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# Nebulin in Z-discs and Costameres

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Deciphering the molecular interactions of proteins forming Z-lines is pivotal for understanding the regulation of myofibril assembly, sarcomeric organization, and mechanical properties of striated muscle. The purpose of this study is to searched for potential novel ligands of the Z-line portion of nebulin. In this study interacting proteins with intra-Z-line region of nebulin were screened using a yeast two-hybrid approach. The interaction was conformed by GST pull-down assay. We identified 269 residues within villin/gelsolin homology domain of supervillin that intreacts with the serine rich region of nebulin. The specific interactions of nebulin and supervillin were confirmed *in vitro* by GST pull-down experiments. Our data suggest that supervillin attaches directly to the Z-line through its interaction with the serine rich domain of nebulin in skeletal muscles. This interaction may link myofibrillar Z-discs to the membrane-associated complexes, costameres.

**Key Words:** Nebulin, Supervillin, Z-line, Yeast-two hybrid, Costamere

## INTRODUCTION

The precise organization of Z-lines, the borders of individual sarcomeres in vertebrate striated muscle, is a remarkable example of supramolecular assembly in eukaryotic cells. Z-lines contain the barbed ends of actin thin filaments, the $NH_2$-terminal ends of titin filaments, the C-terminal ends of nebulin filaments (skeletal muscle), and nebulette (cardiac muscle), as well as a variety of other regulatory and structural proteins. In addition to being a boundary between successive sarcomeres, Z-lines are responsible for transmitting tension generated by individual sarcomeres along the length of the myofibril, allowing for efficient contractile activity[40,46]. Z-line-associated proteins also appear to be crucial for early stages of myofibril assembly since I-Z-I structures (i.e., Z-line precursors) form the earliest identifiable protein assemblies observed during muscle differentiation[8,9,14,17,29]. Thus, deciphering the molecular interactions of proteins forming Z-lines is pivotal for understanding the regulation of myofibril assembly, sarcomeric organization, and mechanical properties of striated muscle. Detailed ultrastructural and biochemical investigations of the Z-disc and its various components have yielded valuable information concerning its structural architecture. The width of the Z-line can vary from ~30 nm in fish skeletal muscle, up to 1 mm in patients with certain forms of nemaline myopathy[11,35,46,51].

The thin filaments from adjacent sarcomeres fully overlap within the Z-line[15,52] and are cross-linked by the direct interaction of actin filaments and the actin filament barbed end-capping protein, CapZ, with α-actinin[12,30]. Both the $NH_2$-terminal region of titin and the C-terminal region of nebulin are also integral components of the Z-line lattice. *In vitro* studies have identified two distinct α-actinin-binding sites within titin's $NH_2$-terminal, 80-kD, Z-disc integral segment. These binding sites (the Z-repeats and titin's $NH_2$-terminal sequences adjacent to the Ig-repeat Z4), may link together titin and α-actinin filaments both inside the Z-line and at its periphery[15,28,39,52].

Nebulin is an unusually large protein that spans the whole length of thin filaments in the sarcomeres of skeletal muscles. Like the majority of myofibrillar proteins, nebulin

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displays a tissue, species and developmental-stage specific diversity. This leads to a considerable variability in its molecular mass which ranges from 600 up to 900 kDa[18,22,48]. The size of nebulin correlates with the length of thin filaments in different skeletal muscles. This, together with the presence of highly periodical features in nebulin's primary structure led to the suggestion that nebulin acts as a molecular ruler that regulates thin filament length[19,20,44].

Supervillin recently described 205-kDa actin-binding membrane protein named supervillin because of C-terminal sequence similarities to the microvillar protein, villin[4] that named supervillin[32]. Supervillin, formerly called p205, was initially identified as a major actin binding protein in bovine neutrophil plasma membranes[31]. Supervillin binds tightly and directly to the sides of actin filaments in F-actin blot overlay assays and also cosediments and coimmunoprecipitates with endogenous actin upon fractionation of purified neutrophil plasma membranes. In adherent cells, supervillin is concentrated with F-actin at sites of cell- cell contact. In nonadherent cells, supervillin appears to be enriched in nuclear and cytoplasmic punctae, as well as being found at the plasma membrane. They reported the molecular cloning of 200 kDa human supervillin[34].

