The N-Terminal Sequence of Paratropomyosin Binding Fragments from β-Connectin

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In order to clarify the position where paratropomyosin binds to connectin in the A-I junction region of a sarcomere, chicken β-connectin was digested by Staphylococcus aureus V8 protease under denaturing conditions and the digested peptides were electrophoretically separated. Five peptides, 150-kDa, 100-kDa, 70-kDa, and 43-kDa fragments, were simultaneously detected by biotinylated paratropomyosin and an anti-connectin monoclonal antibody. The N-terminal sequence of the 43-kDa fragment was found to be YQFRVYAVNK, similar to the sequence of 7556–7565 amino acids in the I51 fibronectin type 3 domain that was located at the A-I junction region of human cardiac titin/connectin. Therefore, we propose that paratropomyosin binds to the 43-kDa fragment from β-connectin at the A-I junction region in both living muscle and in muscle immediately postmortem, and the N-terminus of the 43-kDa fragment is localized in the I51 domain.

Key words: paratropomyosin; β-connectin; A-I junction; myofibrils; postmortem ageing

During postmortem ageing of muscle, a myofibrillar protein, paratropomyosin, weakens the rigor linkages between actin and myosin, and contributes to increased meat tenderness.15 Paratropomyosin was found at the junction of A- and I-bands of sarcomeres in living muscle and in muscle immediately postmortem, and an increase of calcium ion concentration to 0.1 mM in postmortem muscle resulted in the translocation of paratropomyosin from its original position to thin filaments.23 In examining binding of paratropomyosin at the A-I junction region, we have shown3,5 that paratropomyosin bound to both β-connectin/titin 2 and 400-kDa fragment, the proteolytic product of the N-terminal side of β-connectin.6 We have also shown that the interaction of paratropomyosin with β-connectin and the 400-kDa fragment were weakened at a [Ca2+] > 0.1 mM.5 Thus, we have suggested that paratropomyosin bound to connectin at the A-I junction region of sarcomeres. Recently, it has been reported7 that a maximum of 12 moles of calcium ions bound to the 400-kDa fragment to alter the secondary structure. This result coupled with our observation described above lead us to propose that calcium ions may alter the interaction of paratropomyosin with connectin and translocate paratropomyosin on to thin filaments. To test this hypothesis, it is important to clarify the binding site of paratropomyosin on connectin at the A-I junction region.

Connectin (α-connectin/titin 1) is a large, elongated, elastic polypeptide with a molecular mass of about 3,000 kDa, and runs from the Z line up to the edge of the M line in a sarcomere.6 β-Connectin is the part of connectin that includes the A-I junction region and is prepared in a native state from myofibrils.8 For this paper, in order to find which portion of connectin interacts with paratropomyosin at the A-I junction region, β-connectin was prepared from chicken breast muscle and digested by V8 proteases to produce smaller fragments. The peptide fragments that paratropomyosin bound were detected and the N-terminal sequence was analyzed to identify its position in the intact molecule.

Materials and Methods

Reagents. Streptavidin conjugated with alkaline

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CBB R250, Coomassie Brilliant Blue R250; IgG, immunoglobulin G; NFDM, non-fat dry milk; NBT, 4-nitroblue tetrazolium chloride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sulfo-NHS-biotin, sulfo-N-hydroxy succinimidyl biotin; TBS, Tris-buffered saline; 2D-PAGE, two dimensional-polyacrylamide gel electrophoresis
phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 4-nitroblue tetrazolium chloride (NBT) were purchased from Roche Diagnostics (Mannheim, Germany). Sulfo-N-hydroxysuccinimide biotin (sulfo-NHS-biotin) and Staphylococcus aureus V8 protease were bought from Pierce Chemical Co. (Illinois, U.S.A.). Hydroxypatite was purchased from Bio-Rad Laboratories (California, U.S.A.). Mouse anti-titin monoclonal antibody (T11) and goat anti-mouse immunoglobulin G (IgG) antibody conjugated with alkaline phosphatase were bought from Sigma (Missouri, U.S.A.). The other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Preparation of the proteins. Chicken breast muscle was used. Paratropomyosin was purified with a hydroxyapatite column and biotinylated with sulfo-NHS-biotin according to the procedure described in our previous paper.4) β-Connectin was separated by the method of Kimura and Maruyama,5) and purified by the method of Itoh et al.6)

