Binding of a native titin fragment to actin is regulated by PIP2

Catherine Astier*, Fabrice Raynaud, Marie-Christine Lebart, Claude Roustan, Yves Benyamin

Laboratoire de Motilité Cellulaire EPHE, UMR 5539, Université des Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34090 Montpellier, France

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Abstract Titin is a giant protein which extends from Z-line to M-line in striated muscles. We report here the purification of a 150-kDa titin fragment, obtained after V8 protease treatment of myofibrils. This polypeptide was located at the N1-line level, in a titin part known to exhibit stiff properties correlated to an association with actin. By solid or liquid phase binding assays and cosedimentation, we have clearly demonstrated a direct, saturable and relative high affinity binding of the native titin fragment to F-actin. The 150-kDa titin fragment was also shown to accelerate actin polymerization. Furthermore, the actin-titin interaction was found to be inhibited by phosphoinositides.

Key words: Muscle structure; Titin; Thin filament; Actin polymerization; Phosphoinositide

1. Introduction

Striated muscle is a very ordered tissue whose contractile apparatus has been widely studied. In addition to the two sliding filaments, a third elastic filament system was recently described to provide the molecular basis for passive tension [1–5]. Its major component, titin, also referred to as connectin, is the largest polypeptide ever described (around three megadaltons). A single molecule spans half a sarcomere, traversing I- and A-bands with its amino terminus associated to Z-line and its carboxyl terminus to M-line (for recent reviews see [6–9]). Thus, along the sarcomeric structures several proteins, including α-actinin, actin, myosin, muscle-specific calpain p94, C-protein or myomesin, were identified as titin partners [10–14]. These multiple interactions suggest that titin could play an important role in thin and thick filament alignment during muscle contraction and in regulating their assembly during myofibrillogenesis [15].

Recent studies on both skeletal muscle and on individual titin molecules indicate that titin may function as a molecular spring responsible for the elasticity of relaxed striated muscles [16,17]. The I-band region represents the most extensible part of the molecule and contains essentially tandem immunoglobulin-like domains and a PEVK-rich segment [7]. This module was shown to unravel in response to stretch. However, other I-band sections of titin do not extend with a sarcomere increasing length, probably because of their interaction with other myofibrillar structures [18,19,4]. Thus, in the vicinity of the Z-line, a close association of titin with the parallel thin filament could stiffen the segment located between the Z-line and the N1-line (~100 nm long) and stabilize the anchorage of the elastic filament [20,21]. The present work describes, by combining approaches, the interaction of a purified and native titin fragment, located at the N1-line level, with filamentous actin. Moreover, we have also measured the effect of this fragment on actin polymerization, and shown a regulation of this association by phosphoinositides.

*Corresponding author. Fax: (33) (467) 14 47 27.
E-mail: c-astier@crit.univ-montp2.fr

Abbreviations: ELISA, enzyme-linked immunosorbent assay; F-actin, filamentous actin; G-actin, globular actin; GPI, glycerophosphomyoinositol; PI, phosphatidylinositol; PIP_2, phosphatidyl inositol 4,5-biphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Protein or protein fragments preparation

Actin was extracted from rabbit skeletal muscle acetone powder according to the method of Spudich and Watt [22] and further purified as described [23]. For polymerization assays actin was labeled at Cys^{374} with *N*-pyrenyl iodoacetamide [24]. The 150-kDa titin fragment was recovered in the supernatant fraction, after *S. aureus* V8 protease treatment of purified rabbit skeletal myofibrils, and was loaded onto an S-300 HR column (Pharmacia) as described [25]. This titin subfragment was subsequently purified by FPLC onto an anion exchanger column Poros HQ/H (Boehringer). For fluorescence measurements, the 150-kDa titin fragment was labeled with fluorescein isothiocyanate as described elsewhere.