Here, we have used the yeast two-hybrid system to search for components that interact with the C-terminal (intra-Z-line) region of nebulin and thereby anchor it within the sarcomere. This approach identified a direct interaction between the serine-rich region of nebulin and the actin-binding membrane protein, supervillin. It was found, however, that the SH3 domain of nebulin did not appear to interact with supervillin. Our *in vitro* binding data provide that the serine-rich region of nebulin interacts directly with the villin/gelsolin homology domain of supervillin. Nebulin binding region in supervillin is completely conserved in human archvillin, a muscle-specific isoform of supervillin[27].

## MATERIALS AND METHODS

### 1. Yeast two-hybrid interaction studies

For a survey of potential nebulin and supervillin interactions, nebulin cDNA fragments were amplified from human skeletal muscle cDNA library (HL4010AB; Clontech) by PCR. The serine-rich domain (SeR) and SH3 domain (SH3) were amplified with the primer pairs of SeR I/SeR II and hNeSH3 I/ hNeSH3 II, respectively. Amplified PCR fragments were inserted into pGBT9 vector to obtain pGBT9-SeR and pGBT9-SH3, respectively.

The supervillin fragments (SV 977~1217, SV 1036~1336, SV 977~1336 and SV 1336~1788) were digested using restriction enzymes (*Xho* I, *Bam*H I and *Eco*R I) in the clone encoding supervillin (clone B101) and ligated into the pGAD10 vector (Matchmaker system I; Clontech). The products were sized on native agarose gel and purified using GENECLEAN kit (BIO101). The supervillin 1.4 kb cDNA sequence, SV 977~1431 was PCR-amplified from human skeletal muscle cDNA library (HL4010AB; Clontech) using the primer pair Super I/Super II. Amplified PCR fragment was inserted into pGAD424 (Matchmaker system I; Clontech) vector to obtain GAL4-AD fusion.

### 2. Sequence determination and analysis

The identified prey clones were purified QIAprep Spin Miniprep Kit (Qiagen) and sequenced with dideoxy-chain terminators, using Thermo Sequenase Cycle Sequencing Kit (USB) and LI-COR 4200 (LI-COR) as described by the manufacturer. All clones were sequenced by primer walking at LI-COR. Sequence reads were compared and consensus cDNA sequences were constructed using Blast search of NCBI.

### 3. *In vitro* interaction studies

#### 1) Plasmid preparations

Serine-rich domain of the nebulin cDNA was amplified with the primer pair SeR I/SeR II (SeR). The cDNA corresponding to SH3 domain of nebulin was amplified using the primer pair, hNeSH3 I/ hNeSH3 II (SH3). For the isolation of human SeR and SH3 sequences, primer pairs derived from the human sequences (accession no. X83957) were used to PCR-amplify the specific nebulin sequences from human skeletal muscle (HL4010AB; Clontech) under conditions of reduced stringency, allowing primer mismatches during the initial PCR cycles. The PCR products were gel-purified after digestion using restriction enzymes and were inserted into expression vector, pGEX 4T (Amersham Biosciences) to obtain GST fusions (GST-SeR and GST-SH3) for *in vitro* pull-down assay.

Primers were designed from the cDNA encoding human supervillin (accession no. AF051850). The 1.12 kb product encoding exon 33~39 $\frac{1}{2}$ (SV 972~1335) of human supervillin was generated with a degenerate primer. Several

fragments, 0.933 kb, 0.79 kb, 0.78 kb and 1.39 kb products encoding exon 33~38 (SV 972~1274), 33~37 (SV 972~1226), 34~38 (SV 1024~1274), and 39 $\frac{1}{2}$~48 (SV 1335~1788) of human supervillin were amplified by PCR (Fig. 2). All reactions were performed with Advantage-HF 2 PCR kit (Clontech) with human skeletal muscle cDNA library (HL4010AB; (Clontech) as a template. The PCR products were digested using restriction enzymes and inserted into pSP64 Poly(A) (Promega). Prepared plasmids were used for *in vitro* transcription and translation.

