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Nebulin C-terminus Interacts with NCBP51, a New Isoform of RING Finger Protein 125 (RNF125)

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Nebulin, a giant modular protein from muscle, is thought to act as molecular ruler in sarcomere assembly. In skeletal muscle, the C-terminal ~50 kDa region of nebulin extends into the Z-line lattice. The most recent studies implicated highlighting its extensive isoform diversity and exciting reports revealed its expression in cardiac and non-muscle tissues containing brain. Also these novel findings are indicating that nebulin is actually a multifunctional filament system, perhaps playing roles in signal transduction, contractile regulation, and myofibril force generation, as well as other not yet defined functions. However the binding protein of nebulin and function in brain is still unknown. A novel binding partner of nebulin C-terminal region was identified by screening a human brain cDNA library using yeast two-hybrid system. Nebulin C-terminus binding protein 51 (NCBP51) was contained a RING-finger domain and identified a new isoform of RING finger protein 125 (RNF125). The interaction was confirmed using the GST pull-down assay. NCBP51 belongs to a family of the RING finger proteins and its function remains to be identified in brain. The role of nebulin and NCBP51 will be studied by loss-of-function using siRNA technique in brain.

Key Words: Nebulin C-terminus, Human brain cDNA library, Yeast two-hybrid, RNF125, NCBP51

INTRODUCTION

Nebulin is a filamentous protein of modular organization that comprises the fourth filament system of skeletal muscle. A single nebulin molecule associates along the entire length of the thin filament (Wang and Wright, 1988; Wright, et al., 1993), with the C-terminus anchored in the Z-disc and the N-terminus located at the pointed ends of the thin filaments (Millevoi et al., 1998; Herrera et al., 2000; McElhinny et al., 2001). The N-terminus of nebulin contains a unique 8 kDa segment of unknown function and modules M1-M8, which contain a binding site for the thin filament pointed end capping protein, tropomodulin (McElhinny et al., 2001). It has been proposed that the interaction of nebulin with tropomodulin may contribute to the regulation of thin filament lengths in muscle, an idea that supports nebulin's proposed role as a template molecule. Finally, it has been suggested that nebulin also may modulate actomyosin ATPase activity in a Ca^{2+}-dependent manner, perhaps functioning as a unique thin filament regulator (Root and Wang, 1994; Root and Wang, 2001). The central region of nebulin is made up of 185 repeats that are each about 35 amino acid residues in length; these modular repeats are referred to as M1-185 and constitute 97% of the molecule (Labeit and Kolmere, 1995). Within the central region of the molecule (repeats M9 to M162), groups of seven of these modules are arranged into super repeats and share conserved SDXXYK (each repeat) and WLKGIGW (each seven repeats) motifs (Jin and Wang, 1991, Labeit et al., 1991). The segment comprising repeats M160-M170 links nebulin's super repeat region to the C-terminal region modules M171-M185, which are located close to the periphery of the Z-line and are characterized by a highly conserved SSVLYKEN motif. Modules M160-M170 interact with desmin *in vitro*, suggesting that they may function in maintaining the lateral registry of adjacent myofibrils (Bang et al., 2002). The C-terminus is anchored

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in the Z disc of the muscle sarcomere and contains a conserved src homology (SH3) domain (Labeit and Kolmere 1995; Pfuhl et al., 1996). A sarcomeric protein, myopalladin, links nebulin to α-actinin in the Z discs (Bang et al., 2001). There is also evidence that the nebulin SH3 domain may bind titin (Politou et al., 2002). Recently serine-rich identified acrchvillin binding region through the yeast two-hybrid system and *in vitro* bindig assay.

Nebulin indeed is expressed in cardiac muscle. Western blot analyses of chordate muscles revealed that full-length nebulin is present in the hearts of agnathan fishes (Fock and Hinssen, 2002). Consistent with this finding, a transcriptional screen based on the complete mouse nebulin gene revealed the expression of nebulin mRNAs in cardiac tissue, during both early and late stages of development. Furthermore, anti-bodies generated against skeletal muscles nebulin's N-terminal and C-terminal regions labeled rat cardiac myofibrils at the thin filament pointed ends and Z-disc regions, respectively: a molecular layout identical for that of skeletal muscle nebulin (Kazmierski et al., 2003). Recently, it has revealed that nebulin is also expressed in brain, heart, stomach and liver of 15-day-old chicken embryo and in brain of human. Four isoforms have been identified in brain and nine different types of skeletal isoforms are also detected. The presence of nebulin in non-muscle tissue was confirmed by immunofluorescence microscopy studies in 15-day-old chicken embryo. Nebulin localized in the edge of the Z-line in breast muscle, as shown by co-localization with α-actinin. The same labeling pattern was found in cardiac myofibrils, raising the possibility that cardiac nebulin is also anchored in the Z-disc and extends to the pointed ends of the thin filaments. Nebulin mRNA in non-muscle tissue was identified by *in situ* studies (Joo et al., 2004). However the interacting partner and the function of nebulin in brain is still unknown.

