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CLONING AND EXPRESSION OF DNA FRAGMENT ENCODING PARATROPOMYOSIN BINDING CONNECTIN/TITIN DOMAINS AT THE A-I JUNCTION OF A SARCOMERE

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Introduction

A myofibrillar protein, paratropomosin weakens the rigor linkages between actin and myosin, and contributes to meat tenderization. Paratropomyosin was found at the A-I junction of sarcomeres in living muscle and in muscle immediately postmortem, and translocated from its original position to thin filaments by an increase of calcium ion concentration to 0.1 mM during postmortem storage of muscles (Hattori and Takahashi, 1988). We have shown that in chicken breast muscle paratropomyosin bound to both β-connectin/titin 2 and the 43-kDa fragment, the proteolytic product of β-connectin/titin 2, in examining binding of paratropomyosin at the A-I junction region, and determined the N-terminal sequence of the 43-kDa fragment to identify more precise paratropomyosin binding site in the intact molecule (Fei, *et al*., 1999; Yamanoue et al., 2003). The sequence is similar to a part of I51 domain of human cardiac connectin/titin domain structure presented by Labeit and Kolmerer (1995), which is located at the A-I junction region of a sarcomere. So, it is important to clarify whether the domains constituting the 43-kDa fragment are also located at the A-I junction of chicken sarcomere or not.

In the present study, we cloned DNA fragment encoding the 43-kDa fragment and produced recombinant domains constituting the fragment in *E. coli*, and then raising the antibody against a recombinant domain for indirect immunofluorescence microscopy.

Objectives

The objective of this study was to clarify the location of the 43-kDa fragment in chicken sarcomere in order to explain the details of paratropomyosin localization at the A-I junction.

Methodology

Cloning and sequencing of connectin/titin DNA fragment encoding the 43 kDa fragment. Degenerate PCR was performed to amplify connectin/titin DNA fragment

encoding the 43-kDa fragment using chicken cDNA library as a template. Oligonucleotide primers for PCR were synthesized and amplification conditions were 30 cycles of the reaction of 1 min at 94°C, 1 min at 50°C, and 1 min at 70°C after the initial denaturation for 5 min at 94°C, followed by a final 10 min incubation at 72°C. 2nd (nested) PCR product was ligated to pGEM-T-vector (Promega), and the vector was introduced into *E. coli* DH5α strain. Plasmid DNA was purified from selected colonies using the alkaline sodium dodecyl sulfate extraction procedure and the sequence of the plasmid DNA was analyzed by ABI 310 Genetic Analyser (Applied Biosystems).

Expression and purification of recombinantconnectin/tiitn domains. For construction of expression vectors, DNA fragments encoding connectin/titin domains were amplified by PCR using a set of specific primers based on cDNA of 43-kDa fragment and cloned into the *Nde* I - *Bam*H I site of the expression vector, pET- 22b (Novagen). After the expression vectors were introduced into the *E. coli* BL21 (DE3) strain, production of the recombinant proteins were induced by the addition of IPTG. Transformed cells were harvested and sonicated. The cell lysates were centrifuged and the supernatants were applied on a Sephadex G-75 gel filtration. Fractions containing recombinant protein were collected and added solid ammonium sulfate to 1.4 M, then applied on a Butyl-TOYOPEARL 650M or an Ether-TOYOPEARL 650M column equilibrated with 1.4 M ammonium sulfate. Purified recombinant proteins were passed through the column or eluted with a linear gradient of 1.4 to 0 M ammonium sulfate, appearing as single band on SDS-PAGE (Laemmli, 1970) gels.

Indirect immunofluorescence microscopy. Antiserum against purified recombinant domain was raised in a rabbit. A drop of myofibrillar suspension was mounted on a slide and covered with a cover glass. Myofibrils were treated with diluted antiserum against recombinant domain, and then washed thoroughly with a solution containing 75 m NaCl and 10 m sodium phosphate buffer, pH 7.2. The fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (IgG) were reacted with the myofibrils and free IgG were washed out thoroughly. The specimen was observed under a fluorescence microscope and photographed.

Results & Discussion

The sketch shown in Fig. 1 describes the location of the 43-kDa fragment based on human cardiac titin/connectin domain structure. In the agarose gel electrophoresis patterns of the first and the second (nested) PCR products, the bands of about 400 bp, 900 bp and 700 bp corresponding to A, B, and C regions appeared respectively by the second PCR (Fig. 1). After TA cloning of the PCR products and transformation of E.coli, the sequences of plasmid DNAs were analyzed. As the result, nucleotide sequence of 1849 bp from A to C region was determined and DNA fragment of 1158 bp seemed to encode the 43-kDa fragment (data not shown). When the sequence was compared with that of human cardiac connectin/titin, 79.6% similarity was shown. The deduced amino acid sequence of the 43-kDa fragment showed 87.6% similarity to the correspondence part of human cardiac connectin/titin sequence. The 43-kDa fragment was composed of four fibronectin type-3 domains and one immunoglobulin domain as similar as the domain structure of human cardiac connectin/titin.

