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The N-Terminal Z Repeat 5 of Connectin/Titin Binds to the C-Terminal Region of α-Actinin

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Connectin/titin, a 3000 kDa protein, links the Z line to the myosin filament in striated muscle sarcomeres. Using the yeast two-hybrid system, the present work shows that the N-terminal Z repeat 5 region (amino acids 447-472) of connectin binds to the C-terminal region (amino acids 825-897) of α-actinin, the main constituent of the Z line.

The extremely long, elastic connectin/titin molecule spans from the Z line to the M line region of striated muscle sarcomeres (1,2). The complete cDNA (82 kb) sequence of human cardiac connectin was reported by Labeit and Kolmerer (3). Approximately 800 amino acid residues of the N-terminal region of chicken skeletal muscle connectin are involved in the binding to the Z line (4).

It is well known that the main component of the Z line is α-actinin (5-8). We have recently reported that the N-terminal 63 kDa fragment of connectin (amino acids, 3-580) binds to the C-terminal half region of α-actinin (amino acids, 446-897) (9). The present study further reveals that the Z repeat 5 region (amino acids, 447-472) of connectin binds to the C-terminal region (amino acids, 825-897) of α-actinin.

MATERIALS AND METHODS

Detection of the α-actinin binding domain of connectin. The cDNA fragments (CN1-332, CN333-580, CN400-472, CN447-472 and CN473-580 as shown in Fig. 1) were generated by polymerase chain reaction (PCR) using specific primers with additional sequences (*Bam*HI for 5′ primers, *Pst*I for 3′ primers) from the cDNA clone CnH14 (4) encoding the N-terminal region of chicken breast muscle connectin. The cDNA fragments were inserted into the *Bam*HI/*Pst*I site of pGBT9 vector (MATCHMAKER Two-Hybrid System : CLONTECH), designated as pGBT/CNs. pGBT/CN333-459 and pGBT/CN333-472 were generated by deletion of pGBT/CN333-580. These plasmids were cotransformed with pGAD424 vector (CLONTECH) containing the cDNA fragment coding the C-terminal half region of α-actinin (AN446-897, as shown in Fig. 2) into *Saccharomyces cerevisiae* SFY526 for protein expression. The transformants grown on -(Leu, Trp) plates were replicated onto nitrocellurose membrane and the β-galactosidase activity was examined by a filter assay according to the manufacturer's instructions.

Detection of the connectin binding domain of α-actinin. pGAD/AN343-897 and pGAD/AN446-897 were cloned by the yeast two-hybrid system screening with CN63K in our previous work, called clone 1 and clone 2, respectively (9). The cDNA fragments (AN760-795, AN760-824, AN789-824 and AN825-897, as seen in Fig. 2) were generated by PCR from pGAD/AN446-897 using specific primers with additional sequences. AN656-897 was prepared by *Bam*HI digestion of pGAD/AN343-897. The cDNA fragments were inserted into the *Bam*HI/*Pst*I site of pGAD424 vector, designated as pGAD/ANs. pGAD/AN343-655 and pGAD/AN656-876 were generated by deletion of pGAD/AN343-897 and pGAD/AN656-897, respectively. These plasmids were cotransformed with pGBT/CN447-472 (Fig. 1) into SFY526 and the β-galactosidase activity was examined as described above.

Sequencing of chicken skeletal muscle connectin CnF6 clone. Preparation of the chicken breast muscle cDNA library was described previously (4). Using CnD9 clone (4) as a probe, CnF6 was obtained. One strand (2.9 kb) was initially sequenced. Then, the 645 bp *Pvu*II fragment including 258 bp in addition to the original sequence (DDBJ/EMBL/GenBank accession number D83390) was subcloned, and both strands were sequenced with ALFexpress DNA sequencer (Pharmacia). The sequence has been deposited in the DDBJ/EMBL/GenBank database with the accession number AB001536.

RESULTS

α-Actinin Binding Domain of Connectin

The yeast two-hybrid system revealed that the 26 amino acids, 447-472, of the N-terminal region of connectin binds to α-actinin, as shown in Fig. 1. These 26 amino acids form an N-terminal half of the Z repeat 5 (Zr5) described by Gautel *et al.* (10). Thus only a limited region of the CN63K (amino acids, 3-580) previously reported (9), is involved in the binding to α-actinin.

Connectin Binding Domain of α-Actinin

Figure 2 shows that the C-terminal 73 amino acids (825-897) of α-actinin binds to connectin. It is to be noted that the α-actinin fragment (AN656-876) lacking

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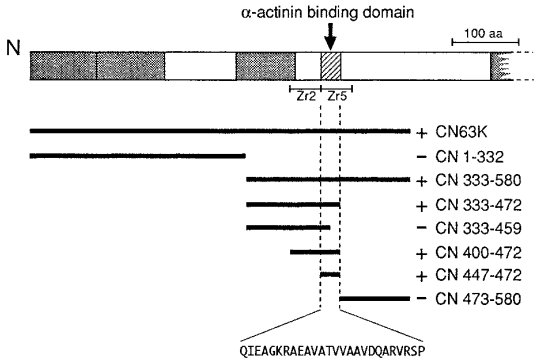

FIG. 1. α-Actinin binding domain of the N-terminal region of chicken skeletal muscle connectin. Shaded block, immunoglobulin-like motif II. CN63K is as reported (9). The thick bars indicate the portions of the clones examined for binding activity to α-actinin. +, positive; −, negative. The amino acid sequence of the binding domain is shown below.

in extreme C-terminal 21 amino acids did not bind to connectin. This was also the case with the α-actinin fragment, AN760-824. We earlier described the binding of the C-terminal half (446-897) of α-actinin to connectin (9).