**2) GST-fusion protein preparation and purification**

The chimeric fusion protein consisting of glutathione S transferase (GST) and human nebulin was expressed after 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction in BL21 (DE3) bacteria for 3 h at 30℃ with shaking. The cells were resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$) containing 1 mM PMSF, 0.1 mM DTT, 10 mM EDTA, and 100 μM leupeptin. The cells were lysed by sonication after addition of 1% Triton X-100 and the cell lysate was clarified by centrifugation at 10,000 g for 10 min. Three ml of the supernatant was mixed with 1.5 ml of 50% glutathione sepharose 4B beads (Amersham Biosciences) for 1 h at room temperature with shaking. The beads were washed three times with 6 ml of 1X PBS and the GST fusion protein was eluted by mixing the beads with 600 μl of glutathione elution buffer (20 mM glutathione, 50 mM Tris-HCl, pH 7.5) at room temperature for 10 min. The elution was repeated twice and pool the three eluates. The purity of the protein was confirmed by SDS-PAGE. Then the elution samples stored at -20℃.

**3) *In vitro* transcription and translation**

All *in vitro* transcription and translation experiments were performed using the TnT Quick Coupled Reticulocyte Lysate System (Promega). To label the peptides, the reactions were performed using the Transcend Non-Radioactive Translation Detection System as described by the manufacturer (Promega). The sizes of *in vitro* transcription and translation proteins were determined.

**4) GST pull-down assay**

The purified GST fusion proteins were incubated for 1 h at room temperature with the resin to immobilize the fusion proteins; glutathione-sepharose 4B beads (Amersham Biosciences) for GST. The beads were washed three times with 1X PBS by 10 folds of bed volume and the GST fusion protein was resuspended in the interaction buffer (PBS, 1% Triton X-100, 1% BSA). For binding experiments, 5 μl of biotin-labeled TnT protein extract was incubated with fusion protein (50 μl of beads, 50% slurry) in the interaction buffer for 1 h 30 min at room temperature with shaking. The beads were washed with PBS containing 1% Triton X-100. Biotin-labeled proteins eluted in sample buffer (0.15 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) were separated by SDS-PAGE using 10~12% gels and transferred to a nitrocellulose membrane. Nitrocellulose blots were blocked with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20) at room temperature for 1 h and probed with streptavidin conjugated to alkaline phosphatase in TBST for 1 h at room temperature with shaking. Blot washed in TBST and water and was used with Western Blue Substrate for alkaline phosphatase for colorimetric detection as described by the manufacturer (Promega).

## RESULTS

**1. Identification of serine-rich region in nebulin required for interaction with supervillin**

To narrow down the supervillin-binding site within nebulin, the interaction of supervillin with two deletion constructs of nebulin was tested. Nebulin cDNA fragments were amplified from human skeletal muscle cDNA library by PCR. Amplified PCR fragments were inserted into pGBT9 vector to obtain GAL4-DNA BD fusions (pGBT9-SeR and pGBT9-SH3), respectively. We performed yeast two-hybrid binding assay with subfragments of pGBT9-Neb-C and identified prey clone, B101 (encoding supervillin) from the screening of 4,000,000 clones.

This analysis showed that supervillin binding region is located in the serine-rich region of nebulin (Fig. 1). Bait constructs were transformed into *Saccharomyces cerevisiae*, strain HF7c. Simultaneously, the cells were transformed with B101 in the pGAD10 prey vector essentially as described by the manufacturer. Cells were grown onto SD/Leu-/Trp-/His-plates supplemented with 1.5 mM 3-amino-1,2,4-triazole (3-AT). β-Galactosidase activity of the cells was measured by colony lift filter assays using X-gal as described by the manufacturer. Neb-C and Neb-SeR are shown positive in β-galactosidase activity and Neb-SH3 is not shown. Neb-SeR contains nebulin's residues 6457-6612 that