The C_3HC_4-type zinc finger domain, also termed RING domain, was first described in the sequence of the human ring 1 gene (Freemont et al., 1991). Since the first description of the RING finger domain over a decade ago, over 400 functionally diverse proteins have been identified as containing this domain (Freemont, 1993). RING, which stands for really interesting new gene, is minimally described as a cysteine-rich motif and follows the general formula $C\text{-}X_2\text{-}C\text{-}X_{(9\text{-}39)}\text{-}C\text{-}X_{(1\text{-}3)}\text{-}H\text{-}X_{(2\text{-}3)}\text{-}C\text{-}X_2\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}X_2\text{-}C$, where X can be any amino acid. The structure of this domain revealed that it did not resemble classical zinc fingers, but utilized its Cys/His residues to bind two zinc atoms in a unique cross-brace arrangement, where the first and third pair of metal ligating residues bind the first zinc and the second and fourth pair bind the second zinc (Borden, 2000). This zinc ligation is required for folding and for subsequent biological actions.

Unlike classical zinc fingers, which are generally limited to the nucleus and function in binding nucleic acids, RING domains are found throughout the cell mediating diverse protein-protein interactions (Saurin et al., 1996; Borden, 2000); however, no general function has been ascribed to this domain. RING finger proteins are found in a wide range of species and have been implicated in diverse cellular processes such as and transcription, cell cycle control. cell growth, cellular differentiation, and apoptosis, as well as in abnormal cellular behavior such as oncogenic transformation (Borden, 2000).

To identify the binding partner of nebulin in brain, we used the yeast two-hybrid system and searched for potential targets of nebulin C-terminus in a human brain cDNA library by using nebulin C-termnal region as a bait. In this work, a new nebulin C-termianl binding protein 51 (NCBP-51) was identified from human brain cDNA library by yeast two-hybrid. It was revealed a new isoform of RNF125, which was unknown the function and contains RING-finger domain. NCBP51 was identified for the first time and it was a novel member of the RING-finger protein that directly associates with the nebulin C-terminus. This interaction was confirmed using *in vitro* interaction assays. Furthermore, it will be provided date for the potential binding region between Neb-C terminal domains and NCBP51 and insight into how the function of nebulin are associated with NCBP-51 in brain.

MATERIALS AND METHODS

1. Yeast two-hybrid screen

1) Bait Plasmid preparation

Human Neb-C was amplified by PCR using human brain Large Insert cDNA library (Clontech) was used directly as template and performed PCR in a volime of 50 μl containing 10X PCR reaction buffer, 10 μM of each primer and 200 μM dNTPs. PCRs were hot-started by adding all reagents except DNA polymerase, heating to 95℃ for 10 min then

holding at 80℃ for 45 min. Enzyme (Ex Taq polymerase, Takara) was then added and amplification was performed 35 cycles (95℃ for 1 min, 56℃ for 30 sec and 72℃ for min). Total PCR products were separated on 1.5% agarose gel. A band was excised from the gel and purified using QIAEX II gel elution kit (Qiagen). The gel-purified band was cloned into the pGEM-T vector (Promega) with T4 DNA ligase. The ligated plasmid was digested with *EcoR* I and *Sal* I and cloned into the pGBKT7 (Clontech) vector. The sequence of bait construct, pGBKT7-Neb-C, was verified by sequencing.

2) Library screening

The yeast two-hybrid screen was carried out using a GAL4-based yeast two-hybrid system (MATCHMAKER Two-Hybrid System 3; Clontech, Palo, Alto, CA); screening and assay were performed as recommended by the manufacturer (Clontech). Bait constructs were transformed into yeast strain AH109 (Meta) and the transformants were plated on dropout medium lacking tryptophan (SD/-Trp). The Pretransformed Human Brain Matchmaker cDNA Library, which is a human brain 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) (aa, 768-881 of GAL4), 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 (SD/-Leu/-Trp) to select for both the bait and prey plasmids. 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 *lacZ*), thereby activating transcription and permitting growth on selection media (His- and Ade-) and the expression of α-galactosidase (*MEL1* product) and β-galactosidase (*lacZ* product). The cotransformants were then plated on dropout medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) to select for colonies that expressed interacting proteins.

