J. Exp. Biomed. Sci. 12 (2006) 73-79

Desmin Binding Property of Nebulin Isoforms

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Nebulin is a giant (600~900 kDa), modular sarcomeric protein proposed to regulate the assembly, and to specify the precise lengths of actin filamints in vertebrate skeletal muscles. Recently, There is an evidence that the nebulin also expressed in non muscle tissue, brain and liver. We identified a new isoform of nebulin from adult brain library by PCR screening. It contains two simple-repeats exon 165, 166 and linker-repeats exon 154~161 except exon 159. The nebulin modules M160 to M170 (exon 150 to exon 161) has been shown to bind desmin. In mature striated muscle, desmin intermediate filaments surround Z-discs and link individual myofibrils laterally at their Z-discs and to other intracellular structures, including the costameres and the intercalated discs of the sarcolemma, sarcoplasmic reticulum, mitochondria, T-tubules, and nuclei. Therefore, it is an interesting possibility that the differential splice pathways within the linker region of nebulin modify the affinity of nebulin's interaction with desmin. The specific interactions of nebulin and desmin were confirmed *in vivo* by yeast two hybrid experiments. To verify in the cellular level the interaction between nebulin isoform and desmin, we transfected COS-7 cell with EGFP-tagged nebulin and DsRed-tagged desmin. Based on evidence showing that despite exon 159 was deleted, the new isoform of nebulin was interact with desmin. This suggest that nebulin in brain may interact with another intermediate filament. The conservation of these ligand-binding capacity in brain and skeletal nebulins suggest that nebulins may have conserved roles in brain and skeletal muscle.

Key Words: Nebulin, Desmin, Intermediate filament, Cytoskeleton

INTRODUCTION

Nebulin is a giant (600~900 kDa) muscle protein expressed predominantly in the thin filaments of striated muscle (Donner et al., 2004). Nebulin's unique properties have established it as the prime candidate for a thin filament template (Littlefield and Fowler, 1998). Single molecules of nebulin span the entire length of the mature thin filament (Trombitas et al., 2001). The C-terminal end of nebulin is partially inserted into the Z-line, whereas its N-terminal end extends to the thin filament pointed ends (McElhinny et al., 2003). Alternative splicing in the central and C-terminal regions results in the expression of various nebulin isoforms in different skeletal muscle types, developmental stages and species, that may be altered in disease (Labeit and Kolmerer, 1995). The molecular size of nebulin isoforms correlates with thin filament length variations in different skeletal muscle types, supporting the hypothesis that nebulin functions as a molecular template to specify the lengths of the thin filaments (Trombitas et al., 2001).

The central region of nebulin is made up of 185 repeats that are each, 35 amino acid residues in length; these modular repeats are referred to as M1-185 and constitute 97% of the molecule (Fig. 1A) (Labeit and Kolmerer, 1995). Within

*Received: March 7, 2006
Accepted after revision: May 10, 2006
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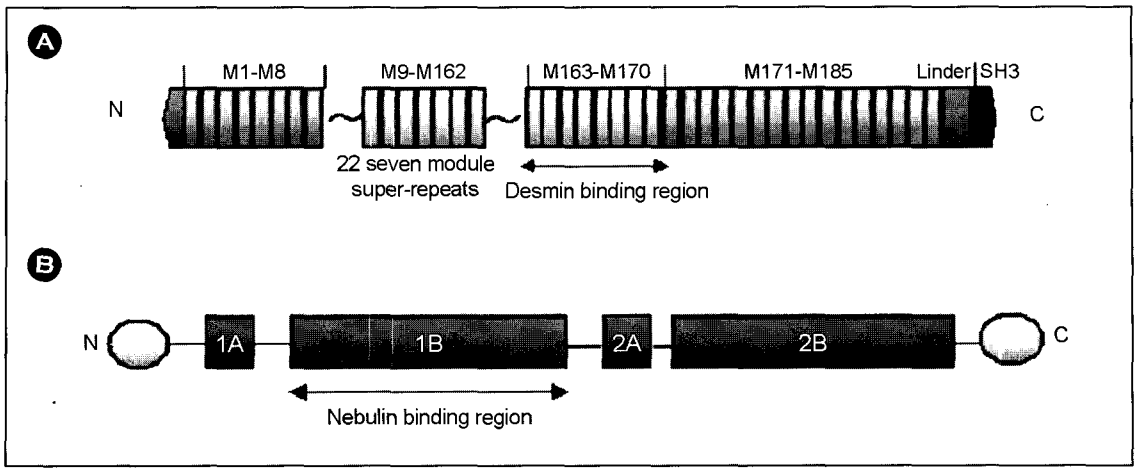

