0.8, 0.16, 0.04 and 0.008 mg/ml of each candidate interacting protein were applied to nitro-cellulose paper and reacted with titin.](image2)

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through side-by-side assembly. Under the conditions used in their work (60 mM KCl, 5 mM phosphate, pH 7) the molar ratio of titin to myosin in the aggregates was as high as 1.5:1 although the ratio \textit{in vitro} is likely to be \textasciitilde 1:50. This high ratio may have been due to self-aggregation of the titin, since an ionic strength only slightly lower was used to precipitate titin during the present work. No attempt was made by Maruyama et al. (1989) to localise the binding region within the myosin molecule, but it was reported that C-protein did not affect this interaction.

The present experiments were conducted near to physiological ionic strength. The solid-phase assay used also has the advantage that it allows interactions with several proteins to be screened conveniently and requires small amounts of material. Since any tendency for proteins to dissociate will be accentuated by the extensive washing used, any binding detected is likely to be relatively tight. Consistent with this, all of the apparent dissociation constants determined using proteins bound to microtitre plates were of the order $10^{-7}$ M. The fact that all the interactions found are consistent with antibody localisation data (except perhaps with actin, see below), together with the failure to detect binding to non-myofibrillar proteins, suggests that the data are meaningful.

The data indicate that titin binds to the LMM region of the tail of the myosin molecule, which is consistent with the proposal that the A-band region of titin is attached to the outside of the thick filament shaft \textit{in vivo} (Whiting et al., 1989). Binding of titin to LMM was also indicated by similar solid-phase assays to the present ones, but using titin constructs expressed from cDNAs (Labeit et al., 1992). Tests with the cDNA constructs also indicated interaction with C-protein. Binding of titin to C-protein may explain the presence of C-protein at 43 nm intervals in the thick filament, since both antibody labelling (Furst et al., 1989) and sequence (Labeit et al., 1992) evidence indicate that titin has substructure with a 43 nm periodicity in the A-band. Since the attachment of H-protein to thick filaments is part of the same 11-stripe pattern in each half A-band as C- and X-proteins (Bennett et al., 1986), it seemed likely that it too would bind to titin. Although no interaction was detected, binding to H-protein under the assay conditions may be weaker. C- and X-proteins are attached to thick filaments (and presumably to titin) at several positions in each half-sarcomere, whereas there is only one H-protein site. Any titin molecule may therefore bind to C- and X-proteins immobilised on nitro-cellulose via several sites, but can presumably attach to H-protein through only one site.

Evidence for an interaction with AMP-deaminase was sought, since antibody localisation has suggested that this enzyme is attached to thick filaments but is located in the I-band, beyond where the myosin-containing part of the thick filament is thought to end (Cooper and Trinick, 1984). Immobilisation of the enzyme in the sarcomere may improve its metabolic efficiency. Since titin molecules extend from the tips of thick filaments across the I-band, it seems possible that AMP-deaminase may be attached to myofibrils through titin. The binding to titin seen here is consistent with this idea.

The observed weak interaction of titin with actin is less easy to understand but has been previously reported by Maruyama et al. (1987). The behaviour of epitopes at different sarcomere lengths suggests that titin is not directly attached to thin filaments, except perhaps near the Z-line. The \textit{in vitro} binding may therefore be an artifact or it may reflect the Z-line interaction. Against the latter possibility, Matsuura et al. (1991) reported that a 1200 kDa I-band fragment of titin did not bundle actin filaments.

The present data support the proposal, based on antibody localisation studies, that the A-band part of the titin molecule is intimately associated with the thick filament. Since the functions of C- and X-proteins are not known the significance of these interactions is unclear, but it seems possible that titin provides a scaffold with which to regulate assembly of the complex structure of the filament.

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REFERENCES


Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. and Kikuchi, M.
123


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