Bait and prey plasmid pairs were also cotransformed into yeast strain AH109 (Clontech). The transformants were initially plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and prey plasmids. Positive interactions were confirmed by cell growth on histidine- and adenine-deplete yeast synthetic medium and by α-galactosidase assays.

3) DNA sequence determination and analysis

The identified prey clones were purified by using QIAprep Spin Miniprep Kit (Qiagen) and sequenced with dideoxy-chain terminators, using Thermo Sequencase Cycle Sequencing Kit (USB) and a LI-COR 4200 Automated DNA Sequencer (LI-COR) as described by the manufacturer. All clones were sequenced by primer walking at LI-COR and homology searches against database sequences were performed using the BLAST algorithm. For yeast two-hybrid interaction study and *in vitro* binding assay, the sequences of all constructs were verified by sequencing.

The RING-finger domain of NCBP51 was predicted using the ScanProsite program (http://www.expasy.org/tools/scanprosite/).

2. Western blot analyses

Protein samples were resolved by electrophoresis on 12% polyacrylamide/SDS gels. Proteins were electroblotted onto nitrocellulose membranes (Amersham Biosciences) for 1 h. The membranes were incubated for 1 h with one of the following primary antibodies in phosphate-buffered saline (PBS): c-Myc monoclonal antibody (Clontech); anti-GST antibody (Amersharm Phamacia Biotech). After being washed three times with PBS containing 0.3% Tween 20 (TPBS), the membranes were incubated for 1 h with one of the following secondary antibodies: rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma); rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma). Blots were washed in TPBS and developed with Western Blue Stabilized for alkaline phosphatase (Promega) for colorimetric detection.

3. *In vitro* transcription/translation and GST pull-down assays

1) Expression and purification of GST-fusion protein

pACT2-NCBP51 was digested with *Sal* I/*Xho* I and inserted into expression vector, pGEX-4T-3 (Amersham Biosciences) to obtain GST fusion for GST pull-down assay. Overexpression of GST-NCBP51 and GST alone (as a control) were induced in *E. coli* strain BL21 (DE3). Overnight cultures of transformed with recombinant pGEX plasmids were diluted 1:10 in L-broth with 100 μg/ml ampicillin and incubated at 30℃ with shaking to an $A_{600\ nm}$ of 0.5.

Isopropyl-β-d-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM. After a further 4 hours of growth, cells were pelleted at 7700 x g for ten min at 4℃ and resuspended in ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Cells were sonicated and then added 1% Triton X-100 and incubated for 30 min at room temperature. Cells were centrifuged at 12,000 x g for ten min at 4℃. To confirm the presence of GST-fusion protein, the blots were tested with anti-GST (Amersham Bioscience; 1:5000) and conjugated rabbit anti-goat IgG-alkaline phosphatase (Sigma; 1:7000). The NBT/BCIP (bromochloroindolyl phosphate/nitro blue tetrazolium substrate) was used as a substrate for detecting colorimetry.

2) *In vitro* transcription and translation

In vitro T7-driven transcription and translation of nebulin C-terminus was done with the TNT $T7^R$ Quick Coupled Transcription/Translation System (Promega) with Transcend Biotin-Lysyl-tRNA (Promega). The pGBKT7-Neb-C (1 μg) and other reagents were mixed following the protocol of the manufacturer. All *in vitro* transcription and translation experiments were performed using the TnT Quick Coupled Reticulocyte Lysate System (Promega). A similar reaction using exception of DNA was also used as a negative control. The reaction mixture was incubated at 30℃ for 90 min. Then, 1 μl of the sample was mixed with 15 μl of SDS-sample buffer and boiled for 2 min. The sample were then analyzed on a 10% or 12% SDS-polyacrylamide gel and electroblotted to a PVDF membrane (Amersham). The membrane was rinsed in TPBS (1X TBS, 0.5% Tween 20), bound with Streptavidin-AP solution and developed with Western Blue Stabilized for alkaline phosphatase (Promega) for colorimetric detection and colorimetrically detected using Western blue stabilized substrate for alkaline phosphatase (Promega).

