Vol. 176, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1991 Pages 189-193

LOCATION OF THE C-TERMINUS OF TITIN AT THE Z-LINE REGION IN THE SARCOMERE

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Received February 6, 1991

SUMMARY Limited proteolysis of titin with trypsin yielded a number of polypeptides which were electrophoresed and transferred to a nitrocellulose membrane. Proteolytic removal of the C-terminal residues on the nitrocellulose-bound polypeptides was achieved by using carboxypeptidase Y. The species of the polypeptides left after the digestion was quantified by immunoblotting with two distinct monoclonal anti-titin antibodies A2 and A12 of which the epitopes were located at 0.74 μm and 0.69 μm away from the center of an A-band, respectively. Two polypeptides (266 kd and 84 kd) reactive to both antibodies were identified in the control group. Fifteen minutes after the digestion, the immunoreactivities of A2 on 266 kd and 84 kd polypeptides were disappeared, while those of A12 on these polypeptides were not affected. The results indicate that the C-terminal end of titin is located near the Z-line region and the N-terminal end at the M-line region in the sarcomere.

Titin (or connectin) is a long, linear molecule with a molecular weight of 3 x 10^6 daltons (1-3). Being a striated muscle-specific protein, titin extends from the M-line region and connects thick filaments to the Z-lines (4 -6). Immunofluorescence staining with different monoclonal anti-titin antibodies on myofibrils revealed that upon stretching of the myofibrils, the positions of titin epitopes in the A-band remained fixed while those in the I-band displayed an elastic behavior (5, 7-9). However, no information is available concerning the molecular organization of titin in a sarcomere.

It is therefore our aim to determine the location of the C- or N- terminus of the titin molecule in the sarcomere with respect to the M- or Z- lines. Titin in myofibrils is highly sensitive to proteolysis as compared with other muscle proteins (10). Furthermore, diverse patterns of polypeptides could be obtained by reacting purified titin with different enzyme solutions (11). In the present study, we prepared a library of titin fragments from native titin by trypsin digestion. They were electrotransferred to the nitrocellulose membrane and further reacted with carboxypeptidase Y. The polarity of the titin molecule in a sarcomere can be deduced from the examination of the

0006-291X/91 $1.50

immunoreactivities of two monoclonal anti-titin antibodies on the dually digested polypeptides.

MATERIALS AND METHODS

Native titin was prepared from chicken breast muscle by the method of Kimura et al. (11). Monoclonal antibodies were generated by using native titin as the immunogen according to Wang & Greaser (12). The specificity of antibodies was examined by immunoblotting as described by Towbin et al. (13). Two monoclonal antibodies, A2 and A12, were selected for the present study. The epitope position was determined on immunofluorescence-stained myofibrils with a measuring magnifier. Trypsin digestion of titin was carried out according to Kimura et al. (11). Titin was incubated with trypsin solution in an enzyme to substrate (w/w) ratio of 1:15 in 0.3M KCl, 10 mM sodium phosphate buffer (pH 7.0) at 25 °C for 15 minutes. Titin fragments were electrophoresed on 12% SDS polyacrylamide gels and transferred to the nitrocellulose membrane (13-14). The membrane strips containing titin polypeptides were incubated in a carboxypeptidase Y solution (0.1 or 1 mg/ml in 75 mM KCl, 20 mM imidazole-HCl, 1 mM ß-mercaptoethanol, 10 mM NaN_3 , pH 7.0) at 35 °C as described by Makuch et al. (15) and Klarskov et al. (16). The reaction was terminated by thoroughly washing of the membrane. The membrane was washed in phosphate buffered saline and then immunoblotted with antibodies A2 and A12; the stain intensity of a selective titin fragment was recorded by a scanning densitometer (Hoefer Scientific Instrutments, GS 300 densitometer).

RESULTS

Titin purified by the method of Kimura et al. (11) is designated as ß-connectin or T II, a degradative form of intact titin (T I). The purity of titin was examined on SDS-polyacrylamide gels (Fig. 1, lane 2). Two monoclonal antibodies, A2 and A12,

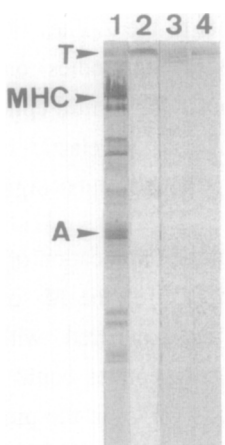

Fig. 1. Purity of native titin and characterization of A12 and A2 monoclonal anti-titin antibodies. Lane 1, myofibrillar proteins. Lane 2, titin purified by gel filtration on a Bio-gel A50 column. Lane 3, immunoblot of myofibrillar proteins with A12. Lane 4, immunoblot of myofibrillar proteins with A2. T, titin. MHC, myosin heavy chain. A, actin.