Fig. 1. The schematic structure of the nebulin and desmin. **(A)** The nebulin molecules is part of and extend the entire length of the thin filaments. **(B)** Desmin is a muscle-specific protein that is decoded by a single gene assigned to human chromosome 2q35.

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. Biochemical, structural, and biophysical studies suggest that a single nebulin module interacts with a single actin monomer and each nebulin super repeat interacts with a troponin-tropomyosin regulatory complex of the thin filament (Holmes et al., 1990; Jin and Wang, 1991; Chen et al., 1993; Pfuhl et al., 1996). 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 motifs. 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). Additionally, nebulin's extreme 20 kDa C-terminus contains a serine-rich domain with potential phosphorylation sites and a src homology 3 (SH3) domain, suggesting that nebulin is involved in signaling events within the Z-line (Labeit and Kolmerer, 1995). In this regard, nebulin's SH3 domain binds to myopalladin, an interaction that appears to be critical for myofibril assembly and/or stability (Bang et al., 2001; Clark et al., 2002). The ~50 kDa C-terminal region of nebulin also extends into the Z-line lattice in skeletal muscle: SH3 domain of nebulin is localized ~25 nm inside the Z-line, whereas the more N-terminal repeating modules of nebulin are located at the periphery of the Z-line (Millevoi et al., 1998).

Nebulin makes up what often is referred to as the fourth filament system of myofibrils. Although nebulin was discovered 2 decades ago, it remain one of the least understood molecules of striated muscle. The full-length nebulin revealed that expression of nebulin mRNAs in cardiac tissue, during both early and late stages of development (Fock and Hinssen, 2002). Furthermore, it has revealed that nebulin is also expressed in brain, heart, stomach and liver of 15-day-old chicken embryo and in hrain of human (Joo et al., 2004) Recently, there is an evidence that the nebulin also expressed in non muscle tissue, brain and liver in the human (Joo et al., 2004). However the function of nebulin in brain is still unknown.

Desmin is the intermediate filaments (IF) protein occurring exclusively in skeletal, cardiac, and smooth muscles and can be more concentrated in some particular structure, such as dense bodies, around the nuclei, around the Z-line or in costameres (Yassemi et al., 1984). Desmin is highly conserved (80%) among different members of the intermediate filament protein gene superfamily. Desmin, like other members of the IF family, is organized into four alpha-helical rods, 1A, 2A, 1B, and 2B, separated by linkers and flanked by non helical head and tail domain (Fig. 1B) (Fuchs and Weber, 1994). Desmin has a less conserved IF head domain, and the end domains are over-all quite variable among IFs and rod domain, which is highly conserved among the various IFs, is important for dimer formation, the first step in filament synthesis.

Many molecules have been reported to associate with desmin, such as other IF proteins, nebulin, the actin and tubulin binding protein plectin, the molecular motor dynein

Table 1. PCR primers

	Gene	Sequence		
Chong24	λTriplEx2 vector	GAGCCCTTCGCGCGGTAACACAACCA		26 mer forward
HCII	Nebulin X83957 to 19733	CGTTGGGTCTCCCTCACCCG		20 mer reverse
Joo-07	Nebulin X83957 from 17283	AGGAATCCTGAAATACAAAGAGAAACATG	EcoRI	30 mer forward
Joo-08	Nebulin X83957 to 18578	CGGGATCCCGCTGACTGGCAATATCAGTAG	BamHI	30 mer reverse
Joo-10	Desmin U59167 from 79	CGGAATTCCCCATGAGCCAGGCCTACTCGT	EcoRI	30 mer forward
Joo-11	Desmin U59167 to 974	CGGGATCCCGCTTCGACTTGTACCACTCCT	BamHI	30 mer reverse

the gene regulatory protein MyoD, DNA, the chaperone αB-crystallin, and protease such as calpain and caspase (Yassemi et al., 1984). Suggested function include myofibrillogenesis, mechanical support for the muscle, mitochondrial localization, gene expression regulation, and intracellular signaling (Yassemi et al., 1984). The identification of an interaction between the nebulin repeats M163-M170 with desmin in the periphery of the Z-line suggests that nebulin is involved in linking the myofibrillar Z-line to the intermediate filament system. This interaction likely contributes to the formation of lateral linkages between individual myofibrils.