3) GST pull-down

The purified GST fusion proteins were incubated for 1 h at room temperature with the resin to immobilize the fusion protein; glutathione-Sepharose 4B beads (Amersham Biosciences) for GST. The beads were washed three times with 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-

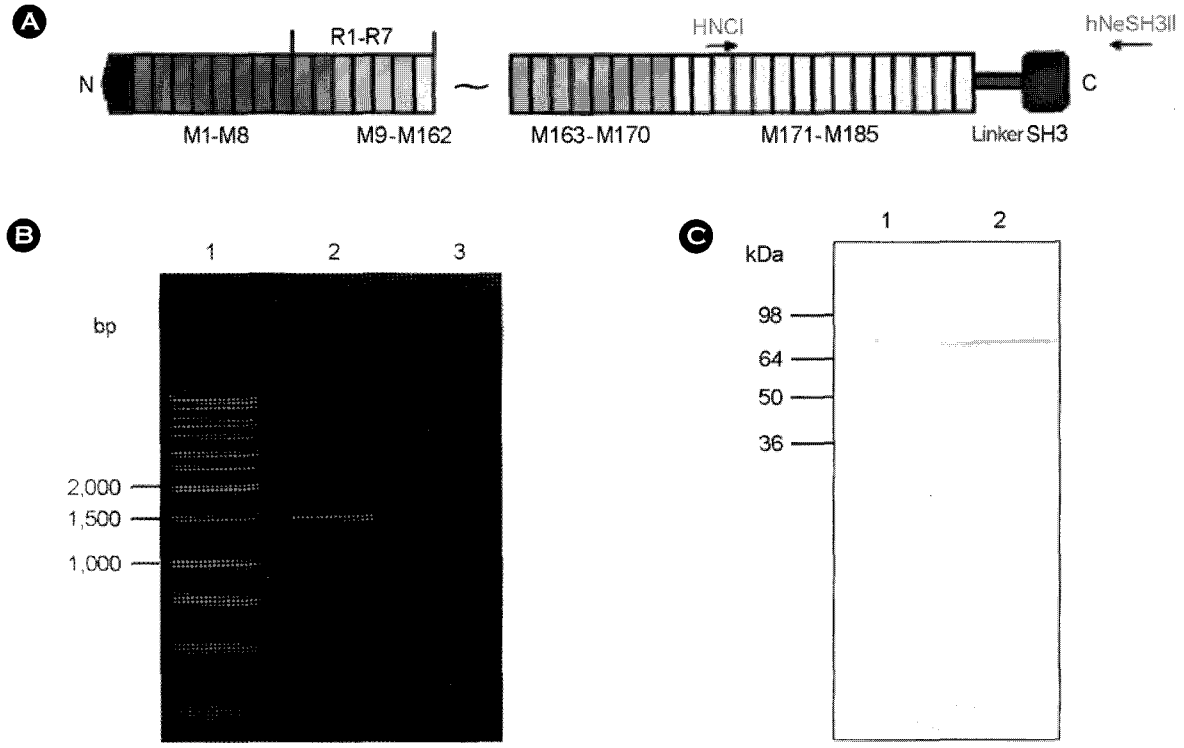

Fig. 1. Preparation of nebulin C-terminal fragment for bait plasmid and Expression of the GAL4 BD-Neb C fusion protein in yeast. **(A)** Nebulin fragment was amplified from human brain Large-insert cDNA library using nebulin-specific primers (HNCI and hNeSH3II). **(B)** Amplified fragment was fractionated on 1.5% agarose gel stained with ethidium bromide. Lane 1, 1 kb DNA ladder; lane 2, amplified Nebulin C-terminus; lane 3, negative control.

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. Beads were washed three times with wash buffer, 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 10% or 12% SDS-PAGE and transferred to a PVDF membrane. The membrane 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 was washed in TBST and water and was used with Western Blue Substrate for alkaline phophatase for colorimetric detection as described by the manufacturer (Promega).

RESULTS

1. Preparation of the human nebulin C-terminal cDNA in brain

The about 1.5 kb of human nebulin C terminus (Neb-C)

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AGGAGGGACCCGGAGTTGCCCGTCACGTCCTTCGACTGCGCCGTGTGCCTTGAGGTGTTACACCAGCCTGTCCGG
R  R  D  P  E  L  P  V  T  S  F  D  C  A  V  C  L  E  V  L  H  Q  P  V  R

ACCCGCTGTGGCCACGTATTCTGCCGTTCCTGTATTGCTACCAGTCTAAAGAACAACAAGTGGACCTGTCCTTAT
T  R  C  G  H  V  F  C  R  S  C  I  A  T  S  L  K  N  N  K  W  T  C  P  Y

TGCCGGGCATATCTTCCTTCAGAAGGAGTTCCAGCAACTGATGTAGCCAAAAGAATGAAATCAGAGTATAAGAAC
C  R  A  Y  L  P  S  E  G  V  P  A  T  D  V  A  K  R  M  K  S  E  Y  K  N