2.2. Antibodies

Rabbit anti-titin antibodies ET19 or KK16 were obtained after injection of synthetic peptides [26], ELLRKTDELLHWTKELT or KEYEKEQALIRKKMAK corresponding, respectively, to residues 1983–2000 or 1169–1185 into the human cardiac muscle sequence [7].

2.3. Electrophoresis and immunoblots

Proteins were fractionated on SDS-PAGE using either 2–10% or 5–18% gradient resolving gels according to the method of Laemmli [27]. Immunoblots were carried out as described [25], and the immunoreactive bands were revealed using peroxidase conjugated secondary antibodies with the enhanced chemiluminescence detection system (Amersham).

2.4. Binding assays

For cosedimentations assays, F-actin and the 150-kDa purified titin fragment were incubated in a 2-mM $MgCl_2$, 100-mM KCl, 1-mM DTT, 10-mM Tris pH 7.8 buffer at 20°C for 30 min. F-actin was pelleted in a Beckman airfuge at 30 p.s.i. for 30 min. Proteins from the resulting supernatants and pellets were visualized onto 5–18% gradient gels after SDS-PAGE and Coomassie blue staining. For ELISA [28], microplates were coated with F-actin (0.05 mg/ml). After saturation with 0.5% gelatin, 3% gelatin hydrolysate in PBS buffer containing 0.1% Tween 20, various amounts of purified 150-kDa titin fragment were allowed to interact. Then, anti-titin ET19 antibodies (1 μg/l) were added to the wells, followed by alkaline phosphatase conjugated anti-rabbit immunoglobulins (Biosys). Assays were monitored at 405 nm with *p*-nitrophenyl phosphate (Sigma). Each assay was conducted in triplicate and mean values were plotted after subtraction of non-specific absorption. Fluorescence experiments were carried out using a Perkin Elmer Luminescence Spectrometer LS 50. Spectra for FITC conjugated titin fragment were performed in 100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl pH 7.8 with an

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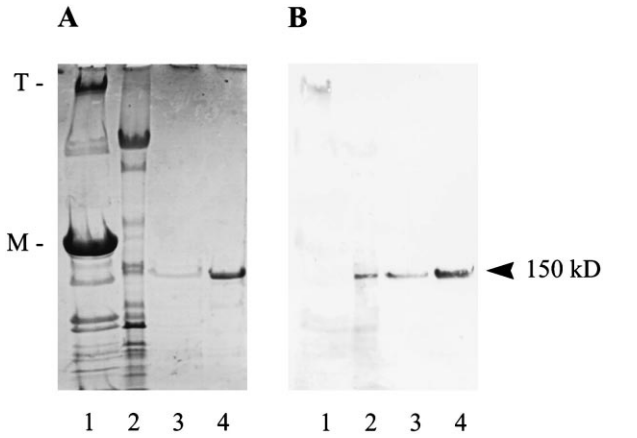

Fig. 1. Purification of a 150-kDa titin fragment. A: Coomassie-blue stains of 2–10% SDS gradient gel. Lane 1: total extract of purified rabbit skeletal myofibrils. Lane 2: supernatant of V8-protease-treated myofibrils (1:2000 (w/w) enzyme/substrate ratio) containing a major product of titin cleavage, an 800-kDa polypeptide. Lane 3: S-300 fraction containing the 150-kDa titin fragment. Lane 4: purified 150-kDa titin fragment after Poros HQ/H (eluted at 300 mM NaCl). B: Western blot of the same gel probed with the affinity purified anti-titin anti-peptide ET19 (0.2 μg/ml).

excitation wavelength at 490 nm. Fluorescence changes induced were deduced from 3 successive records of emission spectra areas.

2.5. Actin polymerization

Enhanced pyrenyl-actin fluorescence was used as an indicator of actin polymerization. Excitation and emission wavelengths were set at 365 and 386 nm, respectively. Polymerization was induced by the addition of 2 mM $MgCl_2$ and 0.1 M KCl.

