Archvillin C-Terminus-Binding Proteins in Human Skeletal Muscle

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Archivillin, a muscle-specific isoform of supervillin, is a component of the costameric cytoskeleton of muscle cells. The purpose of this study was to determine which protein in the skeletal muscle collaborates with archvillin C-terminus. For this purpose, a yeast two-hybrid screening of human skeletal muscle cDNA library was performed using the C-terminal region of archvillin as bait. This study shows that seven human skeletal muscle proteins, namely, nebulin, xeplin, archvillin, GAPDH, TOX4, PITRM1, and YME1L1 interact with archvillin C-terminus. Especially, xeplin is a newly discovered protein interacts with archvillin C-terminus. These results indicate that archvillin C-terminus acts as a bridge between nebulin and xeplin at costameres. Archvillin C-terminal region interacts with nebulin C-terminal region at Z-discs and interacts with xeplin at the vicinity of sarcolemma. I propose that these interactions may contribute to formation of costameric structure and muscle contraction.

Key Words: Archvillin, Yeast two-hydrid, Skeletal muscle, Costamere

INTRODUCTION

Skeletal muscle is an intricate, efficient, and precise machine specializes for rapid force production and has a highly organized protein network system, which maintains the structure and function of muscle cells (Williams et al., 1989; Clark et al., 2002; Kaisto, 2003). The skeletal muscles consist of myofibers made of bundle of multinucleated cells and a myofiber is composed of myofibrils, sarcolemma, and costameres (Gregorio et al., 2000). Costameres, specialized linkages between the subsarcolemmal cytoskeleton and the extracellular matrix, are comprised of various costameric proteins. Costameric proteins play both a mechanical and a signalling role, transmitting force from the contractile apparatus to the extracellular matrix in order to stabilize skeletal muscle fibers during contraction and relaxation (Anastasi et al., 2008).

Archvillin, a 250 kDa muscle-specific isoform of super-

villin, was found in myogenic cell lines and in cardiac and skeletal muscles. Archvillin is derived from the supervillin genomic locus (SVIL) by differential splicing of five conserved exons. The two muscle-specific inserts and five nuclear targeting sequences are encoded within the N-terminal region of archvillin. And three potential actin binding sequences and villin/gelsolin homology domain are encoded in the archvillin C-terminus (Oh et al., 2003). Archvillin, also, was predominantly localized at the cell peripheries in dissected skeletal muscle. In optical and oblique cross sections obtained by epifluorescence and confocal microscopy, archvillin appeared as 'arches' along the sarcolemma. These structures were revealed as alternating circumferential thick and thin bands in en face confocal with occasional longitudinal strand (Oh et al., 2003). This appearance is characteristic of the myofibril-to-sarcolemma attachment sites called costameres (Craig and Pardo, 1983; Pardo et al., 1983; Williams and Bloch, 1999).

Recently study reports that the C-terminal region of archvillin interacts with the nebulin C-terminus at Z-disc in the skeletal muscle (Lee et al., 2004). In addition, archvillin mRNA transcript was specifically expressed in skeletal muscle and heart of human and murine.

On the basis of these data, I found that archvillin is

Table 1. PCR primers used in plasmid constructions

Oligo	Orient*	Gene	Sequences	Restriction site
LYM 13	R	hArchvillin	5'-ACGCGTCGACTCAGAACAGGCCTTTTGC-3'	*Sal* I
LYM 26	F	hArchvillin	5'-CCTGGATCCTGGAGAATGCA-3'	*BamH* I
LYM 27	R	hArchvillin	5'-TGCATTCTCCAGGATCCAGG-3'	*BamH* I
LYM 53	F	hArchvillin	5'-CCGGAATTCATGTCTTCCAACTCCA-3'	*EcoR* I
XIRP2-01	F	hXeplin	5'-GTGATTGTGCAGAGTGCTGAAAAGG-3'	·
XIRP2-02	R	hXeplin	5'-CTCTGTCAGTCTGTTTCTTCAGAGCC-3'	·
Neb-01	F	hNebulin	5'-GGTGAGGGAGACCCAACGGC-3'	·
Neb-02	R	hNebulin	5'-AGTCTCCTGATCTTGGTCATTCCGT-3'	·

*F, Forward PCR primer; R, Reverse PCR primer.

situated at costamere in skeletal muscle and anchors in the Z-disc via the nebulin C-terminus. However, other costameric proteins that interact with archvillin have not been identified and the molecular function of archvillin has not yet revealed in skeletal muscle, either. To identify novel binding partners and molecular functions of archvillin, I performed the yeast two-hybrid system and searched for potential targets of archvillin C-terminus in human skeletal muscle library by using C-terminal region of archvillin as bait.