Protease digestion. Purified β-connectin was mixed at a 10:1 weight ratio with V8 protease and digested from 1 to 36 hours at 25°C under the following conditions, A, in a solution containing 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.1 M Tris-HCl buffer (pH 6.8); B, in 0.15 M NaCl, 4 M urea, and 0.125 M K-phosphate buffer (pH 7.0); and C, in 0.3 M NaCl and 0.25 M K-phosphate buffer (pH 7.0). Digestion was stopped by addition of diisopropyl fluorophosphate (DFP) and digested β-connectin was stored at −80°C until use.

Far-western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli10 using a 4-20% polyacrylamide gradient gel. Two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE) was carried out in the combination of isoelectric focusing (IEF) for the first dimension with DryStrips (Amer sham Biosciences, England) of pH 4-7 and pH 5.5-6.7, and SDS-PAGE for the second dimension with a 7.5-20% polyacrylamide gradient gel. The separated β-connectin fragments were electrotransferred on to a polyvinylidene difluoride (PVDF) membrane (Millipore, U.S.A.) by the method of Towbin et al.11) and fragments that bound to biotinylated paratropomyosin were detected on the membrane by streptavidin conjugated with alkaline phosphatase and biotin according to the method described in our previous paper.4)

Immunoblotting. β-Connectin fragments that corresponded to the A-I junction region were detected by T11 monoclonal antibody for the antigenic probe of the region.12) After β-connectin fragments were electrotransferred on to a PVDF membrane, the membrane was washed with Tris-buffered saline (TBS) and then blocked with 7.5% non-fat dry milk (NFDM) in TBS for 2 hours at room temperature. NFDM was washed out with TBS containing 0.1% Tween 20 (TBS-T) and the membrane was incubated overnight with TBS-T containing T11 monoclonal antibody and 1% NFDM at 4°C. The antibody was washed out with TBS-T and the membrane was overlaid with anti-mouse IgG antibody conjugated with alkaline phosphatase in TBS-T containing 1% NFDM for 1 hour at room temperature, then incubated with the substrate of NBT/BCIP in a reaction solution containing 0.1 M NaCl, 50 mM MgCl2, and 0.1 M Tris-HCl (pH 9.5) for coloring.

Amino acid sequences. β-Connectin fragments that corresponded to the A-I junction region were separated by 2D-PAGE using a DryStrip of pH 5.5-6.7 for the first dimension, and electrotransferred on to a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue R250 (CBB R250) and the spots that paratropomyosin bound were disected out from the membrane. The membrane pieces were set in a sample chamber of a protein sequencer (G1005A, Hewlett Packard, U.S.A.) and the amino acid sequence of the N-terminal side were analyzed by the method of Edman13) using phenyl isothiocyanate (PITC) as the fluorogenic reagent.

Results

β-Connectin was digested by Staphylococcus aureus V8 protease under various conditions to produce smaller fragments, and separated by SDS-PAGE, then electrotransferred on to a PVDF membrane for clarifying the binding position of paratropomyosin on connectin at the A-I junction region of a sarcomere. Figure 1 shows the SDS-PAGE pattern of β-connectin after the digestion and β-connectin fragments detected by probes, biotinylated paratropomyosin and anti-connectin monoclonal antibody (T11), on the membranes. V8 Protease in the presence of 2% SDS digested β-connectin quickly to produce many fragments with smaller peptide sizes (Fig. 1-1A) while the protease in the presence of 4 M urea produced many fragments with larger sizes on the gel (Fig. 1-1B). However, little digestion of β-connectin occurred in 0.3 M NaCl, 0.25 M KPO4 without denaturants, and the larger peptide fragments were not separated clearly on a 4-20% polyacrylamide gel (Fig. 1-1C).

β-Connectin fragments that bound paratropomyosin were detected on the PVDF membrane using biotinylated paratropomyosin (Fig. 1-2) as described in Methods. In the presence of 2% SDS for 1 hour, digestion fragments less than 60 kDa were detected,
Paratropomyosin Binding Fragments from Chicken β-Connectin

Fig. 1. SDS-PAGE Pattern and Overlay of Biotinylated Paratropomyosin and T11 Monoclonal Antibody after Electrottransfer to PVDF Membranes of β-Connectin Digest by *Staphylococcus aureus* V8 Protease.

