

Studies on the interaction between titin and myosin

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Summary. This study examines the interaction of titin and myosin. In order to analyze the domains of myosin contributing to the binding for titin, we conducted a solid phase binding assay. Different portions of myosin (heavy chains, light chains and myosin fragments) were coated on the microtiter wells and reacted with biotinylated titin. Then the binding of biotinylated titin to these polypeptides was detected by using the avidin-biotin-peroxidase method. The results demonstrated that light meromyosin and subfragment 1 were the major domains of myosin interacting with titin. Titin fragments obtained by trypsin digestion were allowed to react with myosin in an affinity column, and the bound fragments were isolated by an acidic elution. Immunoblot analysis of myosin-bound titin fragments revealed that an A-band domain of titin was responsible for the binding of myosin. In addition, biotinylated titin labelled the outer A-bands and Z-bands in intact myofibrils, thus confirming the *in situ* binding of titin to myosin.

Key words: Titin, Myosin, A-band, Interaction

Introduction

Titin is an elastic protein in striated muscles. A single molecule is long enough (0.9 - 1.2 μm) to extend from the Z line near the M-line region (Fürst et al., 1988). The roles of titin are to recenter the A-bands in activated sarcomeres upon relaxation and to produce the resting tension of the myofibers (Horowitz et al., 1989).

Possible interaction of titin with synthetic myosin filaments has been extensively studied (Kimura and Maruyama, 1983a; Kimura et al., 1984a,b; Maruyama et al., 1985, 1989). Titin is found to cause the

aggregation of myosin filaments at KC1 concentration lower than 0.15 M, which normally does not induce myosin aggregation (Kimura and Maruyama, 1983a). The binding molar ratio of titin to myosin is 1.5 to 1 as determined by a cosedimentation assay and gel electrophoresis analysis (Maruyama et al., 1989). The rod portion of myosin molecule is donated with this titin binding ability. S1 and S2 do not demonstrate the ability to bind to titin by turbidity study, flow birefringence and electron microscopy (Maruyama et al., 1985).

The entire titin molecule consists of an A-band and I-band domains. One interesting question is that which domain(s) of titin interacts with myosin. The purpose of this study is to identify the precise portions of myosin that are responsible for the interaction with titin by a solid phase binding assay. The portion of titin bearing myosin binding property will be isolated from a myosin-affinity column and analyzed by two monoclonal anti-titin antibodies.

Understanding the interaction of titin with myosin can shed light on our understanding of how titin is assembled into a sarcomere.

Materials and methods

Purification of Native Titin II. Native titin II was purified from chick breast muscle according to the method used by Kimura and Maruyama (1983b). Titin was then biotinylated and separated from free biotin by gel filtration on a Sephadex G-25.

Purification of myosin subunits and fragments. Crude myosin was obtained by following the procedure of Scordilis and Adelstein (1978). Myosin and C-protein were further separated by a DEAE-Sephadex A-50 column (Margossian and Lowey, 1982). Myosin light chains were dissociated from heavy chains by urea (Perrie and Perry, 1970). Subfragment 1, rod or light meromyosin were produced by chymotrypsin digestion of myosin (Margossian and Lowey, 1982). Subfragment 1 was subsequently purified by DEAE-Sephadex A 50

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column. Subfragment 2 was purchased from Sigma.

Assay of ATPase activity. ATPase activity of S1 was assayed as described by Henkel et al. (1988).

Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Fritz et al. (1989).

Solid Phase Binding Assay. Proteins were coated on ELISA wells at a concentration of 10 $\mu\text{g/ml}$. After blocking with 1% bovine serum albumin, biotinylated

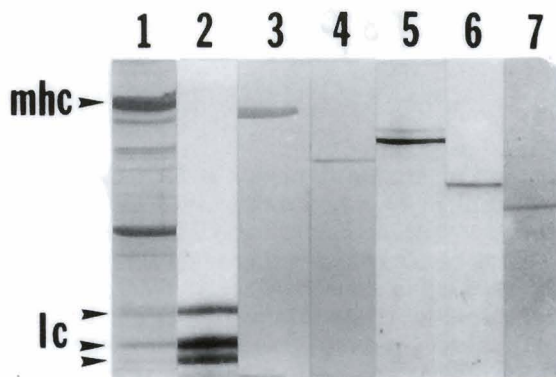


Fig. 1. Electrophoretic pattern of myosin subunits and fragments. 1, myofibrils, 2, purified light chains. 3, purified myosin. 4, purified myosin subfragment 1. 5, gel eluate of myosin rod. 6, gel eluate of light meromyosin. 7, gel eluate of S2.