We identified a new isoform of nebulin from adult brain library by PCR screening. It contains two simple-repeats exon 165, 166 and linker-repeats exon 154~161 except exon 159. The nebulin modules M160 to M170 (exon 149 to exon 161) interact with desmin. Therefore, it is an interesting possibility that the differential splice pathways within the linker region of nebulin modify the affinity of nebulin's interaction with desmin. The purpose of this study is to confirm that deletion of exon 159 influence on binding new isoform of nebulin with desmin and guess the function of nebulin in brain.

MATERIALS AND METHODS

1. Molecular cloning

The reaction was performed on Human brain Large-insert cDNA library (Clontech) with 10X cDNA PCR reaction buffer, 10 μM of each primer (Table 1, chong 24/HC Ⅱ), 200 μM dNTPs and 50X Advantage cDNA Polymerase Mix (Clontech). PCRs were hot-started by adding all reagents except DNA polymerase, heating to 95℃ for 10 min then holding at 80℃ for 45min. Enzyme was then added and amplification was performed 35 cycles (95℃ for 1~2 min, 69℃ for 30 sec and 72℃ for 3 min). The amplified products were cloned into the pGEM-T vector (Promega). The identified clones were purified by using QIAprep Spin Miniprep Kit (Qiagen) and sequenced using Thermo Sequenase Cycle Sequencing Kit (USB) and LI-COR 4200 (LI-COR) as described by the manufacturer. Sequences were identificated using Blast search of NCBI.

2. Yeast two-hybrid interaction studies

Nebulin fragments were amplified from Human brain Large-insert cDNA library (Clontech) and desmin fragments were amplified from Human skeletal muscle cDNA library (Clontech) by PCR. The Joo-07/Joo-08 primers (Table 1) designed from the human nebulin cDNA sequence (accession no. X83957) and amplify the exon 150~161 region of nebulin, encoding the linker region of human nebulin. The Joo-10/Joo-11 primers (Table 1) designed from the human desmin cDNA sequence (accession no. U59167) and amplify the exon1-4 of desmin. Generated DNA products were inserted into pGADT7 and pGBKT7 vector (Matchmaker system 3; Clontech) to obtain GAL4-DNA AD fusions and GAL4-DNA BD fusions. The lithium acetate (LiAc)-mediated method was performed to transform DNA into yeast. The yeast competent cells (AH 109) were prepared and suspended in LiAc solution with the plasmid DNA to be transformed, along with excess carrier DNA. Polyethylene glycol (PEG) with an appropriate amount of LiAc was added and the mixture of DNA and yeast was incubated at 30℃. After incubation, DMSO was added and the cells were heat shocked at 42℃. Positive colonies appearing after 5~10 days at 30℃ were assayed for X-α-galactosidase activity on SD/-Ade/-His/-Trp/-Leu plates.

3. Fluorescence microscopy

To generate the EGFP-tagged and DsRed-tagged expression vectors, fragments of the nebulin cDNA and desmin cDNA were amplified by PCR using seqence specific pri-