MATERIALS AND METHODS

Bait plasmid preparation

The LYM53, LYM27, LYM26 and LYM13 primers (Table 1) designed from the human archvillin cDNA sequence (accession no. AF109135) to generate human archvillin C-terminal region (hAvC). hAvC was amplified from human skeletal muscle cDNA library (HL4010AB; Clontech) as a template in PCR reactions with Advantage-HF 2 Taq DNA polymerase Kit (Clontech). PCR products were separated on 1.5% agarose gel and purified using QIAEX II gel elution kit (Qiagen). The gel-purified band was cloned into the pGEM-T vector (Promega) and sequenced. The ligated plasmid was digested with *EcoR* I and *Sal* I, and the DNA fragment was subcloned into the pGBKT7 vector with a selectable *TRP1* marker for expression of fusions with the GAL4 DNA binding domain (BD) (Clontech). The bait construct, pGBKT7-hAvC, was also sequenced to ensure the maintenance of the proper reading frame.

Library screening

The yeast two-hybrid screening was carried out using a GAL4-based yeast two-hybrid system (MATCHMAKER Gold Yeast Two-Hybrid System, Clontech) and procedures recommended by the manufacturer (Clontech). Bait constructs, pGBKT7-hAvC, were transformed into yeast strain Y2H Gold (MATa) and the transformants were plated on dropout medium lacking tryptophan (SD/-Trp). The Pretransformed Human Skeletal Muscle MATCHMAKER cDNA Library, which is a human skeletal muscle cDNA library that has been cloned into a pACT2 vector with a selectable *LEU2* marker for expression of fusions with the GAL4 activation domain (AD) and which has been pretransformed into yeast strain Y187 (MATα), was purchased from Clontech. The two transformant cultures were mated to each other and initially plated on dropout medium lacking leucine and tryptophan supplemented with X-α-gal and aureobasidin A (SD/-Leu/-Trp/X/A). In this two-hybrid system, the GAL4 BD binds to the GAL upstream activating sequence and, if the fusion proteins interact, the AD is brought into proximity with the promoters of four reporter genes (*HIS3*, *ADE2*, *MEL1*, and *AUR1-C*), thereby activating transcription and permitting growth on selection media (-His and -Ade) and the expression of α-galactosidase (*MEL1* product) and inositol phosphoryl ceramide synthase (*AUR1-C* product). Aureobasidin A, highly toxic drug, was used to lower background activity. Many clones were suppressed by toxicity of this drug but when bait and prey protein interact, GAL4-responsive *AUR1-C* expression confers strong resistance (AbA^R) to the otherwise highly

toxic drug aureobasidin A. To select for colonies that expressed interacting proteins, all the blue colonies that grew on SD/-Leu/-Trp/X/A onto higher stringency dropout medium lacking adenine, histidine, leucine, and tryptophan supplemented with X-α-gal and aureobasidin A (SD/-Ade/-His/-Leu/-Trp/X/A) were patched out using a sterile toothpick.

Yeast colony PCR

Yeast colonies were used directly as template and performed all PCRs in a volume of 20 µl containing 2X ExPrime Taq Premix (GENET BIO) and 0.5 µM of each gene specific primer pairs (xeplin, XIRP2-01 and XIRP2-02; nebulin, Neb-01 and Neb-02) (Table 1). After an initial hot start at 94℃ for 5 min, amplification was performed for 30 cycles (94℃ for 30 sec, 55.5℃ for 30 sec, and 72℃ for 30 sec), followed by 5 min of extension at 72℃. PCR products were identified on a 1.5% agarose gel with

ethidium bromide staining.

DNA sequence determination and analysis

The identified prey clones were purified by using QIAprep Spin Miniprep Kit (Qiagen) and sequenced with fluorescent dye terminators, using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit V.3.1 (Applied Biosystems) and an ABI 3730XL Capillary DNA Sequencer (Applied Biosystems) as described by the manufacturer. All clones were sequenced by primer walking at ABI 3730XL and homology searches against database sequences were performed using the BLAST algorithm.