3. Results

3.1. Purification and characterization of an N1-line fragment of titin

In a previous report we have demonstrated that exogenous proteases applied to purified myofibrils induced a splitting of titin at the Z-line proximity, allowing a release of titin subfragments [25]. We describe here the purification of a 150-kDa titin fragment solubilized after a V8 protease treatment of rabbit skeletal myofibrils (Fig. 1A). After gel filtration onto an S-300 Sephacryl column, the fractions containing the 150-kDa proteolytic product were loaded onto an anion exchanger column, and the 150-kDa polypeptide eluted at 300 mM NaCl gradient. Its sarcomeric location was defined by using two anti-titin anti-peptide antibodies (cf. Section 2) previously checked for their reactivity on native rabbit skeletal titin. Thus, only the anti-peptide antibodies corresponding to residues 1983–2000 (ET19) in the human cardiac sequence, showed a strong reaction with the titin purified fragment (Fig. 1B) while by immunofluorescence it gave a single stripe signal around the N1-line (data not shown). Moreover, this titin 150-kDa fragment was found to react with the T12 anti-titin monoclonal antibody, defined to label each half sarcomere approximately at 100 nm from the Z-line [29].

3.2. Binding of titin fragment to actin

The ability of the titin fragment to interact with filamentous actin was first assayed by cosedimentation. A purified 150-kDa titin fragment at a concentration of 0.06 mg/ml, as estimated by UV measurements, was incubated with 0.6 mg/ml of

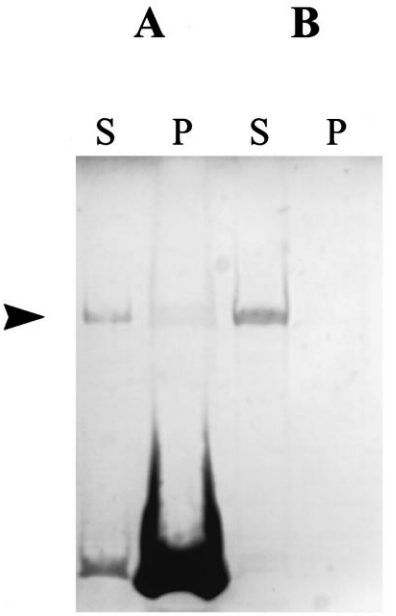

Fig. 2. F-actin cosedimentation assays of a purified titin fragment. Coomassie-stained SDS gels of (A) a purified 150-kDa titin fragment incubated with F-actin, (B) a control titin fragment in the absence of actin. S, supernatant; P, pellet. The 150-kDa titin fragment is indicated by an arrowhead.

F-actin. Densitometric analysis of the results shown in Fig. 2 indicated that about 20% of the titin fragment cosedimented with F-actin under these conditions. Thus, even if the binding was not particularly strong, it was considered to be significant with regard to control (Fig. 2, lanes B). The interaction of the titin fragment with actin was then verified by direct ELISA for which actin was immobilized onto microplates and titin binding followed with the specific ET19 antibodies. The titration curves showed a saturable association with an apparent dissociation constant of 0.31 ± 0.06 μM (Fig. 3), that appeared