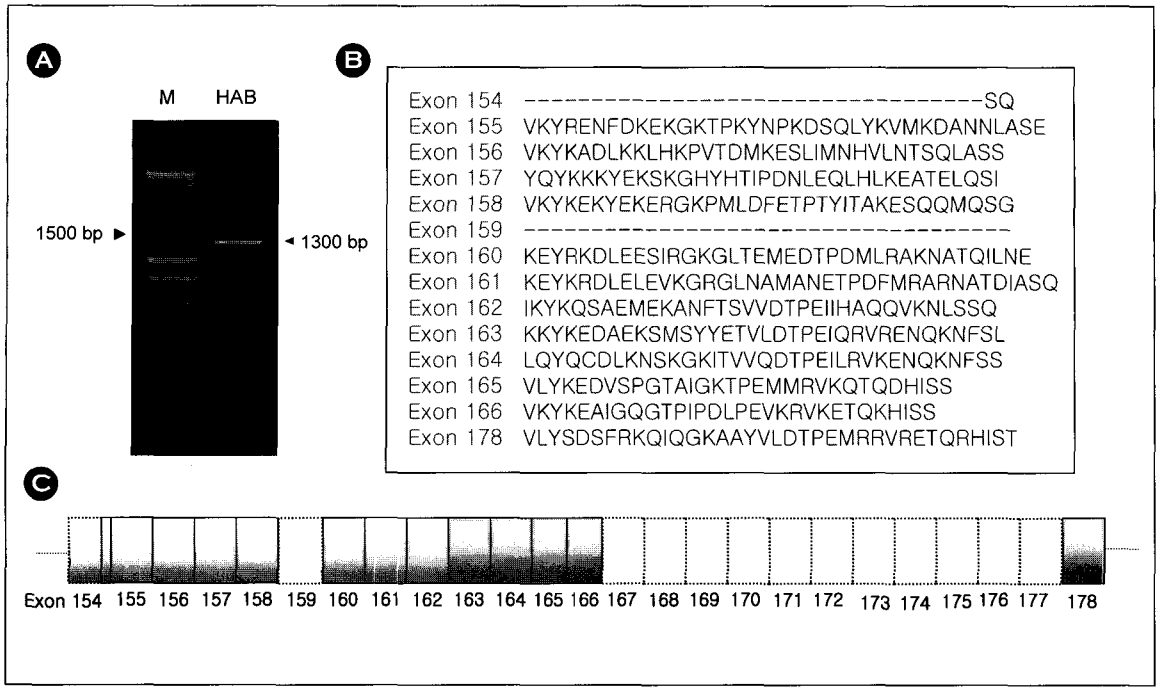

Fig. 2. Cloning of human nebulin isoform in brain. **(A)** A 1.3 kb of nebulin fragment was obtained from PCR product of human adult brain library cDNA. **(B)** Alignment of amino acid of 1.3 kb nebulin fragment. Exon 159 was spliced in human brain. **(C)** The exons of 1.3 kb nebulin fragments were aligned. Exon 167~177 were also spliced in muscle but exon 159 was only spliced in brain.

mers. Generated DNA products were inserted into pEGFP C2 vector and pDsRed1 C1 vector (Clontech). COS-7 cells, a monkey kidney fibroblast, was purchased form the Korean Cell Line Bank (KCLB #.21651). The cells were maintained in DMEM (Cambrex Bio Science Walkersvile, Inc.) supplemented with 10% fetal bovine serum. COS-7 cells were transfected with EGFP-nebulin fusion and DsRed-desmin constructs using the FUGENE 6 Transfection Reagent (Roche) according to the manufacture's instructions. The cells were incubated under 5% CO_2 atmosphere at 37℃ for approximately 24 h before processing. After fixation, cells were observed on a fluorescence microscope BX50 (Olympus). Recording of the samples was done electronically using a digital camera DP70 (Olympus) connected to DP70-BSW Version 01.02 software.

RESULTS

1. Identification of nebulin isoform in brain

A 1.3 kb fragment for nebulin was obtained from human adult brain library by PCR using primers chong 24 (λTripl-Ex2 vector sequence primer) and HCII (nebulin sequence specific primer). The identity of the PCR product was confirmed by sequencing. The partial brain nebulin sequence was identical to the skeletal muscle cDNA as determined by Blast alignment of National Center for Biotechnology Information. It contains two simple-repeats exon 165, 166 and linker-repeats 154~161 except exon 159 (Fig. 2). The alternatively spliced exon 159 gives rise to two different transcripts. Because this isoform is not present in muscle, the use of alternative transcripts might explain specific function of nebulin in brain.

