Binding of the N-terminal 63 kDa portion of connectin/titin to α-actinin as revealed by the yeast two-hybrid system

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Abstract Connectin/titin is a 3000 kDa protein which links the myosin filament to the Z-line in vertebrate striated muscle sarcomeres. To search for the Z-line proteins to which connectin binds, the yeast two-hybrid system was applied using cDNA coding the N-terminal 63 kDa fragment of connectin. Two clones coding the C-terminal half region of α-actinin (amino acids, 343–897 and 446–897) were obtained. Enzyme-linked immunosorbent assay clearly demonstrated the interactions of α-actinin and the N-terminal 63 kDa fragment of connectin in vitro. Thus it is concluded that the N-terminal 63 kDa portion of connectin binds to α-actinin in the Z-line of myofibrillar sarcomeres.

Key words: Connectin; Titin; α-Actinin; Muscle structure; Z-line; Two-hybrid system; Enzyme-linked immunosorbent assay

1. Introduction

Connectin/titin [1,2] is the largest peptide, ~3000 kDa, hitherto known [3]. Connectin positions the myosin filament at the center of a sarcomere in vertebrate striated muscle myofibrils by linking the myosin filament to the Z-line [4]. The connectin molecule is a long, thin, elastic filament and is responsible for passive tension generation of stretched sarcomeres [5].

We have recently shown that some 800 amino acid residues of the N-terminal region of connectin are involved in the binding to the Z-line [6]. The present work describes the binding of the N-terminal 63 kDa peptide of connectin to α-actinin as revealed by the yeast two-hybrid system and enzyme-linked immunosorbent assay (ELISA). α-Actinin [7,8] is localized in the Z-line of striated muscle sarcomeres [9]. The amino acid sequence was determined by Arimura et al. [10].

2. Materials and methods

2.1. Screening by the yeast two-hybrid system

The cDNA fragment coding the N-terminal region of chicken breast muscle connectin (CN63K, amino acids 3–580, 63 kDa) [6] was constructed in pGBT9 vector (MATCHMAKER™ Two-Hybrid System: CLONTECH) for protein expression, designated as pCN63K. The library was constructed by inserting the chicken breast muscle cDNA fragments into the *Eco*RI site of pGAD424 vector (CLONTECH). The preparation of the cDNA fragments was previously described [6], using oligo(dT) as well as random hexamers as primers.

For screening, CN63K was co-expressed in *Saccharomyces cerevisiae* HF7c with the chicken breast muscle cDNA library as described by the manufacturer. The plasmids were rescued from colonies grown on -(His, Leu, Trp) plates, sequenced, and retransformed with pCN63K into *S. cerevisiae* SFY526 to confirm positive binding.

2.2. Expression and purification of CN63K

The fragment coding CN63K was ligated inframe to pRSET (Invitrogen) and the construct was transformed to the *E. coli* strain BL21(DE3)pLysS. The 6×His-tagged protein was induced by the addition of isopropyl β-D-thiogalactopyranoside and purified through Ni-NTA-agarose (QIAGEN) column.

2.3. ELISA

Two sets of experiments were carried out. The 63 kDa fragment or egg albumin (Sigma) was coated on 96-well multiplates for 2 h at room temperature. After the plates were blocked with 1% bovine serum albumin (Sigma) in Tris-buffered saline (TBS), various amounts of α-actinin purified from chicken skeletal muscle [11] were added to each well and incubated at room temperature. In the second series, the well plates were coated with α-actinin or egg albumin and then treated with the 63 kDa fragment as described above.

After washing the plates with TBS containing 0.05% Tween 20, anti-α-actinin (TRI, the first series) or PcCOM1 antibodies [6] (the second series) was added to the wells, followed by treatment with peroxidase-conjugated anti-rabbit immunoglobulins (Bio-Rad). The interactions were visualized with 0.7 mg/ml orthophenylenediamine in 100 mM citrate buffer, pH 4.5.

3. Results

3.1. Binding of the N-terminal 63 kDa fragment of connectin to α-actinin revealed by the two-hybrid system

To identify any muscle protein bound to the N-terminal 63 kDa fragment of connectin (Fig. 1), a chicken breast muscle cDNA library was screened by the yeast two-hybrid system.

Screening of approximately 2×10^5 yeast HF7c transformants resulted in 25 clones grown on -(His, Leu, Trp) plates. Fifteen of these clones exhibited positive β-galactosidase activities. It was shown that 13 had the same 1.9 kb insert (clone 1) and 2 had the identical 1.6 kb insert (clone 2). The combination of pGBT9 (DNA-binding domain vector) and clone 1 or clone 2 was β-galactosidase activity-negative, as well as the combination of pCN63K and pGAD424 (activation domain vector).

Sequencing revealed that both inserts were cDNAs coding the C-terminal half region of α-actinin [10], as shown in Fig. 2. The clone 1 insert encoded amino acids 343–897 of α-actinin (1–897) and the clone 2 insert encoded amino acids 446–897 of α-actinin. Interestingly, both α-actinin cDNAs cloned lacked the actin-binding domain of α-actinin [10]. It is concluded that expressed 63 kDa fragment binds to the C-terminal half region of α-actinin in yeast cells.