An Isoform of Chicken Breast Muscle Connectin Contains Four Z Repeats

During analysis of the primary structure of chicken breast muscle connectin (4), we obtained a cDNA clone, CnF6, which has 258 bp more than the original sequence (D83390, for CnF6 AB001536). By analyzing the sequence of CnF6, it was found that the additional portion encodes two Z repeats, thus there are four Z repeats in CnF6 (Fig. 3A). The C-terminal repeat, which is able to bind to α-actinin, showed the highest homology with human cardiac connectin Z repeat 5 (Zr5) especially in the 26 amino acids responsible for the binding (77% identity, Fig. 3C). Therefore, the C-terminal motif of chicken Z repeats corresponds to human Zr5. The N-terminal motif of chicken Z repeats shows the highest homology with human Zr2 (Fig. 3C). The additional two repeats found in CnF6, designated ZrX and ZrY (Fig. 3A), are different from Zr1 and Zr5. However, which of the other human Z repeats corresponds to ZrX or ZrY has not yet been determined (Fig. 3C).

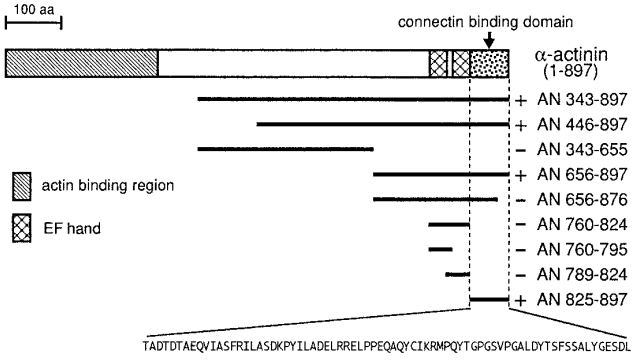

FIG. 2. Connectin binding domain of α-actinin. α-Actinin sequence is taken from ref. (8). The amino acid sequence of the binding domain is shown below.

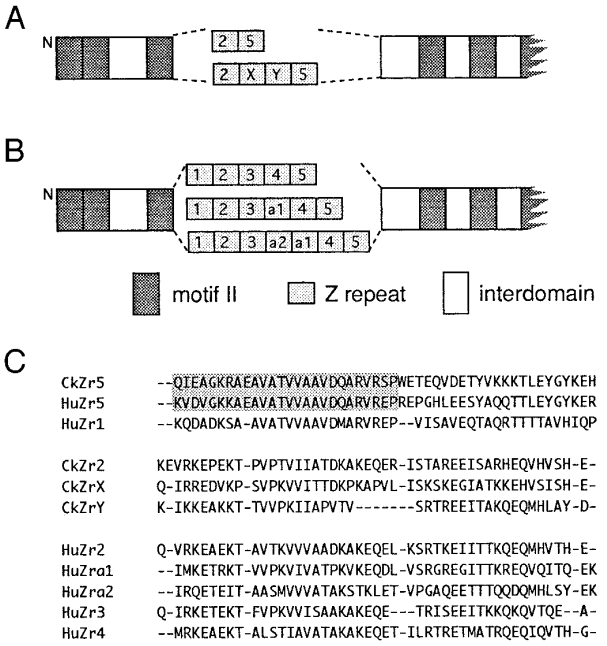

FIG. 3. Z repeat region of the N-terminal portion of connectin. (A) Chicken skeletal muscle connectin. (B) Human cardiac connectin. Adapted from Ref. (10), Company of Biologists, Ltd. (C) Multiple alignments of the amino acid sequences of Z repeats. Ck, chicken skeletal muscle connectin. Hu, human cardiac connectin (10). The shaded sequences show the α-actinin binding region.

DISCUSSION

Identification of the α-actinin binding domain (Zr5) of connectin requires a careful sequence examination. As described by Gautel *et al.* (10), there are several isoforms in the 45 amino acid residue repeat (Z repeat) region of human cardiac connectin (Fig. 3B). In chicken skeletal muscle connectin, there are two Z repeat isoforms: Zr2 - Zr5 and Zr2 - ZrX - ZrY - Zr5 (Fig. 3A). We cannot assign the ZrX and ZrY from the sequence analogy, whereas Zr2 and Zr5 of chicken skeletal muscle connectin have 57 and 63 % sequence identity with Zr2 and Zr5 of human cardiac connectin, respectively (10). It should be emphasized that the two isoforms of chicken connectin and all the human cardiac connectin isoforms (10) contain the α-actinin binding region (Zr5). Turnacioglu *et al.* (11) described that the KIKK sequence in the 46 kDa portion of zeugmatin (a proteolytic fragment of connectin) might be involved in the α-actinin binding because this sequence of ICAM-1

binds to α-actinin (12). The KIKK sequence is present in the ZrY but not in Zr5. Since the 46 kDa portion of zeugmatin contains Zr2-ZrX-ZrY-Zr5, the Zr5 may be responsible for its α-actinin binding. Hence, the KIKK sequence is not the α-actinin binding domain of connectin.

The α-actinin binding domain of connectin is rich in hydrophobic residues (15 amino acids out of 26). These residues may be involved in the binding to the hydrophobic residues of α-actinin (34 amino acids out of 73). It is to be noted that the actin binding region, spectrin-like repeats and EF hand region of α-actinin (8) are not related to the connectin binding (Fig. 2). Rather, the C-most-terminal region is involved.

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