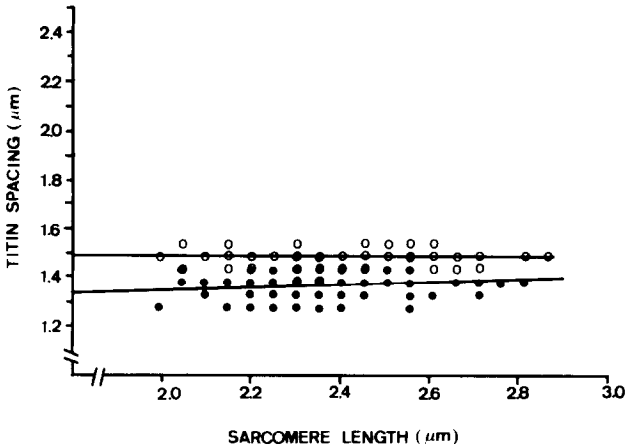

Fig. 2. The location of epitopes defined by antibodies A2 and A12 in the sarcomere. The average center-to-center distance (μm) between antibody-decorated bands is plotted against the sarcomere length (μm). It shows that epitopes of A2 and A12 remain at the same position as the sarcomere length varies, and are situated at 0. 74 μm and 0.69 μm away from the M-line , respectively. o---o, A2. ●---●, A 12.

were generated against native titin and demonstrated to be specific to titin (T I and T II) in skeletal muscle by immunoblot analysis (Fig. 1, lanes 3-4). Each of two antibodies recognized a distinct and nonrepetitive epitope on the titin molecule as evidenced by their reaction with a diverse set of titin fragments (Fig. 3) and their different staining positions on myofibrils (Fig. 2). Within a sarcomere, each antibody stained a pair of bands symmetric to the M-line. The distance between antibody-

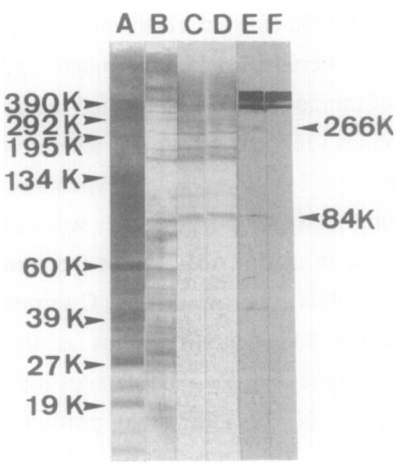

Fig. 3. Immunoblot analysis of titin fragments by antibodies A2 and A12 after carboxypeptidase Y digestion. A-B, silver-stained gels. A, molecular weight standard. B, titin fragments. C-D, immunoblots with antibody A12. E-F, immunoblots with antibody A2. Titin fragments was reacted with carboxypeptidase Y for 0 min (C and E) and 15 min (D and F), Arrowheads (on the right panel) indicate two polypeptides (266 kd and 84 kd) reacting with both antibodies. The staining intensity of these polypeptides by antibody A12 remains the same while that by antibody A2 was decreased to an undetected level.

labeled bands within a sarcomere was plotted against the sarcomere length (Fig. 2). It showed that the positions of titin epitopes located by antibodies A2 and A12 were independent of the sarcomere length, and they were localized at 0.74 μm and 0.69 μm away from the center of the A-band, respectively.

Digestion of titin with trypsin released a number of polypeptides (Fig. 3, lane B). Two polypeptides (266 kd and 84 kd) were identified to react with antibodies A2 and A12 in the zero min control (Fig. 3, lanes C and E). The immunoreactivity of A2 on 266kd and 84 kd polypeptides were completely abolished by the enzyme treatment (Fig. 3, lane F). In contrast, the immunoreactivity of A 12 on these two bands remained unchanged (Fig. 3, lane D). Thus, carboxypeptidase Y preferentially removed the epitope of A2, which was located at the C-terminal ends, from 266 kd and 88kd titin fragments.

DISCUSSION

By low-angle rotary shadowing electron microscopy, titin is revealed as a long, linear molecule with periodic globular domains (2-3,17), and one end of the molecule contains the binding site for two M-line proteins (18). The polarity of the molecule, however, cannot be determined on the basis of the morphological data. Especially for a gigantic molecule like titin, it is rather difficult to define which end of the molecule is the C-terminus. The present study was an attempt to locate the N- or C-terminus of the titin molecule in a sarcomere. Our strategy was to use two distinct monoclonal anti-titin antibodies in a study of the effect of carboxypeptidase Y on titin fragments. This enzyme attacks the C-terminal end of a polypeptide. Several polypeptides were not affected by the enzyme treatment, which might be due to the fact that the epitopes of A2 and A12 were not in the C-terminal region of these polypeptides or that the C-terminal ends of the polypeptides contained indigestible amino acids (16). We focused on two polypeptides (266 kd and 84 kd) which covered two titin epitopes of A2 and A12. The epitope of A2 on these two polypeptides was rapidly excised by carboxypeptidase Y, suggesting that the epitope of A2 was near the C-termini of these two polypeptides as compared with that of A12. Since the epitope of A2 was closer to the Z-line than that of A12, we thus conclude that the C-terminus of the titin molecule is located at the Z-line region and the N-terminus at the M-line region in the sarcomere. It is reasonable to expect that the binding sites for the M-line proteins or α-actinin might be located in the N- or C-terminal portions of the titin molecule, respectively. Further studies are needed, however, to verify this speculation.

The functional domains of titin have not yet been studied in detail. Kimura et al. (11) have isolated a 400 kd polypeptide from the chymotrypsin digestion of titin, thereby demonstrating the myosin-binding property of this fragment. It is interesting to map this fragment on the titin molecule and examine whether it is a N-terminal peptide. Labeit et al (19) have successfully cloned parts of the titin gene which might code for the myosin-binding domain, as witnessed by its similarity to gene sequence of many

proteins interacting with myosin. It may be feasible to define the functional regions of the titin molecule in the near future on the basis of the present finding.

Acknowledgments: This work was supported by grants from National Science Council of the Republic of China (NSC 78-0412-B002-138 and NSC 79-0412-B002-139).

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