TGCGCTGAGTGTGACACCCTGGTTTGCCTCAGTGAAATGAGGGCACATATTCGGACTTGTCAGAAGTACATAGAT
C  A  E  C  D  T  L  V  C  L  S  E  M  R  A  H  I  R  T  C  Q  K  Y  I  D

AAGTATGGACCACTACAAGAACTTGAGGAGACAGCAGCAAGGTGTGTATGTCCCTTTTGTCAGAGGGAACTGTAT
K  Y  G  P  L  Q  E  L  E  E  T  A  A  R  C  V  C  P  F  C  Q  R  E  L  Y

GAAGACAGCTTGCTGGATCATTGTATTACTCATCACAGATCGGAACAGAGGCCTGTGGTAAGGATTTTTGTTACA
E  D  S  L  L  D  H  C  I  T  H  H  R  S  E  Q  R  P  V  V  R  I  F  V  T

TGTATTACAGCAATGTCTGAATTCAGGACACATTAGGAAATCCATTTAATAGATTTATATTTATAGATACCTTAT
C  I  T  A  M  S  E  F  R  T  H  *

AAAGGATAATTATTTAAGTTAAGGTTATGGTAACTCTGTGACTGTTTACAATGTGTGAAAGTGTTCAGTTGAAAC
TGATAAGAACGACTAATGTGTAATCACTCATTTATGAAATTACACATTATCTCTAACAGGTAATCATGGGCATAA
TCAACTAAAATGATCCAAAATGCACAGTTAAGATCTAAGTTTACCAGCTGGGCCAATATGGTGAAACCCCATCTC
TACTAAAAATACAAAAACTAGCTGGGTACATTGGCGGGCATGTGTAATCCCAGCTACTAGGGAGGCTGAGGCAGA
AGAATCGCTTGAACCTGGGAGGCAGAGGTTGCAGTGAGCCGAGATGGAGCCACTGTACTCCAGCCTGGGCAACAG
AGCAAGACTGTCTCAAAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequences of NBCP51 cDNA. NBCP51 was identified by the yeast two-hybrid system. The RING-finger domain is indicated as shaded gray boxes. The amino acid sequence is shown in a one-letter code beneath the coding sequence. An asterisk denotes the termination codon.

was amplified by PCR using Human Brain Large Insert cDNA library (Clontech) as template and performed PCR using appropriate primers (HNC I and hNeSH3 II) containing *EcoR* I/*Sal* I restriction enzyme site designed from the human nebulin cDNA sequence (accession No. X83957). The Neb-C sequence was identical to the skeletal muscle cDNA as determined by Blast alignment of National Center for Biotechnology Information. It contains two simple-repeat (exons 165 and 166), linker, serine-rich and SH3 domain (Fig. 1 (A) and (B)).

2. Interaction of a COOH-terminal nebulin in yeast two-hybrid experiments

To identify potential interaction partner of the nebulin, a human brain cDNA library was screened using the yeast two-hybrid technique and the C-terminal fragment of the nebulin cDNA as bait. Initially, the pGBKT7-Neb-C vector was transformed into yeast strain AH109. It was expressed and assayed the transformants for *ADE2*, *HIS3*, *MEL1* and *lacZ* activation. It was confirmed that GAL4 BD-Neb C did not auto-activate these reporter genes. Furthermore, to verify the protein expression of GAL4 BD-Neb C, the transfomants was analyzed by Western blot using the anti-c-Myc monoclonal antibodies along with untransformed yeast which served as a control. Fig. 1 (C) shows a Western blot of the control, untransformed strain (lane 1) and for a GAL4 BD-Neb C fusion protein strain (lane 2). The pGBKT7-Neb C expression construct yielded a fusion protein of the expected molecular size (about 70.5 kDa), indicating that the GAL4 BD-Neb C fusion was suitable for use as bait in our two-hybrid protein interaction screening.

To identify proteins that interact with Neb-C, a human brain cDNA library cloned into pACT2 was screened using the yeast two-hybrid technique and the C-terminal fragment of the nenulin (Neb-C) into pGBKT7 as the bait. Out of 3.8×10^7 transformants, it was obtained >1,000 colonies that were positive for the expression of the selection markers (*HIS3*, *ADE2*, and *MEL1*). Primarily, about 400 colonies was selected and grown on selective plates. The pACT2 plasmids containing the cDNA sequence of the putative Neb C interaction partner were recovered form the yeast cells. From these, thirteen clones were analysed by activity of the α-galactosidase assays. DNA sequencing revealed that one of the colonies identified to a novel RNF125 isoform. A database search revealed that the partial cDNA contains 914 bp which encode for a protein of 161 amino