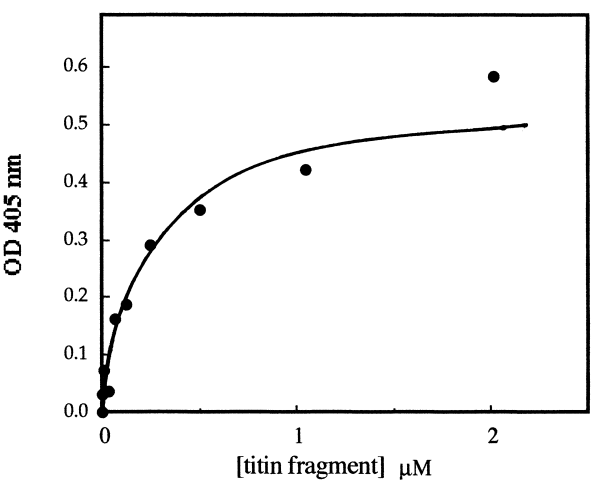

Fig. 3. Interaction of a titin fragment with actin as revealed by ELISA. Serial dilutions of the 150-kDa titin fragment were incubated with F-actin (50 μg/ml) and immobilized onto microplates. Titin fragment binding was followed by using affinity purified anti-titin anti-peptide antibody ET19 (1 μg/ml). The assay was performed in triplicate wells, and the data were corrected for non-specific binding.

higher under these assay conditions than for cosedimentation measurements.

3.3. Titin fragments accelerate actin polymerization

We further extend our investigations onto actin-titin binding to test whether the titin fragment affected the actin polymerization parameters by using fluorimetric measurements. As illustrated in Fig. 4 for the initial part of polymerization curves, it was found that the 150-kDa titin fragment significantly increased the rate of actin polymerization induced by 2 mM $MgCl_2$ and 0.1 M KCl without modifying the lag time length. No effect of the purified titin fragment on pyrenyl G-actin was detected under the conditions employed.

3.4. PIP2 dependence of titin-actin interaction

As for many actin-binding proteins which regulate polymerization of actin, PIP_2 was found to be a potential regulator of actin-titin interaction. Thus, the actin binding on titin fragment was drastically reduced in the case of preincubation of the purified titin fragments with 5 μg/ml of PIP_2 (Fig. 5). By this method, the estimated apparent K_d of the actin-150-kDa titin fragment interaction was in the micromolar range. Furthermore, an effect of dissociation was also measured onto preformed actin-titin fragments complexes with the addition of increasing amounts of PIP_2 (Fig. 5, inset). In this case, the concentration of PIP_2 needed to obtain a half-decrease of the initial fluorescence of the actin-titin fragment complex was comprised between 2 and 10 μg/ml. In the same conditions, PI or GPI were found to be 10-fold less efficient for dissociation of actin-titin fragment complexes. Thus, taken together, these results indicated that the actin binding ability of titin fragments was strongly inhibited by PIP_2. Moreover, it should be mentioned that phosphoinositide binding to titin fragments could not be detected by this method, while no modification of fluorescence diffusion or polarization was registered after their addition to the labeled titin fragment (data not shown).

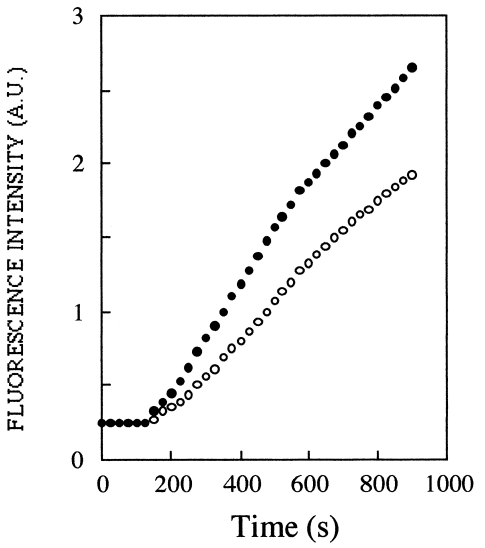

Fig. 4. Effect of a titin fragment on actin polymerization. The increase of a fluorescence signal resulting from polymerization of pyrene labeled G-actin (7 μM) was followed in the presence (●) or absence (○) of the purified 150-kDa titin fragment, after addition of 0.1 M KCl and 2 mM $MgCl_2$ at zero time. Fluorescence intensity was expressed in arbitrary units.