β-Connectin was digested by V8 protease in a 1:10 weight ratio to β-connectin in various solutions for 1-36 hours at 25°C. Digested β-connectin was separated by SDS-PAGE using a 4-20% polyacrylamide gel and the gel was stained with CBB R250 (1). After electrottransfer of digested β-connectin onto PVDF membranes, the membranes were incubated with biotinylated paratropomyosin (2) and T11 antibody (3) overnight at 4°C. Bound biotinylated paratropomyosin and T11 antibody were detected by the procedures described in Materials and Methods. Arrows show the bands detected mainly in digestions in the presence of 4 M urea. A, digestion with 2% SDS; B, with 4 M urea; C, in K-phosphate buffer (pH 7.0). a, digestion for 1 hr; b, 3 hrs; c and e, 6 hrs; d, f, and h, 12 hrs; g and i, 24 hrs; j, 36 hrs; b-C, β-connectin; MW, molecular weight marker.

and were digested to smaller fragments with increasing time (Fig. 1-2A). When β-connectin was digested by V8 protease in the presence of 4 M urea for 1 hour, biotinylated paratropomyosin mainly bound to bands of 150 kDa, 100 kDa, 70 kDa, 50 kDa, 43 kDa, and 20 kDa. Additional incubation time had little effect (Fig. 1-2B, arrows). In the absence of both SDS and urea only peptides above 150 kDa were detected by biotinylated paratropomyosin and those fragments were poorly resolved (Fig. 1-2C). Also a few bands around 50 kDa were observed.

Proteolytic fragments constituting the A-1 junction region of β-connectin were detected using T11 antibody (Fig. 1-3). Few fragments reacted with the monoclonal antibody following digestion by V8 protease in the presence of 2% SDS. A single fragment of 20 kDa was observed in 1 hour of digestion (Fig. 1-3A). On the other hand, many bands were observed with T11 antibody following protease digestion in the presence of 4 M urea. The major bands were 150-kDa, 100-kDa, 70-kDa, 50-kDa, 43-kDa, and 40-kDa bands (Fig. 1-3B, arrows). In digestions without SDS and urea, bands above 150 kDa were detected by T11 antibody (Fig. 1-3C). Digestion of β-connectin by trypsin and α-chymotrypsin produced little fragments that were detected by both probes, except for a 45-kDa fragment produced from digestion by α-chymotrypsin for 36 hours at 25°C (data not shown).

Based upon these results, digestion of β-connectin by V8 protease in the presence of 4 M urea was used to make a 2D-PAGE peptide map for detection of paratropomyosin binding fragments by biotinylated paratropomyosin and T11 monoclonal antibody as described in Methods. Approximately 370 fragments were separated as protein spots stained by the silver stain method (Fig. 2-1). Following transfer on to a PVDF membrane, many fragments higher than 35 kDa in the area of pH 5-7 were detected by biotinylated paratropomyosin as shown in Fig. 2-2. Peptides with isoelectric points in the area of pH 6-7 were predominant. T11 antibody bound fragments from 35 to 45 kDa in the area of pH 5-6, but few larger fragments were observed in the area of pH 6-7.

Arrows in Figs. 2-2 and 2-3 indicate peptides detected by both biotinylated paratropomyosin and antibody probes. Peptides of 150 kDa, 100 kDa, 70 kDa, and 43 kDa were mainly observed, but not the one with a mass of 50 kDa. The four peptides of 43 kDa were smaller than the others, so amino acid sequences of their N-terminal sequences were analyzed. The spots, C1, C2, C3, and C4 (named in turn from acidic side), were separated by 2D-PAGE using a DryStrip of pH 5.5-6.7 to enlarge the separation pattern, and stained with CBB R250 after transfer on to a PVDF membrane (Fig. 3). Each spot of C1, C2, C3, and C4 was dissected out from 10 membranes for protein sequencing. Amino acid sequences of the N-terminal sides of C1, C2, and C4 spots were EGKKHFL, EGKKHFL, and YQFRVYAVNK, respectively (Fig. 4). C1 and C2 spots appeared to be the same fragment. The difference of isoelectric point between C1 and C2 fragment may be due to some
Fig. 2. 2D-PAGE Pattern and Detection of β-Connectin Fragments by Biotinylated Paratropomyosin and T11 Monoclonal Antibody on PVDF Membrane of β-Connectin Digested by Staphylococcus aureus V8 Protease with 4 M Urea.