RESULTS

A 2.5 kb of human archvillin C-terminus (hAvC) DNA fragment was amplified using Human Skeletal Muscle cDNA library (Clontech) as a template. The fragment
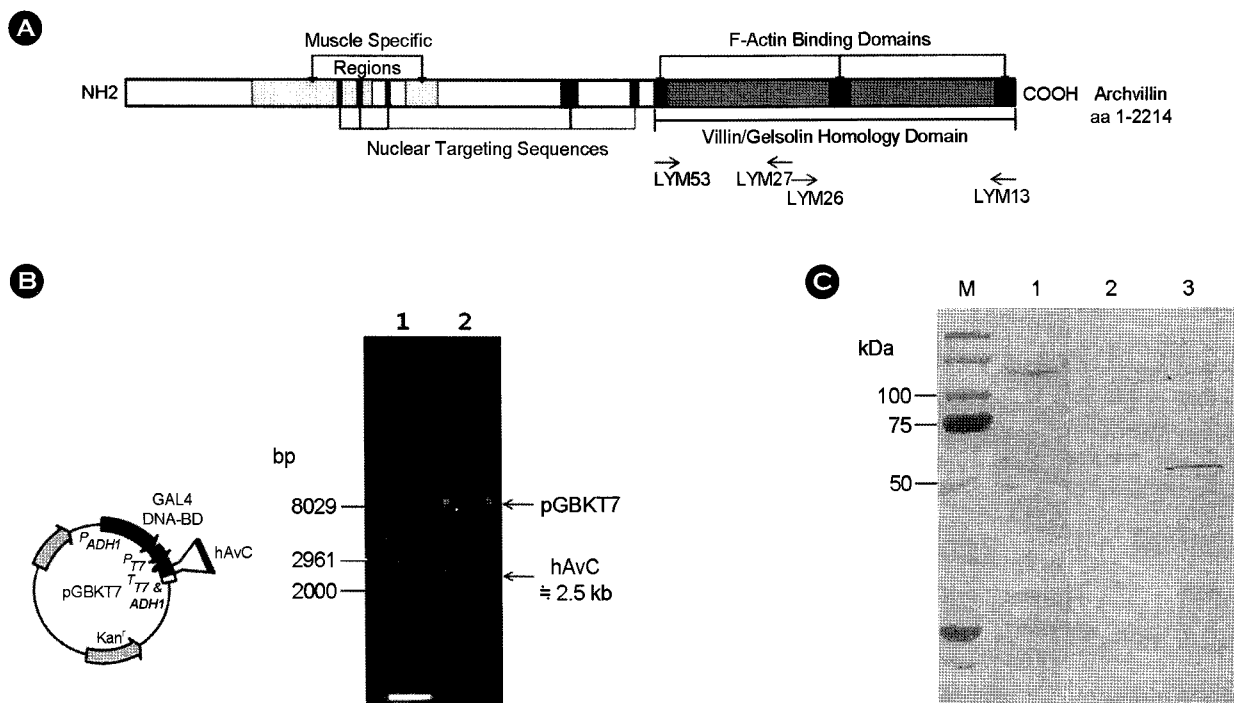

Fig. 1. Preparation of archvillin C-terminus fragment for bait plasmid construction and Expression of the GAL4 BD-hAvC fusion protein in yeast. (A) Archvillin C-terminus fragment was amplified from human skeletal muscle cDNA library using nebulin-specific primer pairs (LYM53/LYM27 and LYM26/LYM13). (B) Schematic diagram of pGBKT7 plasmid cloned human archvillin C-terminus (hAvC) (left). pGBKT7-hAvC plasmid cloned treated with Nde I and Sal I (right). (C) Yeast strain Y2H Gold was transformed with pGBKT7-hAvC. Transformants were selected on the appropriate SD medium. Protein samples were resolved by electrophoresis on a 10% polyacrylamide/SDS gel. Proteins were electroblotted from the gel to a PVDF membrane, which was probed with a anti-c-Myc monoclonal antibody, followed by an AP-conjugate of goat anti-mouse IgG. Lane 1, Y2H Gold transformed with pGBKT7-hAvC; lane 2, untransformed Y2H Gold as negative control; lane 3, Y2H Gold transformed with pGBKT7-p53 as positive control.

contains villin/gelsolin domain and F-actin binding domains (Fig. 1A). The fragment was digested with *EcoR* I and *Sal* I, and subcloned into the pGBKT7 vector (Fig. 1B). The hAvC sequence was confirmed by Blast alignment of National Center for Biotechnology Information.