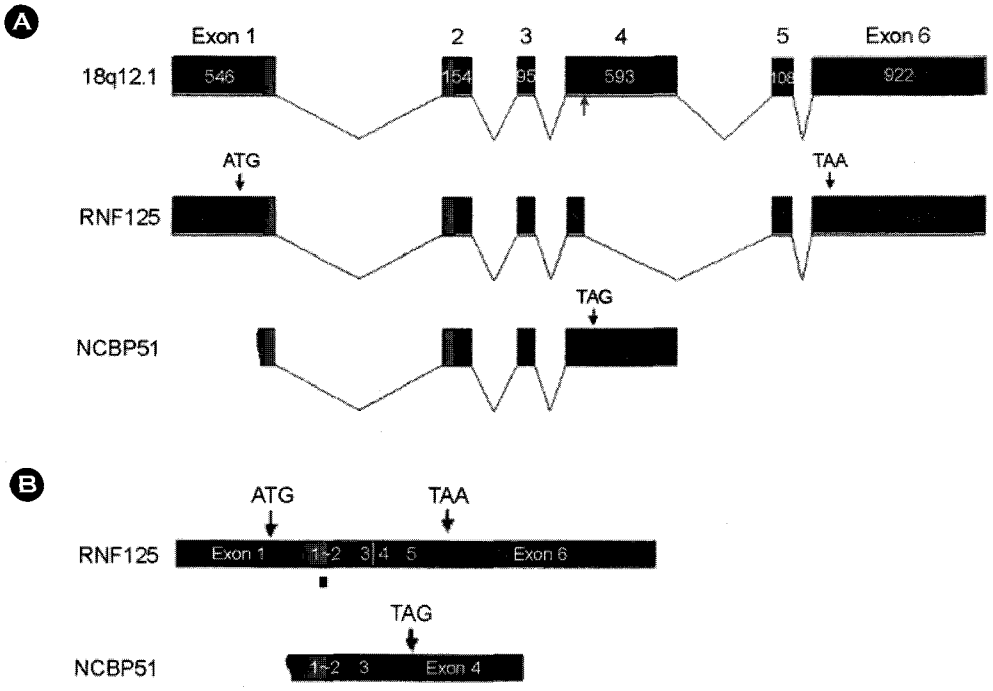

Fig. 3. Schematic presentation of genomic and cDNA of RNF125 and NCBP51. **(A)** The RNF125 is composed of six exons and five introns. The exon sizes (in bp) are shown in the six boxes. The locations of the translation start and stop codons (marked by black arrows) are within the first and sixth exons in RNF125 and stop codon is the fourth exon in NCBP51. **(B)** The relative locations of the RING domain is shown in red box.

acid (aa) containing a RING-finger domain and 3'-UTR of 431 bp (Fig. 2).

3. Sequence analysis of NCBP51

RNF125 was located on chromosome 18q12.1 and composed of six exons and five introns. The translation start and stop codons are within the first and sixth exons in RNF125 and the stop codon is the fourth exon in NCBP51. Especially, RNF125 was spliced in fourth exon (Fig. 3).

4. Human Neb-C interacts with NCBP51

To further confirm that Neb-C interacts with NCBP51, yeast strain AH109 was cotransformed with a bait plasmid (either pGBKT7-Neb-C or pGBKT7) and a prey plasmid (either pACT2-NCBP51 or pACT2). Double transformants were fist plated on dropout medium lacking leucine and tryptophan (SD/-Leu/-Trp) to select for both the bait and prey plasmids. The cotransformants were then plated on dropout medium lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp/X-α-gal). As shown in Fig. 4, yeast cells transformed with both pGBKT7-Neb-C and pACT2-NCBP51 grew on the SD/-Ade/-His/-Leu/-Trp/X-α-gal plate, as a positive control (p53, SV40), while the other double transformants did not.