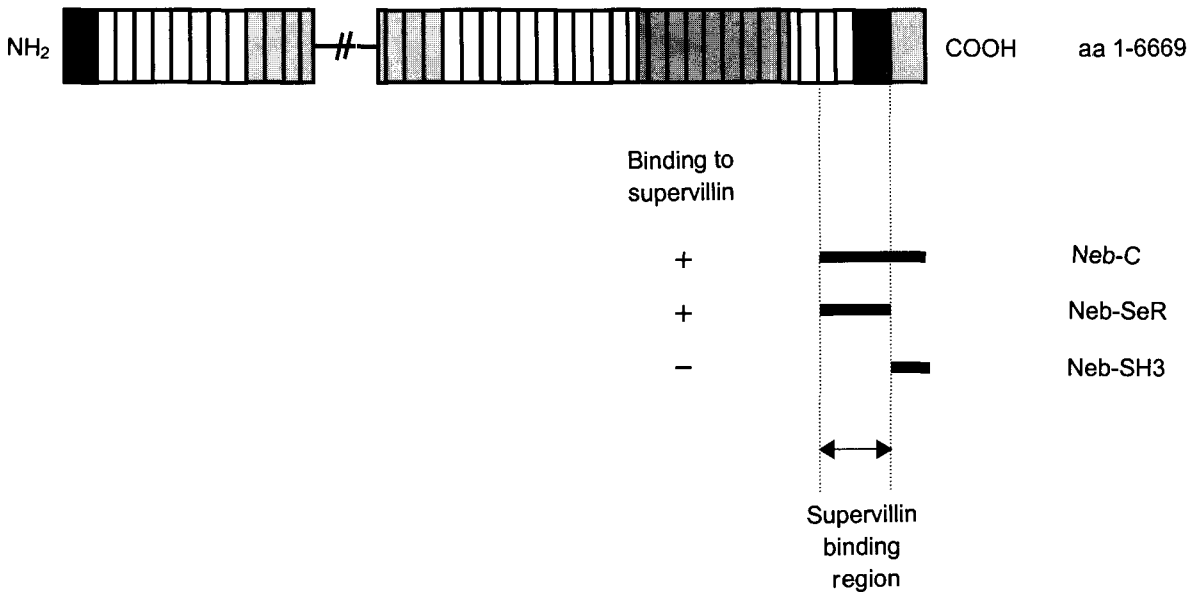


**Fig. 1.** Association of supervillin with the serine-rich region of nebulin . A series of nebulin deletion constructs (lines below the nebulin domain structure) were tested for interaction with C-terminus of supervillin in the yeast two-hybrid system. This assigned the supervillin-binding domain within nebulin to its serine-rich domain. (+) and (−) denote the presence or absence of the growth of yeast colonies on SD/Trp-/Leu-/His-plates supplemented with 1.5 mM 3-AT.

is required for binding.

### 2. Identification of the nebulin binding site in supervillin

To identify more precisely the supervillin sequences responsible for the nebulin-supervillin interaction, the supervillin fragments were digested using restriction enzymes the clone encoding supervillin (clone B101). Then they were ligated into the pGAD10 vector to obtain GAL4-AD fusion (pGAD-SV 977~1217, SV 1036~1336, SV 977~1336, and SV 1336~1788). pGAD-SV 977~1431 was amplified from human skeletal muscle cDNA library (HL-4010AB) by PCR. Amplified PCR fragment was inserted into pGAD424 vector. We performed yeast two-hybrid binding assay with subfragments of the clone, B101 and pGBT9-Neb-C.

Truncation analysis of supervillin indicated that residues 993~1261 (269 residues) within villin/gelsolin homology domain are sufficient for the interaction with nebulin's M184-185 + Serine rich domain (Fig. 2). pGBT9-Neb-C was transformed into HF7c. Simultaneously, the cells were transformed with the prepared prey constructs as described by the manufacturer. Cells were grown onto SD/Leu-/Trp-/His-plates supplemented with 1.5 mM 3-amino-1,2,4-triazole. β-Galactosidase activity of the cells was measured by colony lift filter assays using X-gal. SV 977~1336, SV 993~1261 and SV 977~1431 were positive in terms of β-galactosidase activity and SV 977~1217, SV 1036~1336, and SV 1336~1788 were negative. The additional presence of residues, however, further enhanced yeast growth and β-galactosidase activity and may therefore be responsible for strengthening the interaction.