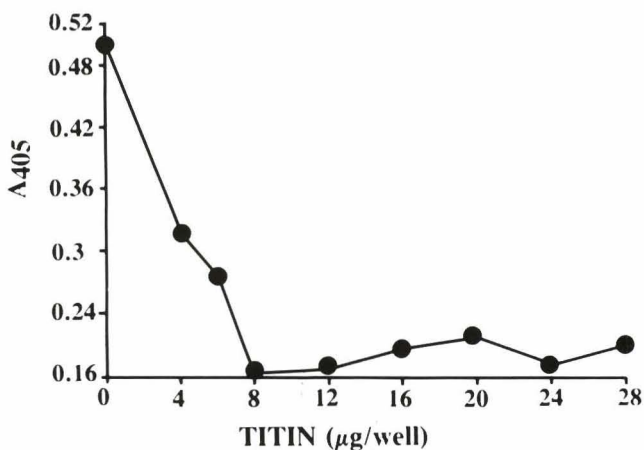


Fig. 2. Inhibition of the binding of biotinylated titin to myosin by unlabelled titin. Biotinylated titin (10 $\mu\text{g/well}$) was mixed with unlabelled titin (0-24 $\mu\text{g/well}$) before adding to the myosin-coated wells.

titin at different concentrations was added to each well and incubated for one hour. The wells were washed with PBS-Tween (0.05% Tween 20 in phosphate buffered saline), reacted with 1:500 diluted avidin-biotin peroxidase mixture (Vector, Lab, PA, USA) for one hour, and developed with a substrate solution using 2,

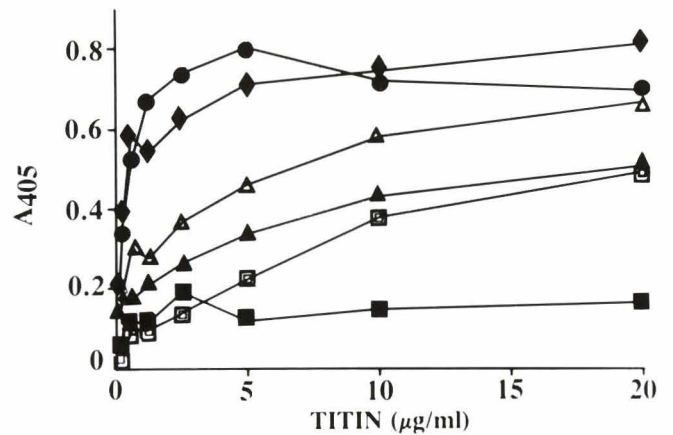


Fig. 3. Interaction between titin and subunits and subfragments of myosin. Biotinylated titin was added to the wells coated with different proteins. It shows that biotinylated titin has strong binding to light meromyosin (solid diamonds), rod (solid circles) and heavy chains (open triangles). Titin also binds to S1 (solid triangles) and light chain (open squares) but not to S2 (solid squares).

2'-azinobis-di-3-ethylbenthiazolinesulfonate as the chromagen. The absorbance of wells was measured by a MicroELISA reader (Dynatech, USA).

Affinity chromatography. Titin was digested in a trypsin solution according to the method described by Kimura

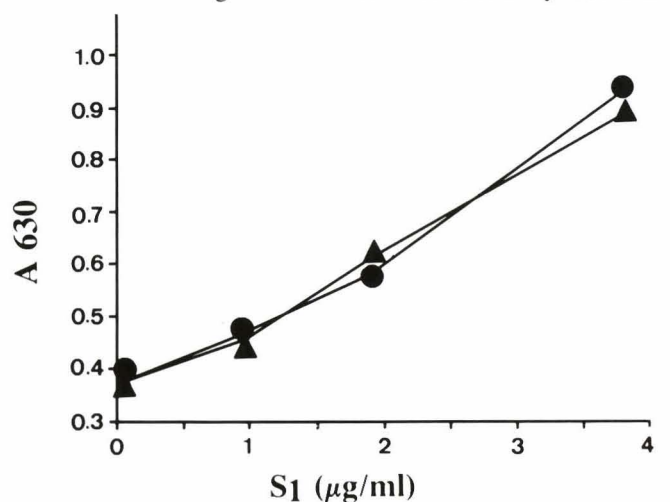


Fig. 4. Effect of titin on the calcium-activated ATPase activity of myosin subfragment 1. Solid triangles, ATPase activity of subfragment 1. Solid circles, ATPase activity of subfragment 1 in the presence of titin (1.2 $\mu\text{g/ml}$).