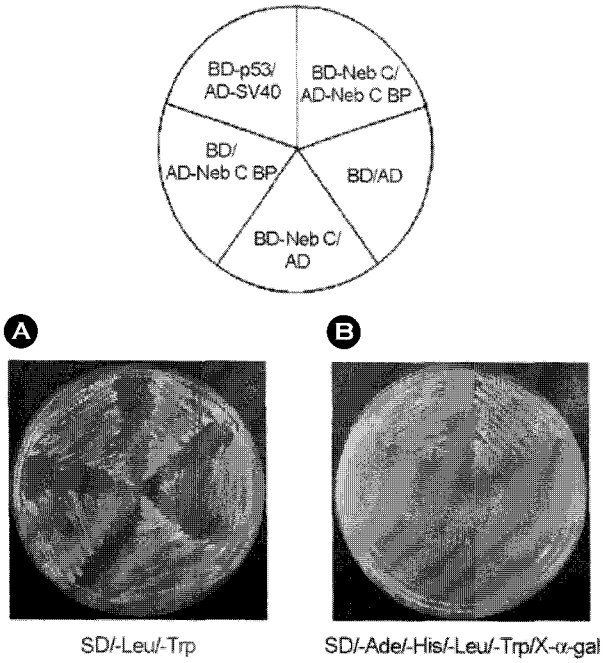

Fig. 4. Yeast two-hybrid analyses of the interaction of human Neb-C and NCBP51. Growth of transformed yeast on selective plates. Yeast strain AH109 was cotransformed with pGBKT7-X and pACT-Y incorporating the indicated constructs (up). Plasmid pGBKT7-X encodes a fusion of the GAL4 BD (aa 1-147) and a protein X, whereas plasmid pACT2-Y encodes a fusion of the GAL4 AD (aa 768-881) and a protein Y. Plasmids pGBKT7-53 and pACT2-SV40 large T antigen were transformed as a positive control because p53 protein is known to interact with SV40 large T-antigen. Transformed AH109 cells containing both plasmids were streaked out on plates lacking leucine and tryptophan (SD/-Leu/-Trp) or plastes lacking adenine, histidine, leucine, and typtophsn (SD/-Ade/-His/-Leu/-Trp/X-α-gal).

5. GST pull-down assays of human Neb-C and NCBP-51

To characterize the interaction of Neb-C and NCBP51 *in vitro*, it was carried out GST pull-down assays using GST-fused NCBP51. *In vitro* transcription/translation, Neb-C gene was cloned into the pGBKT7 expression vector and sequenced before expression. One microgram of plasmid was used in the TNT Quick coupled transcription/translation system and detected by the Transcend non-radioactive translation detection system. The proteins were migrated at an approximately size of 57 kDa. GST-NCBP51 or GST alone was incubated with the *in vitro* translation products of Neb-C and glutathione-Sepharose 4B beads. After centrifugation, the proteins bound to the glutathione-Sepharose beads were separated by 12% polyacrylamide/SDS gels. Proteins were transferred to PVDF membranes followed by detection with

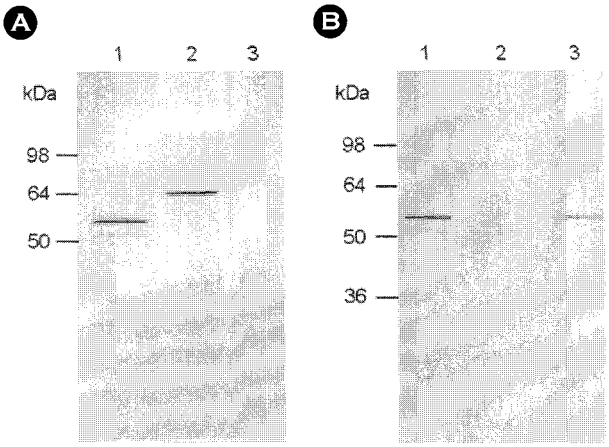

Fig. 5. *In vitro* transcription and translation and GST pull-down assays. **(A)** *In vitro* transcription/translation. Neb-C gene was cloned into the pGBKT7 expression vector and sequenced before expression. One microgram of plasmid was used in the TNT Quick coupled transcription/translation system and detected by the Transcend non-radioactive translation detection system. Lane 1, product of Neb-C; lane 2, control product of T7 Luciferase Control DNA; lane 3, negative control. **(B)** GST pull-down assay. *In vitro*-translated Neb-C bound to glutathione-Sepharose 4B beads in the presence of GST-NCBP51 fusion protein. Lane 1, In vitro-translated Neb-C (Input); lane 2, *in vitro*-translated Neb-C with alone GST as negative control; lane 3, *in vitro*-translated Neb-C with GST-NCBP51.

streptavidin-AP. Under these conditions, the Neb-C bound to GST-NCBP51 but not to control GST (Fig. 5).