### 3. The exon 33~38 of supervillin binds to the serine-rich region of nebulin

The interaction of nebulin serine-rich domain with supervillin was confirmed by GST pull-down assays. For purification of GST-nebulin fusion proteins, nebulin's fragments were inserted into pGEX 4T expression vector and were induced to overexpress with 0.4 mM IPTG. The molecular weight of the fusion protein was about 26 kDa (GST), 33.6 kDa (GST-SH3), and 45.3 kDa (GST-SeR), respectively. GST-SH3 fusion protein included the nebulin SH3 domain (Neb-SH3) and GST-SeR fusion protein included the nebulin serine-rich region (Neb-SeR). The purity of the proteins was confirmed by SDS-PAGE. After the cell lysates were mixed with glutathione sepharose 4B beads, unbound proteins were removed and analysed on SDS-PAGE. The beads were washed with PBS. GST-nebulin fusion proteins were eluted from the glutathione Sepharose 4B with glutathione

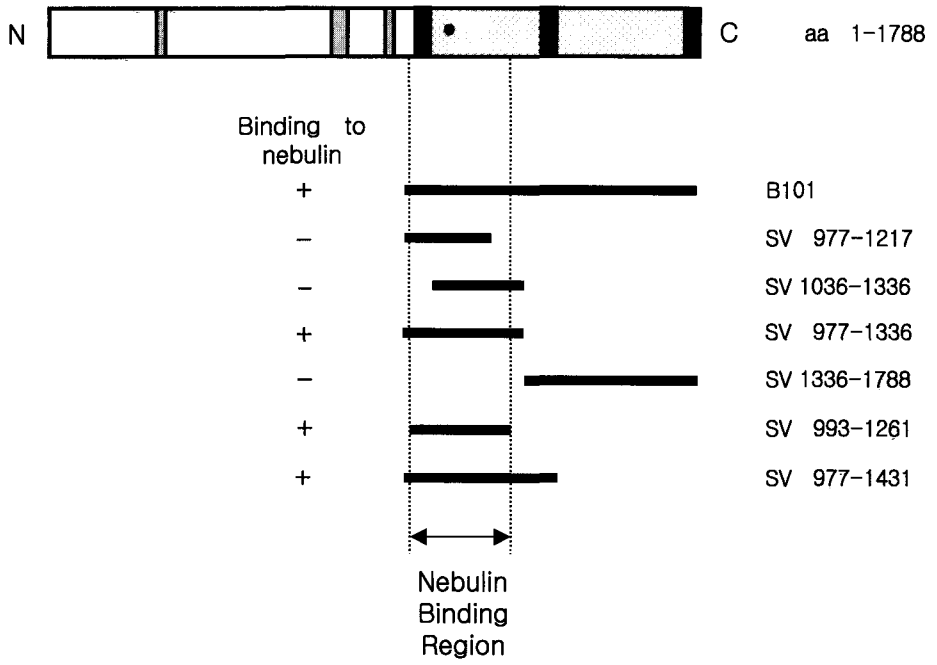


**Fig. 2.** Minimal region of supervillin required for interaction with nebulin by yeast two-hybrid assay. Fragments of the supervillin were tested for the ability to interact with the bait nebulin in the two-hybrid system. The experiment demonstrated that the region of supervillin between aa 993 and 1261 is capable to interact with nebulin. (+) and (−) denote the presence or absence of the growth of yeast colonies on SD/Trp-/Leu-/His-plates supplemented with 1.5 mM 3-AT.