2. Binding of desmin and nebulin isoform

In order to identify potential interaction with desmin and nebulin isoform, deleted the exon 159, pGADT7-nebulin and pGBKT7-desmin subfragments were cotransformed into yeast AH109. The cotransformants were grown onto SD/-Ade/-His/-Trp/-Leu plates. These results show that both nubulin linker region containing exon 159 and new isoform deleted exon 159 interact with desmin (Fig. 3A). To further confirm that desmin interacts with nebulin isoforms, α-galactosidase assay was performed (Fig. 3B). These data suggest that exon 159 wasn't necessary to interact with desmin and therefore nebulin in brain also may interact with intermediate filaments.

3. Nebulin isoform colocalization with desmin

To study in the cellular level the interaction between nebulin isoform and desmin, the EGFP-tagged nebulin and DsRed-tagged desmin were transfected into COS-7 cells. In cells expressing only EGFP, fluorescence was observed throughout the cell, like in cells expressing only DsRed. In DsRed-tagged desmin, the fluorescence signal was concentrated at the cytoplasm and nucleolus. In cells expressing EGFP-tagged nebulin, it is displayed a diffused cytosolic localization. In cells cotransfected with desmin, EGFP-nebulin staining was distributed almost completely to the same sites where desmin was present. This suggest that nebulin linker region associates with desmin (Fig. 4). In agreement with previous results in yeast two-hybrid, colocalization was observed when desmin was cotransfected with the nebulin.

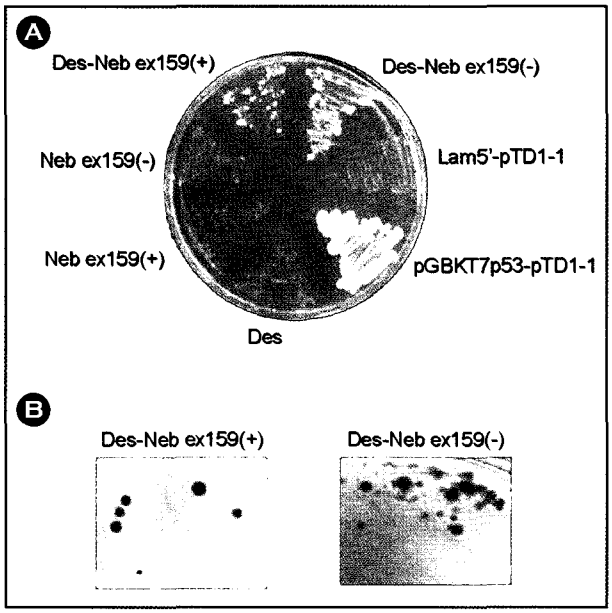

Fig. 3. Interaction of the nebulin isoform with desmin. **(A)** Nebulin isoform also interacts with desmin. As a positive control, binding of pGBKT7 p53 + pTD1-1 was tested and Lam5' + pTD1-1 as a negative control. **(B)** X-α-galactosidase assay.

DISCUSSION

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 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 (Hu et al. 1986; Locker and Wild, 1986; Wang and Wright, 1988). Recently various isoforms of nebulin have been found in human (Donner et al., 2004; Joo et al., 2004). There are four regions with alternatively spliced exons, that is, exons 63~66, 82~105, 143~144, 166~177, giving rise to a number of different transcripts. The alternatively spliced exons 143~144 give rise to two different transcripts varying bet-