DISCUSSION

Nebulin is a giant (600~900 kDa), modular sarcomeric protein proposed to regulate the assembly, and to specify the precise lengths of actin (thin) filaments in vertebrate skeletal muscles (Wang and Wright, 1988; Wright, et al., 1993). Nebulin recently was detected in cardiac muscle in a molecular layout identical to skeletal muscle nebulin, although at lower levels (Fock and Hinssen, 2002; Kazmierski et al., 2003; Donner et al., 2004). Cardiac and skeletal nebulins have many, if not all, conserved functions. Interestingly, our previous studies showed that the nebulin transcripts were detected in non-muscle, brain and liver in the human (Joo et al., 2004). Joo et al. (2004) identified four isoforms (Type IIa, III, VIIb and VIII) of simple repeat region in human brain. In this studies, nebulin C-terminus was identified newly in human brain cDNA library, it contains two 31-residue modules (exons 165 and 166: Type IIa) in simple repeat, serine rich and SH3 domain (Fig. 1). The nebulin function of brain, structurally extremely different to cardiac and skeletal muscle, has been unknown.

To date, several binding partners of nebulin have been identified in skeletal muscle. Several groups identified nebulin as the prime candidate molecule for functioning as a ruler to specify the precise lengths of the thin filaments in skeletal muscle. The three extreme N-terminal modules (M1-M2-M3) of nebulin bind to tropomodulin (McElhinny et al., 2001), the nebulin modules M160 to M170 interact with desmin (Bang et al., 2002), and nebulin SH3 domain binds to myophallodin (Bang et al., 2001) and titin (Politou et al, 2002; Ma and Wang, 2002). Linker + SeR fragment was revealed that it binds to archvillin by yeast two-hybrid recently. McElhinny et al., (2003) suggest that this connection may be critical for many important cellular processes including thin filament length regulation, muscle contraction, signaling events, and force generation, as well as other not yet defined functions. So far, it remained unknown which proteins are interacted with nebulin in brain.

Nebulin C-terminus, contains simple repeat, serine-rich and SH3 domain, was identified that it interacts with NCBP-51 by using a yeast two-hybrid screening (Fig. 4). A blast search revealed that the partial cDNA was similar to the RNF125 (RING finger protein 125). RNF125 has been just known the sequence and located in chromosom 18 and it composed of six exons and five introns. As the results of genomic cDNA analysis, NCBP51 revealed a new isoform of RNF125 and it consists of exon 1-4 contained 3'UTR (Fig. 3).

RING was first described in the sequence of the human ring 1 gene (Freemont et al., 1991) and was later found in a large number of proteins through sequence comparison analysis. These proteins are involved in variety of functions such as oncogenensis, signal transduction, gene regulation, cellular differentiation, peroxisome biogenesis, viral infection, development, tansciptional repression, and ubiquitination (Saurin et al., 1996; Borden, 2000).

Another family of RING finger proteins is directly involved in the regulation of vesicular transport. The yeast RING finger protein Vac1 has been implicated in the regulation of vesicle docking and fusion via interactions with members of the syntaxin and Sec1 family (Burd et al., 1997). EEA1 and Hrs are early endosome-associated RING finger protein (Mu et al., 1995; Komade et al., 1997). Neurodap1 has been implicated in protein sorting from Golgi to the postsynaptic density in neurons (Nakayama et al., 1995). Rim1 is a neuron-enriched RING finger protein localized to presynaptic active zones and has been implicated in nuerotransmitter exocytosis (Wang et al., 1997). ARD1 family of proteins involved in the regulation of vesicular trafficking (Mishima et al., 1993). MURF-1, MURF-2 and MURF-3 are a specific class of RING finger proteins that are expressed in striated muscle tissues. MURF-1 has been suggested to act as an ubiquitin ligase, thereby controlling proteasome-dependent degradation of muscle proteins. *In vitro*, the RING finger domain of MURF-1 acts as an E3-dependent ubiquitin ligase, while studies *in vivo* in a MURF-1-deficient mouse model demonstrated that limb muscle degeneration after lesion of the ischiatic nerve was decelerated by 36%. The most recent MURF-1 studies suggested that it catalyze titin, nebulin, MLC-2, troponin-I ubiquitination (Witt et al., 2005).

In this study, NCBP51 was identified as a new isoform of RNF125 and interacts directly with nebulin C-terminus. NCBP51 biding to the C-terminus of the human brain nebulin was demonstrated in the yeast two-hybrid screening (Fig. 4), a finding that was confirmed by GST pull-down assays (Fig. 5).

This is the first report to demonstrate the interaction of human brain nebulin with NCBP51 and to identify a new isoform of RNF125. NCBP51 belongs to a family of the RING finger proteins and the function of NCBP51 in brain remain to be identified. Studies are now in progress to elucidate the physiological interaction between these proteins and the loss-of-function of RNF125 or nebulin using the SiRNA technique in brain. Further studies will be necessary to clarify the role of the nebulin and NCBP51 in barin.

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