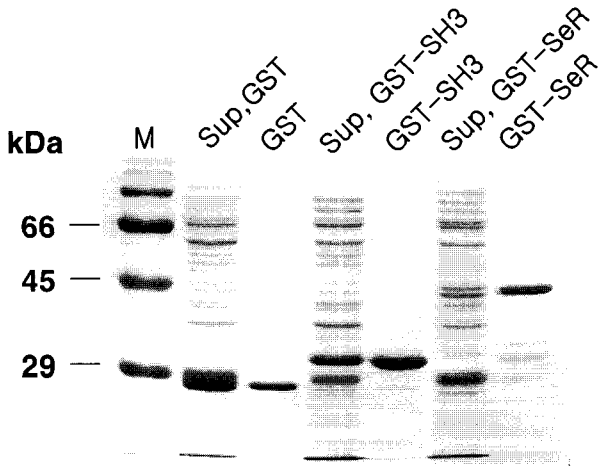


**Fig. 3.** Preparation of GST-nebulin fusion proteins. GST-fusion proteins were inserted into pGEX 4T expression vector and transformed into BL-21 (DE3) and subsequently subjected to SDS-PAGE analysis. M denotes high molecular weight marker. Cells were induced to overexpress GST-nebulin fusion protein with 0.4 mM IPTG at 30℃ for 3 h. The expected molecular weights of the fusion proteins were about 26 kDa (GST), 33.6 kDa (GST-SH3), and 45.3 kDa (GST-SeR). The cell lysates were mixed with glutathione Sepharose 4B beads. After incubation at room temperature for 1 h, unbound proteins (Sup) were removed and analysed on SDS-PAGE. The beads were washed with PBS. GST-nebulin was eluted from the glutathione sepharose 4B with glutathione elution buffer (20 mM glutathione, 50 mM Tris-HCl, pH 7.5) at room temperature for 10 min.

elution buffer (Fig. 3).

Human skeletal muscle supervillin cDNA (accession code AF051850) sequence was assigned to 48 exons. The ~2.5 kb coding information for human C-terminal supervillin is contained in 15 exons encoding the villin/gelsolin homology domain. For *in vitro* bindind assay, supervillin's fragments were translated *in vitro*. Primers were designed from the cDNA encoding human supervillin (accession no. #AF051850). Supervillin' fragments were amplified with primers flanking the exon 33~39$\frac{1}{2}$ (SV 972~1335), 33~38 (SV 972~1274), 33~37 (SV 972~1226), 34~38 (SV 1024~1274), and 39$\frac{1}{2}$ ~48 (SV 1335~1788) from a human skeletal muscle cDNA library by PCR. The amplified products of 1,120 bp, 933 bp, 790 bp, 780 bp and 1,390 bp were analyzed by agarose gel (Fig. 4A). The PCR products were digested using restriction enzymes and inserted into pSP64 Poly(A). Prepared plasmids were used for *in vitro* transcription and translation. All *in vitro* transcription and translation experiments were performed using the TnT Quick Coupled Reticulocyte Lysate System and the Transcend Non-Radioactive Translation Detection System. *In vitro* translated proteins were sized by SDS-PAGE and blotting (Fig. 4B).

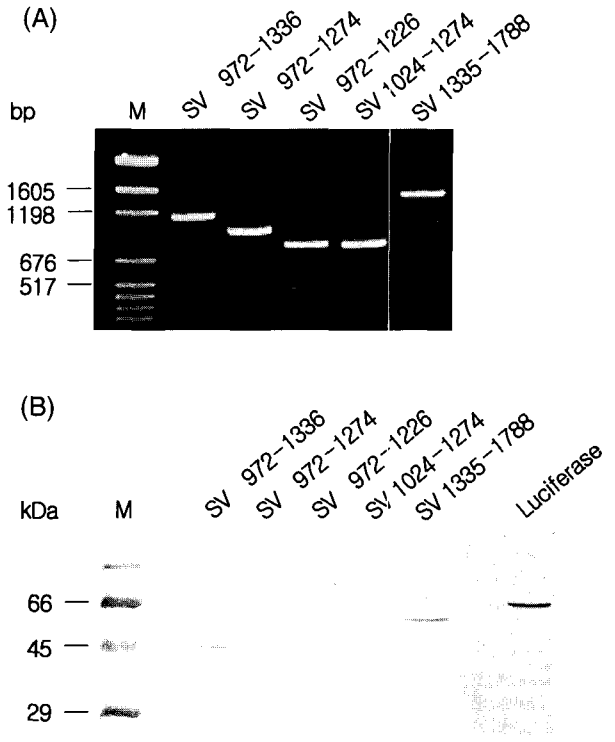


**Fig. 4.** Supervillin fragments were translated *in vitro*. (**A**) SV 972~1336, SV 972~1274, SV 972~1226, SV 1024~1274, and SV 0335~1788 of human supervillin were amplified from a human skeletal muscle cDNA library with PCR. (**B**) Supervillin fragments were translated *in vitro* and detected using the Transcend Non-Radioactive Translation Detection System.