et al. (1984b). The total titin fragments were applied on a myosin-coupled Sepharose 4B column equilibrated in a buffer (0.1 M NaCl, 10mM Tris, 5mM β -mercaptoethanol, pH 7.2). Glycine buffer (0.1 M, pH 2.5) was used to elute the column. The bound titin

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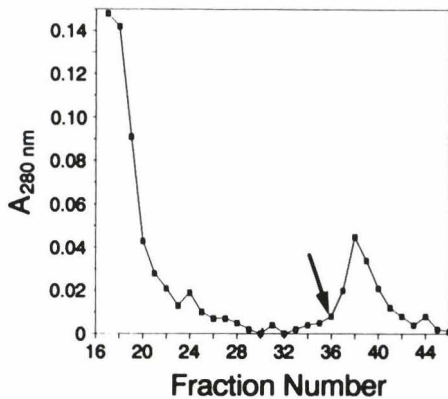


Fig. 5. Elution profile of titin fragments purified by a myosin-affinity column. Arrow indicates the change of buffer.

fragments were analyzed by immunoblotting with monoclonal anti-titin antibodies A12 and A2. The specificity of these two antibodies has been described in our previous paper (Wang et al., 1991). Each antibody stained different positions at the outer A-band.

Labelling of biotinylated titin to the A-bands. Chicken skeletal myofibrils, intact or extracted with a formamide solution (7.5 M formamide, 75 mM KCl, 15 mM sodium phosphate, 1 mM EDTA, pH 7.0) to release isolated A-bands, were incubated with biotinylated titin (10 $\mu\text{g}/\text{ml}$ in PBS) for one hour. The myofibrils were then washed with PBS and reacted with 1:20 diluted FITC-conjugated goat anti-biotin (Sigma) or 1:200 diluted avidin-biotin-peroxidase for one hour, followed by reacting with a substrate solution which contains 3, 3'-diaminobenzidine as chromagen.

Results

Evidence for the interaction between titin and myosin subfragment 1 and rod

The purities of myosin subunits and subfragments were examined by gel electrophoresis (Fig. 1). The interaction pattern of biotinylated titin and myosin on the microtiter wells was similar to that of myosin heavy chain and biotinylated titin as shown in Fig. 3. In the following experiment, 10 $\mu\text{g}/\text{ml}$ of biotinylated titin mixed with unlabelled titin of different amounts was added to myosin wells. The binding of biotinylated titin to the myosin wells was significantly inhibited by unlabelled titin (Fig. 2). The result indicated that labelled and the unlabelled titin compete with each other for a saturable number of binding sites on the myosin. The binding sites of myosin for titin were further identified by examining different subunits and subfragments of myosin in a solid-phase binding assay. The result demonstrated the strong binding of titin to light meromyosin of the rod, a moderate degree of binding to S1 and light chains and no binding to S2

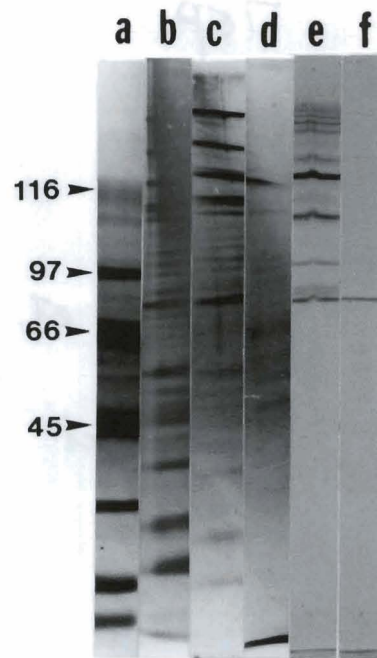


Fig. 6. Immunoblot analysis of myosin-bound titin fragments. a, molecular weight standard. b, total fragments. c, unbound fragments. d, bound fragments, e and f, bound fragments immunoblotted with monoclonal antibodies A12 and A2 respectively.