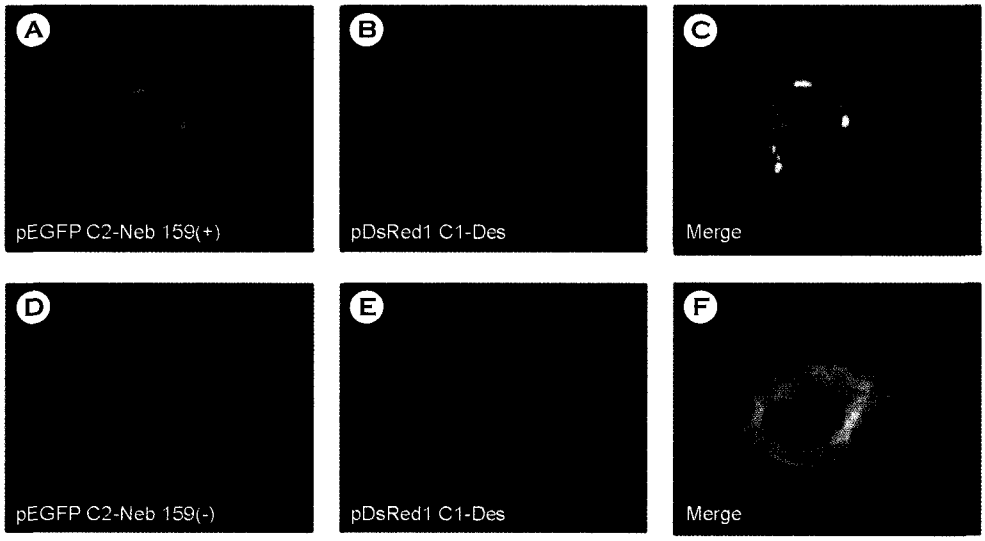

Fig. 4. Colocalization of EGFP-tagged nebulin with DsRed-tagged desmin in transfected COS-7 cells. COS-7 cells were cotransfected with pEGFP C2-Neb 159(+) and pDsRed1 C1-Des. And they were also cotransfected with pEGFP C2-Neb 159(−) and pDsRed1 C1-Des. Composite image show the superimposition of the EGFP (*green*) and DsRed (*red*) signals; areas of overlap appear *yellow*.

ween muscle types and between developmental stages (Donner et al., 2004). The alternatively spliced exons 166~177 express at least 24 different transcripts in human muscle alone (Donner et al., 2004; Labeit and Kolmerer, 1995).

In the present study, we report an identification of a new isoform of nebulin by PCR screening method in brain. It contains two simple-repeats exon 165, 166 and linker-repeats exon 154~161 except exon 159. The alternatively spliced exon 159 gives rise to two different transcripts. Because this isoform is not present in muscle, the use of alternative transcripts might explain specific function of nebulin in brain.

The exon 159 was a part of desmin binding region in nebulin and Bang et al. (2002) reported evidence for a nebulin/desmin Z-line linkage system based on *in vitro* interaction and *in situ* colocalization studies. This interaction likely contributes to the formation of lateral linkage between individual myofibrils (Bang et al., 2002). So it is an interesting possibility that the differential splice pathway modify the affinity of nebulin's interaction with desmin.

Therefore, we confirm that deletion of exon 159 influence on binding of the new isoform of nebulin with desmin. The specific interactions of nebulin and desmin were confirmed *in vivo* by yeast two hybrid experiments. To verify the colocalization between nebulin and desmin *in vivo*, we transfected COS-7 cell with EGFP-tagged nebulin and DsRed-tagged desmin.

Studies *in vivo* indicate that both nebulin linker region containing exon 159 and the new isoform deleted exon 159 interact with desmin. In cells cotransfected with desmin, EGFP-nebulin staining was distributed almost completely to the same sites where desmin was present. In agreement with the previous results in yeast two-hybrid, colocalization was observed when desmin was cotransfected with the nebulin. Based on evidence showing that despite exon 159 was deleted, the new isoform of nebulin was interact with desmin. The conservation of these ligand-binding capacity in brain and skeletal nebulins suggest that nebulins may have conserved roles in brain and skeletal muscle. The identification of an interaction between the nebulin repeats M163-M170 with desmin in the periphery of the Z-line suggests that nebulin is involved in linking the myofibrillar Z-line to the intermediate filament system (Bang et al., 2002). Therefore nebulin in brain also may interact with intermediate filament and contributes to the formation of cytoskeleton.

It appears that the new isoform of nebulin interacts with desmin. But desmin is intermediate filament protein of muscle and endothelial cells. So it is not present in non-muscle, containing the brain. There is nebulin binding region in desmin's rod domain, 1B. Rod domain is highly conserved among the various IFs. Therefore nebulin in brain may interact with another intermediate filament, vimentin, nestin, synemin, paranemin etc. The ligand of the nebulin new isoform in brain remain to be identified. The analysis of ligand binding to nebulin isoform should lead to further progress in elucidating their function in brain.

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