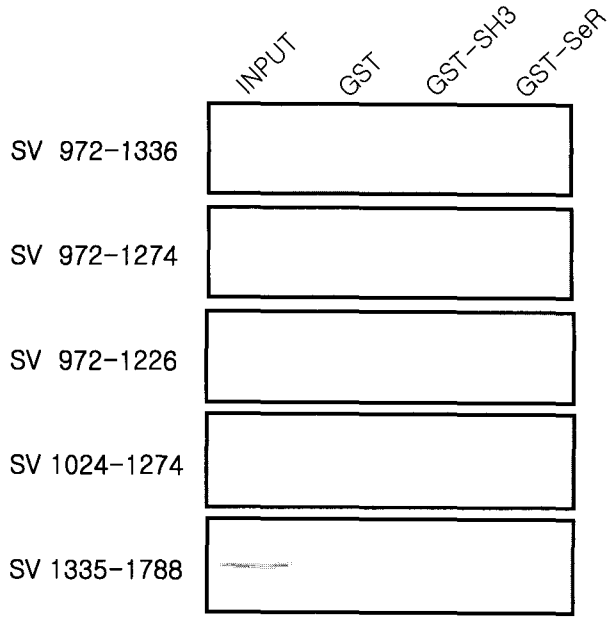


**Fig. 5.** Supervillin interacted with nebulin in GST pull-down assays. Supervillin fragments (SV) were translated *in vitro* (INPUT, lane 1). *In vitro*-translated SV 972~1336 and SV 972~1274 bound to glutathione-Sepharose 4B beads in the presence of GST-nebulin serine-rich region fusion peptide (GST-SeR, lane 4). No bindings were observed in the presence of GST-nebulin SH3 domain fusion peptide (GST-SH3, lane 3). As a negative control, binding of GST and SVs to beads were tested (lane 2).

The interaction of nebulin C-terminal region and supervillin C-terminal region were tested by GST pull-down assays. SV 972~1336 and SV 972~1274 coprecipitated with the GST-SeR but do not coprecipitated with GST alone and GST-SH3 (Fig. 5). Three supervillin's fragments do not also coprecipitated with GST-negulin fusion proteins. This confirmed that the nebulin serine-rich domain specifically binds to supervillin's exon 33~38 involving residues 993~1261. Therefore, this region contains six exons that encode ~34 kDa in C-terminus of supervillin. This negulin binding region is located within supervillin's central villin/gelsolin homology domain and contains two gelsolin homology domain.

## DISCUSSION

In this study, we aimed to further dissect the molecular components required for the coordinated organization of Z-line components into regular, hexagonal lattices. We took the approach of searching for protein interactions that anchor the C-terminal region of nebulin within the sarcomere. This allowed us to identify the specific binding of nebulin and supervillin. The nebulin serine-rich region is required for interaction with supervillin. Nebulin binding region in supervillin is located in its C-terminal region.

A muscle-specific isoform of supervillin, archvillin, was found by cloning and characterizing a 250 kDa protein from human and mouse skeletal muscle[27]. This protein is derived from the supervillin genomic locus (SVIL) by differential splicing of five conserved exons, four of which encode muscle-specific protein sequences distributed as two 'inserts' within the function-rich N-terminus of the protein. Because of the likelihood of muscle-specific conserved functions, they suggested that the isoform of supervillin found in muscle, the principal source of supervillin in the body, be called 'archvillin' (Latin, archi; Greek, archos; 'principal' or 'chief'). Archvillin is the isoform found in myogenic cell lines and in cardiac and skeletal muscles, which are the tissues in which supervillin/archvillin messages are the most abundant. Supervillin interacts with vinculin-