To identify potential binding partners of hAvC, a human skeletal muscle cDNA library was screened using the yeast two-hybrid technique (Fig. 1). Initially, the pGBKT7-hAvC vector was transformed into yeast strain Y2H Gold. Transformation and protein-protein interactions were monitored by *ADE2*, *HIS3*, *MEL1* and *AUR1-C* activation. GAL4 BD-hAvC alone did not auto-activate the reporter genes. Western blot analysis (Fig. 1C) using anti-c-Myc monoclonal antibodies on Y2H Gold strains confirmed GAL4

BD-hAvC expression.

I used a large-scale two-hybrid mating strategy to screen for interacting partners of hAvC. Strain Y2H Gold (MATa) was transformed with pGBKT7-hAvC. This bait-containing strain was mated with strain Y187 (MATα), expressing a human skeletal muscle cDNA library. Mating generated 7 × 10⁴ diploid cells, from which 1,053 positive clones were obtained. Sequence analyses of 100 of the positive clones revealed that 36 of these 100 clones represented a portion of nebulin and 42 of these 100 clones represented a portion of xeplin. To eliminate overlapping clones containing nebulin or xeplin AD/library plasmid, I performed colony PCR for 953 remaining positive clones (Fig. 2). Through this analysis, 853 of 953 overlapping clones were eliminated.
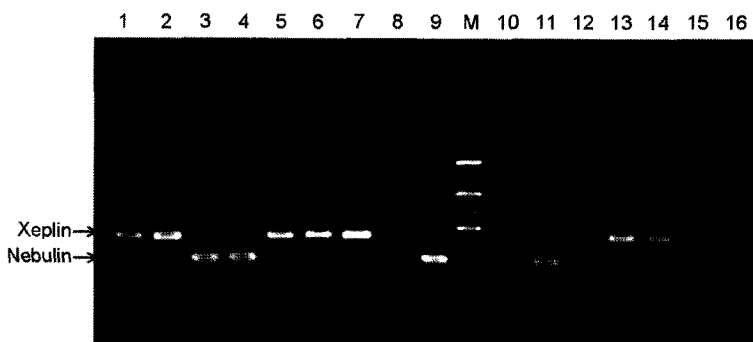
Fig. 2. Screening for xeplin- and nebulin-positive clones by colony PCR. Clone No. 371~386, were analyzed by colony PCR. Only 5 of the 16 clones were negative. Specific primers for xeplin and nebulin (xeplin, XIRP2-01/XIRP2-02; nebulin, Neb-01/Neb-02) amplify respectively 370 bp and 208 bp of each gene. M, molecular size marker.
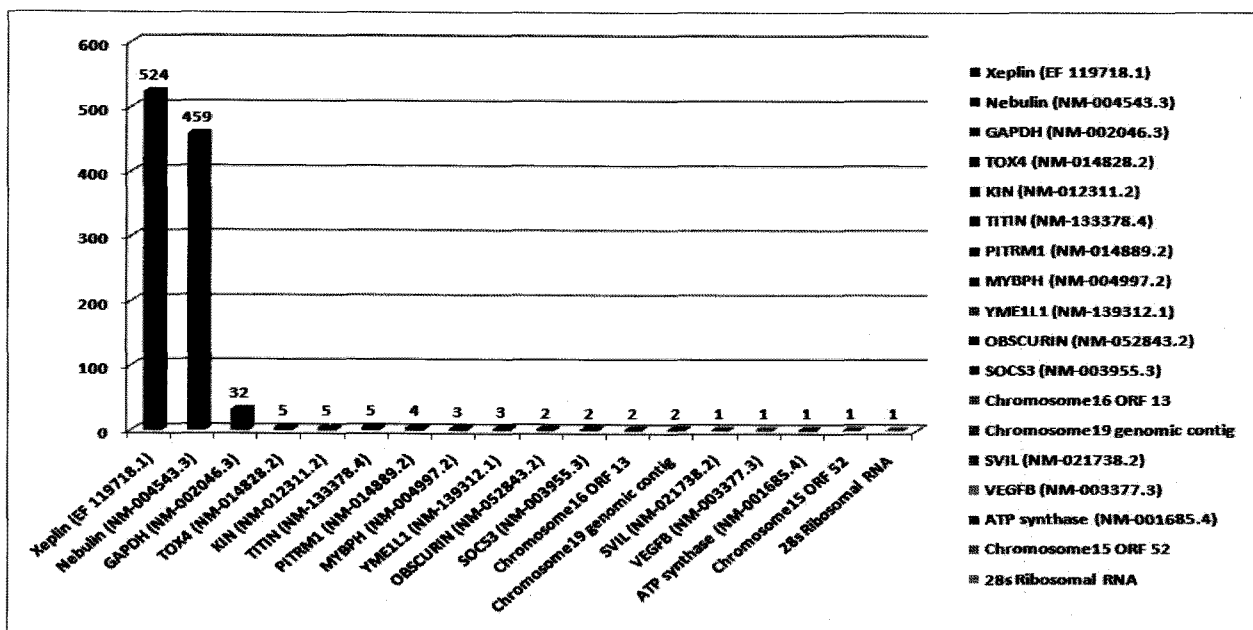
Fig. 3. Putative positive proteins interact with the C-terminal region of archvillin. Sequence analysis and colony pcr revealed that 18 kinds of protein were found to be associated with the C-terminal region of archvillin.