(Fig. 3). In order to examine the effect of titin on ATPase activity of S1, a fixed amount of titin (1.2 $\mu\text{g}/\text{ml}$) was incubated with S1 solutions of different concentration (0-4 $\mu\text{g}/\text{ml}$), which was then assayed for calcium-activated ATPase. The binding of titin to S1, however, did not alter the calcium-activated ATPase activity of S1 (Fig. 4).

The elution profile of titin fragments from a myosin-column was shown in Fig. 5. Several bound fragments were reacted to A12 antibody and only one of them to A2 antibody (Fig. 6). The result indicated that an A-band domain of titin near the epitope positions of antibody A12 and A2 showed the affinity for myosin binding.

Biotinylated titin labelled the A-band and Z-line in Myofibrils

In the control, myofibrils were not labelled with biotinylated BSA (Fig. 7a,b). In long sarcomeres, biotinylated titin was added to the A band (about outer one fourth of an A band) and the Z line (Fig. 7c,d). In short sarcomeres, the labelling was mainly on the Z line. In formamide-extracted myofibrils, the binding of biotinylated titin was found in the isolated A bands, especially the outer A band where the greater intensity was observed by avidin-biotin-peroxidase method (Fig. 7e,f) or immunofluorescence (Fig. 7g,h).

Discussion

Using a turbidity assay and electron microscopy,

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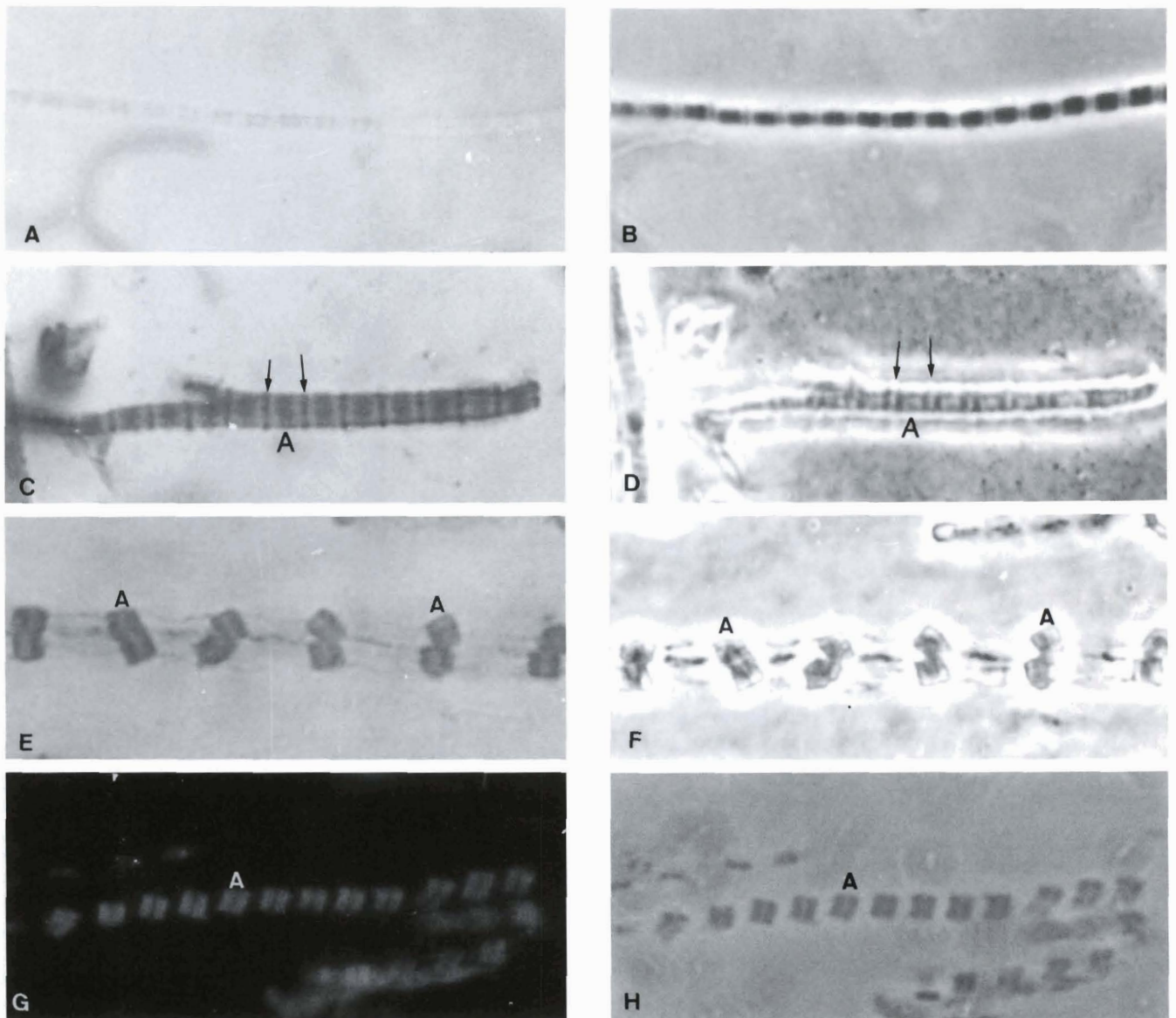