β-Connectin in a solution containing 4 M urea, 0.15 M NaCl, and 0.125 M K-phosphate buffer (pH 7.0) was digested by V8 protease in a 1:10 weight ratio to β-connectin for 24 hours at 25°C. β-Connectin fragments were separated in two dimensions by isoelectric focusing at pH 4-7 and SDS-PAGE using a 7.5-20% polyacrylamide gel, and the gel was stained by the silver stain method (1). After electrotransfer of β-connectin fragments onto PVDF membranes, the membranes were incubated with biotinylated paratropomyosin (2) and T11 antibody (3) overnight at 4°C and stained by the procedures described in Materials and Methods. Arrows show the protein spots detected by both biotinylated paratropomyosin and T11 antibody. MW, molecular weight marker.

chemical modification of a particular amino acid residue. The sequence of the C3 fragment has not been determined successfully, probably because the total protein amount of C3 fragments dissected out from 10 membranes was too low.

Discussion

It has been shown that paratropomyosin was found at the A-I junction region of sarcomeres in living and immediately postmortem skeletal muscle and translocated to thin filaments by the increase of calcium ion concentration during postmortem ageing of muscle. In previous papers, we have demonstrated that paratropomyosin bound to β-connectin, and that the interaction of paratropomyosin with β-connectin was influenced by calcium ion in vitro. From these results, we suggested that paratropomyosin bound to connectin at the A-I junction region. To clarify the position where paratropomyosin binds to...
Paratropomyosin Binding Fragments from Chicken β-Connectin

**Fig. 3.** Four Spots of 43-kDa Fragments Stained with CBB R250 after 2D-PAGE for Determination of the N-Terminal Sequence. β-Connectin was digested by V8 protease as shown in the legend of Fig. 2. The digested β-connectin was separated in two dimensions by isoelectric focusing at pH 5.5–6.7 and SDS-PAGE using a 7.5–20% polyacrylamide gel. Four spots of 43-kDa fragments were numbered in turn from acidic side as C1, C2, C3, and C4. Half a membrane of acidic side is shown. MW, molecular weight marker.

**Fig. 4.** N-Terminal Sequences of C1, C2, and C4 Fragments and Comparison of the Sequence of C4 Fragment with 7556-7565AA of Human Cardiac Titin/Connectin.

Each spot of C1, C2, and C4 in Fig. 3 was dissected out from the 10 membranes and analyzed the N-terminal sequences with a protein sequencer. Asterisks indicate identical amino acids between the N-terminal sequence of C4 fragment and 7556-7565AA in Is1 domain of human cardiac titin/connectin.17 Amino acid residue of the N-terminal side of 7556-7565AA is glutamic acid (E).

β-connectin at the A-I junction region, we digested β-connectin with a protease to produce smaller fragments, and searched for fragments that paratropomyosin exclusively bound. SDS-PAGE showed that digestion by V8 protease in the presence of 4 M urea was the best method to produce various fragments from β-connectin (Fig. 1-1B), since β-connectin was not readily attacked by the protease under nondenaturing conditions (Fig. 1-1C). V8 protease specifically attacks the carboxy-terminal sides of aspartic and glutamic acid residues in phosphate buffer, even in the presence of denaturants such as SDS and urea.15 Probably more proteolytic sites are exposed under these conditions to produce smaller fragments than under native conditions.