Table 2. Positive clones showing the interactions with archvillin C-terminus

GenBank number	Protein name	Total clones
EF119718.1	Xeplin	524
NM_004543.3	Nebulin	459
NM_002046.3	GAPDH	32
NM_014828.2	TOX4	5
NM_014889.2	PITRM1	4
NM_139312.1	YME1L1	3
AF 109135	SVIL	1

Subsequently, 100 of 953 non-overlapping clones were analyzed by rescue plasmids from yeast and sequencing. Finally, 18 candidate proteins that interact with archvillin C-terminus were identified (Fig. 3). After autoactivation test in yeast two hybrid systems, only 7 of the 18 kinds of proteins were found to be associated with the C-terminal region of archvillin (Table 2). The rest 11 kinds of protein were false positive.

DISCUSSION

Archvillin is localized at costamere and composed of many functional domains and sequences. From this fact, archvillin is expected to perform important roles by interacting with other costameric proteins at costamere. This study shows that seven human skeletal muscle proteins, namely, nebulin, xeplin, archvillin, GAPDH, TOX4, PITRM1, and YME1L1 interact with archvillin C-terminus. As expected, nebulin interacts with archvillin. The C-terminal region of nebulin interacts with C-terminal of archvillin at Z-line in skeletal muscle and the colocalization of nebulin and archvillin was identified in COS-7 cells (Lee et al., 2008). Nebulin, which consists of an acidic N-terminal domain, 35-residue module repeats, a linker domain, and a C-terminal SH3 domain, is thought to determine the length of the actin filament in skeletal muscle (Labeit and Kolmerer, 1995; Wang et al., 1996). According to the functional properties, each portion of nebulin interacts with specific partner, respectively. In particular, the ca. 50 kDa C-terminal region of nebulin extends into the Z-line lattice in skeletal muscle and contains an SH3 domain and a Ser-rich region (Bang et al., 2002).

Furthermore, recent studies suggest that the SH3 domian of nebulin may directly interacts with the spring-like PEVK domain from the I-band region of titin (Ma and Wang, 2002).

In human, xeplin, is derived from XIRP2 gene located on chromosome 2q24.3. Xeplin exists as multiple isoforms (six types of isoform) that arise through alternative splicing of XIRP2 gene which contains eleven protein-coding exons and ten intervening noncoding introns. Translated xeplin (also known as XIRP2, CMYA3, and beta-xin) mRNA was expressed in skeletal muscle, heart, brain, stomach and lung. However the function of the xeplin in human skeletal muscle and heart has been unknown.

Archvillin binds directly to F-actin, co-isolates with other costameric proteins such as dystrophin and caveolin-3 in low-density sarcolemmal membranes. In myoblasts, archvillin is concentrated at the plasma membrane with F-actin, non-muscle myosin II and vinculin (Oh et al., 2003).

The functions or features of GAPDH, TOX4, PITRM1 and YME1L1 in human skeletal muscle and heart has not been known. To investigate the molecular basis for the interaction between archvillin and these proteins, and to definite the function of these proteins, further studies are required.

These data therefore provide a logical hypothesis that archvillin C-terminus acts as a bridge between nebulin and xeplin at costameres. Archvillin C-terminal region interacts with nebulin C-terminal region at Z-discs and interacts with xeplin at the vicinity of sarcolemma. In addition, it is thought that archvillin form the inverse dimer formation, and it is expected that this formation help to avoid the overlap of binding site which is needed to interact with nebulin and xeplin.

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