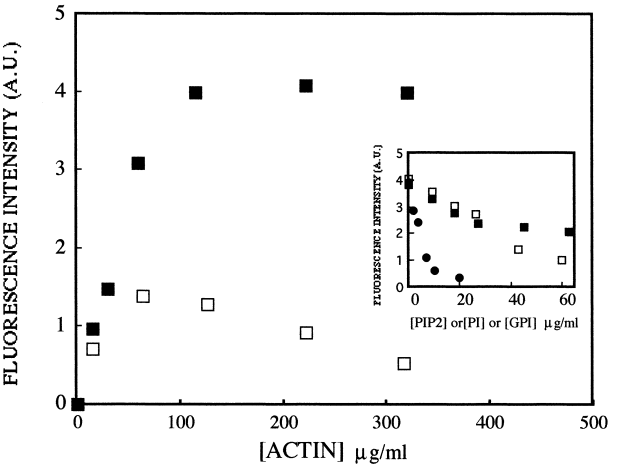

Fig. 5. Effect of phosphoinositides on actin-titin interaction. Increasing concentrations of F-actin were added to an FITC-labeled 150-kDa titin fragment, respectively after addition (□) or not (■) of PIP2 (5 μg/ml), and the fluorescence variations were monitored. Inset: After preincubation of the FITC-labeled 150-kDa titin fragment with 220 μg/ml of F-actin, increasing amounts of PIP2 (●), PI (□) or GPI (■) were added to the mixture.

4. Discussion

When the giant titin molecule was discovered, its sarcomeric location with a Z-line anchorage and its position in parallel to thin filaments were simultaneously described [30]. Recently, a molecular association between the elastic and thin filament which could be supported in vivo by an interaction between titin and actin was shown [31]. This association seemed not only to depend on the filamentous and native structure of actin, since it was not detected by binding assays involving denaturing conditions, but also to involve preferentially the N-terminal part of the titin molecule in the Z-line proximity [20,21]. Thus, microscopy observations after in situ specific titin epitope decoration in different stretching conditions [4], in conjunction with cosedimentation assays realised with recombinant fragments corresponding to immunoglobulin-like domains from the stiff N1-line region, showed a close association of this titin segment with actin [21]. On the other hand, the extreme susceptibility of the titin molecule to endogenous proteolysis led to restricted in vitro binding assays of actin to purified T2 form of titin, i.e. lacking part of the I-band and the near-Z-line segment of the molecule. In this case, it was also demonstrated that the truncated titin form could interact with actin as with reconstituted thin filaments [32].

By different approaches, the present work showed the ability of a native and purified 150-kDa proteolytic fragment of titin, belonging to the Z-line proximal part of the molecule, not only to interact strongly with filamentous actin (K_{dapp} in the micromolar range) but also to accelerate the actin polymerization process. Furthermore, for the first time we have demonstrated that actin-titin interaction is regulated by phosphoinositides, since PIP_2 was shown to inhibit the association of actin to the 150-kDa titin fragment or to dissociate preformed complexes. In muscle, the specific localization of PIP_2 at the Z-line level [33] could indicate that in this dense multiproteic structure, thin filaments should be independent of titin. Thus, the anchorage of the N-terminal part of titin, known to involve an interaction with α-actinin, would be efficiently reinforced by molecular contacts of titin with actin

filaments and putative other ligands, generating the stiffness observed for the elastic filaments only in the Z-line vicinity. In addition, the stability of the titin-actin complexes at the N1-line level was previously illustrated by a higher resistance to gelsolin extraction of this part of the thin filament [34]. Thus, this direct and probably lateral association between thin and elastic filaments could prevent sliding or damage of this structure highly exposed to repeated tensions. Concerning the titin ability to facilitate actin filament assembly, it could be postulated that, as previously suggested, titin acts as a template during myofibrillogenesis and/or in muscle cell regeneration, allowing the I-Z-I brush formation by its association with the Z-line components and its effect onto thin filament elongation, while both titin and actin filaments are then responsible for the capture of thick filaments [35]. Thus, in mature structures, the specificity of contractile filaments association with the third filamentous system could essentially be assigned to titin specific sequences which remain to be characterized.

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