When β-connectin digested by V8 protease under the various conditions was separated by SDS-PAGE and overlaid with biotinylated paratropomyosin on a PVDF membrane, biotinylated paratropomyosin bound to many proteolytic fragments of β-connectin in all solutions (Fig. 1-2). We have previously shown16 that a large amount of paratropomyosin bound to β-connectin by measuring the turbidity of a paratropomyosin and β-connectin mixture. In this study biotinylated paratropomyosin bound to many β-connectin fragments on the membrane. Thus, biotinylated paratropomyosin may be able to bind to β-connectin in domains other than the A-I junction region. T11 monoclonal antibody, an antigenic probe of the A-I junction region, was a useful tool to detect the region specifically.12 We used the antibody to detect the β-connectin fragment that corresponded to the A-I junction region (Fig. 1-3), assuming that it binds on the membrane specifically to amino acids that reside at the A-I junction (we have observed that T11 antibody binds specifically to a 400-kDa fragment including the A-I junction region on the membrane). From the results of detection by both biotinylated paratropomyosin and T11 antibody, it was considered that the bands of 150 kDa, 100 kDa, 70 kDa, 50 kDa, and 43 kDa contained β-connectin...
fragments corresponding to the A-I junction region, and also that paratropomyosin bound to those fragments.

\( \beta \)-Connectin has a unique structure as described above, so many fragments with the same mass produced by V8 protease digestion were not able to be separated by SDS-PAGE. One band detected by both biotinylated paratropomyosin and T11 antibody on PVDF membrane possibly contains some different \( \beta \)-connectin fragments. 2D-PAGE is based on two independent properties of poly peptides, isoelectric point and molecular size, and is one of the best techniques to separate protein components.\(^{16}\) We adopted the 2D-PAGE method in order to analyze fragments that constituted the bands detected simultaneously by both probes. Many protein spots were visible by 2D-PAGE and it should be noticed that many fragments with the same mass were separated (Fig. 2-1). The two dimensionally separated fragments were electro transfers to on PVDF membranes and detected by biotinylated paratropomyosin and T11 antibody. As a result, protein spots of 150 kDa, 100 kDa, 70 kDa, and 43 kDa were simultaneously detected and could be the fragments that contained the paratropomyosin binding site on \( \beta \)-connectin at the A-I junction region (Figs. 2-2 and 2-3, arrows). Therefore, we proposed that paratropomyosin bound to some part on the 43-kDa fragment at the A-I junction region in living muscle and in muscle immediately postmortem. The other fragments above 70 kDa that react with both biotinylated paratropomyosin and T11 antibody may include the 43-kDa fragment in their structures.

Other than a small region near the N\(_2\) line,\(^{17}\) amino acid sequences of only the N- and C-termini of chicken connectin have been reported,\(^{18,19}\) and the sequence around the A-I junction region has not been analyzed. So the N-terminal sequences of C1, C2, and C4 fragments were analyzed (Fig. 4) and compared with the sequence of human cardiac titin/connectin deduced from complete cDNA.\(^{20}\) The homology search found that the N-terminal sequence of C4 fragment was similar to cardiac titin/connectin at the sequence of 7556-7565AA and was located in the I51 domain of the structure proposed by Labeit and Kolmerer.\(^{21}\) V8 protease specifically cleaves the carboxy-terminal side of aspartic and glutamic acid residues of the target protein.\(^{19}\) Indeed the amino-terminal side of 7556-7565AA was a glutamic acid residue, so it seems reasonable to locate the C4 fragment in a stretch of the domain structure from the I51 domain. On the other hand, the N-terminal sequences of C1 and C2 fragments were not similar to the A-I junction region of cardiac titin, and the reason is not clear. The N-terminal sequences of the other fragments above 70 kDa detected by both probes might give additional information on the paratropomyosin binding site on connectin, but they were not analyzed in this study.

We have previously suggested that, if the primary structure of chicken connectin was similar to that of human cardiac titin/connectin, paratropomyosin interacted with purified \( \beta \)-connectin at 149 to 154 domains, which consisted of a complex domain architecture with a stretch of fibronectin-3-type domains at the A-I junction region.\(^ {5} \) The fibronectin-3-type domain is one of the domains of fibronectin contributing to cell adhesion and binding specifically to a number of biologically important macromolecules.\(^ {22}\) This characteristic of the fibronectin-3-type domain might localize paratropomyosin at the A-I junction region where it would correspond to the gap in myosin cross bridges near the end of the thick filaments.\(^ {23}\) The primary structure of chicken connectin around the region needs to be analyzed in order to specify the paratropomyosin binding site.

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Paratropomyosin Binding Fragments from Chicken $\beta$-Connectin