3.2. Binding of α-actinin to the N-terminal 63 kDa fragment of connectin detected by ELISA

In order to confirm in vitro interactions of the N-terminal 63 kDa fragment of connectin and α-actinin, ELISA was

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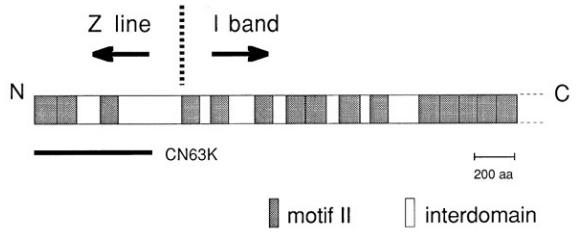

Fig. 1. The N-terminal region of chicken breast muscle connectin involved in the binding to the Z-line. The thick line indicates the portion of CN63K (cf., [6]).

adopted using antibodies to the 63 kDa fragment or α-actinin. As shown in Fig. 3A, added chicken breast muscle α-actinin bound to the 63 kDa fragment but not to egg albumin; conversely, added 63 kDa fragment bound to α-actinin but not to egg albumin (Fig. 3B). This demonstrated that the N-terminal portion of connectin directly interacts with α-actinin in vitro.

4. Discussion

In a previous report [6] we showed by immunoelectron microscopy that a portion of some 800 amino acids of the N-terminal region of connectin binds to the Z-line in chicken breast muscle sarcomeres. This finding suggests that the N-terminal portion of connectin binds to some protein(s) constituting the Z-line. The present work clearly shows that the 63 kDa fragment of connectin binds to α-actinin.

α-Actinin is well known to be the main component of the Z-line of vertebrate striated muscle [9,12]. Therefore, it is not unexpected that the N-terminal portion of connectin binds to α-actinin in the Z-line. The α-actinin dimer is thought to cross-connect actin filaments in the Z-line [13]. The N-terminal portion of connectin thus appears to bind to the cross-connecting α-actinin dimer.

Here, we should mention rather contradictory reports on the interactions of connectin with α-actinin. Nave et al. [14] showed that α-actinin did not bind to connectin on nitrocellulose blots separated by SDS gel electrophoresis, while Takahashi and associates [15] stated that α-actinin did bind to connectin on nitrocellulose blots. This discrepancy has not yet been explained. However, the present results, both in vitro and in vivo, clearly show that the N-terminal region of connectin binds to the C-terminal half region of α-actinin. Furthermore, Sanger and associates have recently reported that zeugmatin is a Z-line portion of connectin and the 46 kDa fusion protein binds to α-actinin both in vitro and in vivo [16].

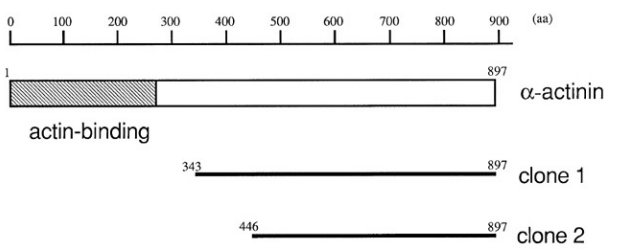

Fig. 2. The region of α-actinin bound to the N-terminal 63 kDa fragment of connectin.

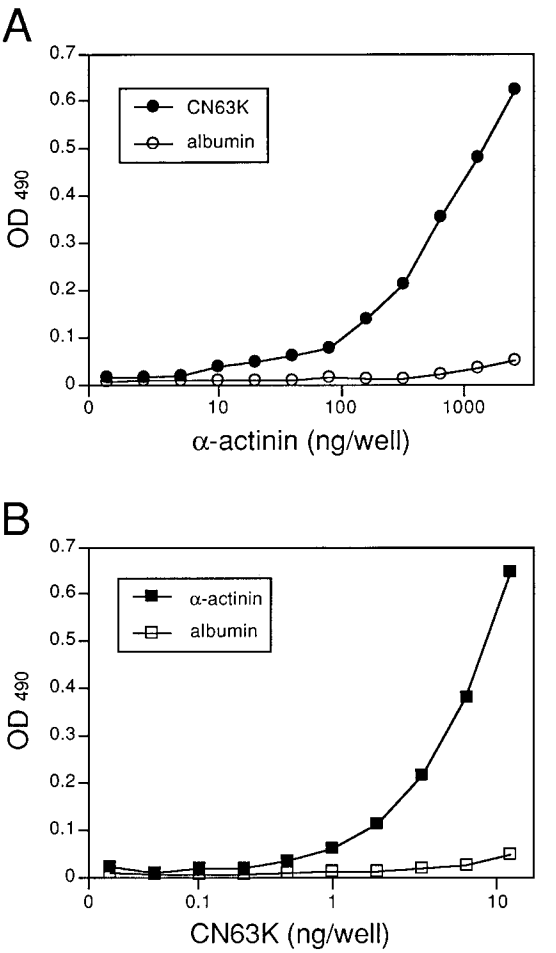

Fig. 3. Interactions of the N-terminal 63 kDa fragment of connectin and α-actinin as revealed by ELISA. 96-Well multiplates were coated with 50 μl of 10 μg/ml CN63K (●) or egg albumin (○) (A) and α-actinin (■) or egg albumin (□) (B). Various amounts of α-actinin (A) or CN63K (B) were added to each well.

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