DNA fragments encoding each domain of 43-kDa fragment were expressed in *E. coli* and recombinant domains were purified from cell lysates by the method described in ***Methodology***. Figure 2 shows SDS-PAGE pattern of a chicken recombinant domain, CK-I53 domain, based on human cardiac connectin/titin domain structure. The recombinant domain appeared in *E. coli* after addition of IPTG and was purified by hydrophobic interaction chromatography. The antibody against recombinant CK-I53 domain was raised in a rabbit for indirect immunofluorescence microscopy. When the anti-serum was reacted with chicken myofibrils, the fluorescence was recognized at the A-I junction as a result detected by the FITC labeled second antibody (Fig. 3), indicating that CK-I53 domain is located at the junction of a sarcomere.

Conclusions

Chicken DNA fragment encoding the 43-kDa fragment from β-connectin/titin 2 was cloned and expressed in *E. coli*. Antiserum against a recombinant domain constituting the 43-kDa fragment reacted at the A-I junction of a sarcomere. Thus, it was confirmed that the 43-kDa fragment is located at the junction where paratropomyosin is localized in living muscle and in muscle immediately postmortem.

References

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Tables and Figures

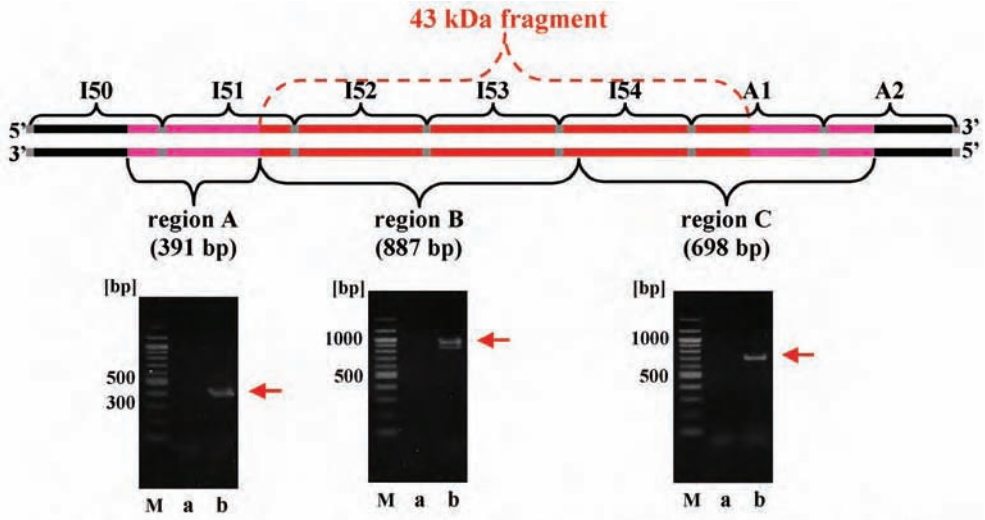

Fig. 1. PCR amplification of DNA fragments encoding chicken connectin/titin 43-kDa fragment.
Lane M, marker; a, 1st PCR product; b, 2nd PCR product.

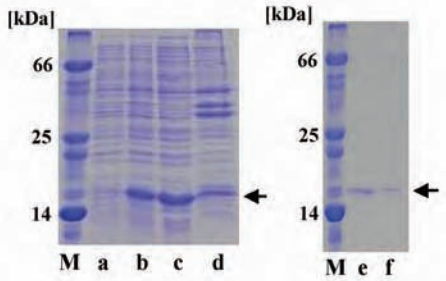

Fig. 2. SDS-PAGE patterns of expression and purification of recombinant CK-I53 domain.
Lane M, marker; a, without IPTG; b, with IPTG; c, supernatant from cell lysate; d, precipitate from cell lysate; e, Sephadex G-75 fraction; f, Butyl-TOYOPEARL 650M fraction. Arrows indicate recombinant CK-I53 domain.

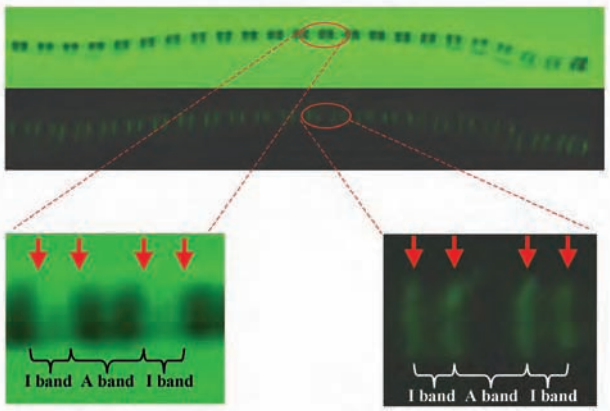

Fig. 3 Localization of connectin/titin I53 domain in chicken breast myofibrils.
Arrows indicate the A-I junction.