Fig. 7. Binding of biotinylated titin in skeletal myofibrils. B, D, F and H are the phase images of A, C, E and G. Biotinylated titin is localized by the avidin-biotin-peroxidase method (C-F) or the immunofluorescence (G-H). In the control panel (A-B), biotinylated bovine serum albumin did not label any myofibril. Biotinylated titin is added to A- and Z-bands in intact myofibrils (C-D) and A-bands in formamide-extracted myofibrils (E-H). A, A-band. Arrows indicate the Z-bands.

Maruyama et al. (1985) demonstrated that titin binds to the myosin rod but not to S1 and S2. The addition of titin to an S1 solution does not affect the ATPase activity of S1 (Kimura et al., 1984a). However, in our study, surprisingly, we observed a moderate affinity of binding between titin and S1. This finding was in diametrical contradiction with that reported by Maruyama et al. (1985). This discrepancy between the two studies might be due to the fact that the solid-phase binding assay is a more sensitive method in detecting protein interaction as compared with the methods employed by Maruyama et al. (1985). The binding of titin to subfragment 1, however, did not affect the

calcium-activated ATPase activity of S1 as examined in this study. Thus, it appears that titin binds to the non-enzymatic part of the subfragment 1. Our findings that titin binds to the myosin rod but not to S2 is consistent with Maruyama et al. (1985). Titin also bound to myosin light chains, the significance of which fact, yet, remains to be established.

It is important to know whether the property of myosin binding is ascribed to the A-band domain of titin or the whole titin molecule. A 400 kd titin fragment is shown to possess the ability of myosin binding (Kimura et al., 1984b). This 400 kd fragment, however, has not been mapped onto the titin molecule. A recent study by

Labeit et al. (1990) shows that part of a titin gene possesses a gene sequence similar to those gene sequences of many myosin-binding proteins. This gene product, a titin fragment, has been located by a monoclonal antibody to be in the A-band domain of titin which was near 0.33 μm from the M-line (Labeit et al., 1990). With affinity chromatography and immunoblot analysis, we demonstrated that an A-band domain of titin in a region near 0.7 μm from the M-line possessed the myosin-binding property. It appeared that several titin domains in the A-band bind to myosin. However, none of these myosin-bound titin fragments were analyzed with other anti-titin antibodies recognizing the I-band domain of titin in the present experiment. Thus, the possibility that an I-band domain of titin may also bind to myosin could not be excluded.

The interaction of titin and myosin *in vitro* was further supported by the binding of biotinylated titin to the A-bands. The binding of titin to the Z-bands might be due to the interaction between titin and α -actinin (Wang et al., 1990). Titin, has been proposed to integrate I-Z-I brushes and A-bands during myofibrillogenesis (Fürst et al., 1989). The present observation of the interaction between titin and A-bands validates the above hypothesis. In conclusion, our results confirm that titin is a myosin-binding protein and this binding is reinforced by its interaction with light meromyosin, S1 and light chains.

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