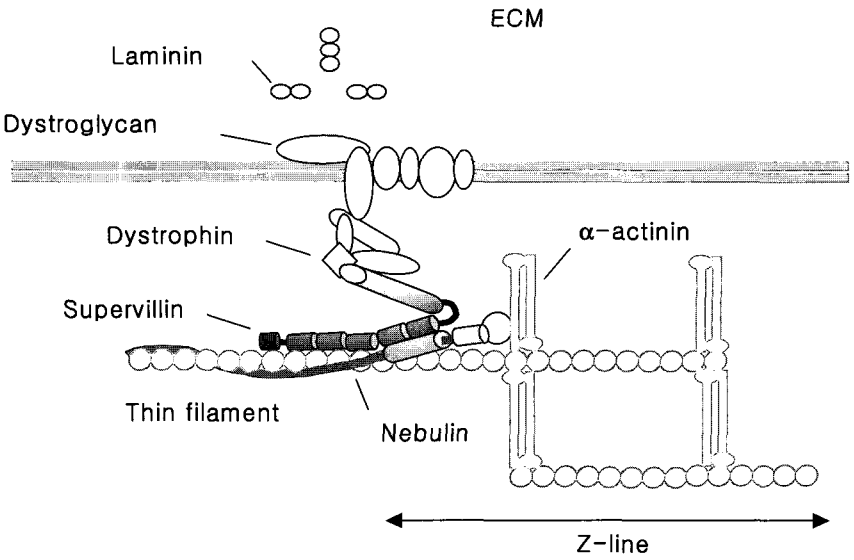


**Fig. 6.** Model of interaction between nebulin and supervillin in costameres. Nebulin acts as a molecular ruler that regulates thin filament length in sarcomere. Supervillin binds directly to F-actin and colocalizes with dystrophin at costameres in skeletal muscle.

containing focal adhesions[50] and co-isolates with spectrin, non-muscle myosin II and a-actinin[25,32]. Because archvillin contains all the sequences found in supervillin, a nonmuscle protein, archvillin is likely to participate in the crosstalk between the spectrin- and focal adhesion-based membrane skeletons at costameres in muscle[27].

In skeletal muscle, archvillin is primarily sarcolemmal, localizing at costameres although a structural basis for this has remained elusive. Previous studies have also led to the identification of nebulin modules M160-M183, from the peripheral (I-band side) region of the Z-line[23], with the intermediate filament protein, desmin[1]. Their binding studies favor a model in which desmin also directly links together adjacent Z-lines by its specific interaction with nebulin modules in the periphery of the Z-line. Ultrastructural studies have suggested the presence of lateral connections by a microfilament system located between Z-discs and the sarcoplasmic reticulum, and by intermediate filaments, which link adjacent Z-lines[26,33,47]. More recent immunolabeling studies have further confirmed the presence of a lateral Z-line linkage system and have identified desmin and plectin as components of it[16]. Consistent with a role in the lateral registration of myofibrils, desmin is localized in the periphery of the Z-line[13,24]. Desmin can function to mediate inter-Z line linkages independently of plectin. Consistent with this model, plectin maintains its localization in desmin knockout mice[6]. Interestingly, even in the absence of desmin, interlinking filaments between myofibrils and the sarcolemma are observed, suggesting that components other than desmin must also interlink myofibrils and attach them to the sarcolemma[21].

We found a component other than desmin by screening of binding partners of nebulin-C terminal region. Our binding experiments showed that nebulin binds directly to supervillin *in vitro*. Furthermore archvillin, muscle supervillin, is localized at costameres in skeletal muscle. This is the region where the myofibrils and form a mechanical connection to a protein complex that allows force to be transmitted beyond the cell interior to the extracellular matrix[10,36,43]. We propose a model in which nebulin, a component of the myofibril, binds to archvillin, while the C-terminal region of archvillin binds to the complex of proteins localized at the interior surface of the cell membrane in these specialized regions in skeletal muscles (Fig. 6).

Organized complexes of membrane-associated cytoskeletal proteins (membrane skeletons) maintain the integrity and organization of the plasma membrane (sarcolemma) of striated muscle cells during the mechanical stresses associated with load-induced stretching and muscle contractions [2,3,38,42,49]. Lateral forces across the sarcolemma to the basal lamina are resisted primarily by costameres, cables of filamentous cytoskeletal material that connect the sarcolemma to Z- and M-lines in underlying myofibrils[7,33,41]. Current information suggests that membrane skeleton proteins in

motile cells control the formation or stabilization of dynamic cell surface extensions, such as microvilli and pseudopods, and/or are involved in transient interactions with the substratum and with other cells[5,37,45].

Nebulin acts as a molecular ruler that regulates thin filament length in sarcomere and archvillin binds directly to F-actin, co-isolates with dystrophin and caveolin-3 in low-density sarcolemmal membranes, and colocalizes with dystrophin at costameres in skeletal muscle[27]. Our data suggest that nebulin binds to archvillin and is involved in linkage of the myofibrils and the sarcolemma in skeletal muscles.

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