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Nogo-A inhibits necdin-accelerated neurite outgrowth by retaining necdin in the cytoplasm

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ABSTRACT

Nogo-A has been identified in the central nervous system as an inhibitor for axonal regeneration. Previous works have mainly focused on Nogo-A in oligodendrocytes and the roles of neuronal intracellular Nogo-A remain elusive. To gain deep insight into the physiological functions of Nogo-A, a yeast two-hybrid screening was performed with Nogo-66 as bait. We identified a new interaction between Nogo-66 and necdin. Mutagenesis analysis revealed that the central region of necdin was indispensable for the interaction of necdin with Nogo-66. The interaction was further confirmed by co-immunoprecipitation in neural tissues and cultured cortical neurons. Morphological evidence showed that Nogo-A and necdin highly colocalized in rat cortical and dorsal root ganglia neurons. Ectopic expression of Nogo-A in HEK293 cells led to retention of necdin from the nucleus to the cytoplasm. Furthermore, overexpression of Nogo-A in PC12 cells and cultured cortical neurons inhibited necdin-accelerated neurite outgrowth. Meanwhile, necdin was found to be significantly sequestered in the cytoplasm of PC12 cells stably overexpressing Nogo-A. Together, these data suggest that Nogo-A is a novel necdin binding protein and inhibits necdin-accelerated neuronal neurite outgrowth by sequestering necdin in the cytoplasm.

Introduction

The regeneration of central nervous system (CNS) axons following injury is drastically restricted by the presence of inhibitory molecules within myelin (Caroni and Schwab, 1988; Bandtlow and Schwab, 2000). Several myelin-associated proteins with axon outgrowth inhibitory activity have been identified including Nogo, which is a member of the reticulons (RTNs) and occurs in three forms, Nogo-A, Nogo-B and Nogo-C, that are generated from alternate splicing or differential promoter usage (GrandPre et al., 2000). All the three isoforms of Nogo share a 66-amino acid residue extracellular domain (Nogo-66) and a C-terminal domain, among which Nogo-A is the longest isoform (Brittis and Flanagan, 2001; GrandPre et al., 2000; Chen et al., 2000). Nogo-66 and the large specific N-terminal of Nogo-A have been well documented to possess the ability to inhibit neurite outgrowth in vitro (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000; Oertle et al., 2003).

Nogo-A was found in the developing and adult nervous system, particularly in several types of neurons. Expression of Nogo-A mRNA and protein were detected in neurons as early as E14.5 in the embryonic mouse forebrain. The widespread and embryonic expression pattern of Nogo-A, particularly expression during early developmental stages, suggests that Nogo-A proteins might have additional functions (Huber et al., 2002; Mingorance-Le et al., 2007). Several intracellular functions for Nogo-A have been reported. Overexpression of Nogo-A induced a progressive disassembly, retraction and loss of the inhibitory Purkinje cell terminals (Aloy et al., 2006). Nogo proteins have been demonstrated to play an essential role in ER formation (Voeltz et al., 2006). The absence of Nogo resulted in faster polarization and an increase in neurite branching in vitro (Mingorance-Le Meur et al., 2007). Dodd et al found that Nogo-A, -B and -C interact on the cell surface and suggested that Nogo proteins could form a channel or transporter in the ER and/or on the cell surface (Dodd et al., 2005). Thus, the important features of intracellular Nogo-A are beginning to be understood. However, intracellular functions for Nogo-A on neuronal development remain elusive.

To gain insight into physiological functions of Nogo-A, it is of considerable importance to identify new binding-partners of Nogo-A. Therefore, using Nogo-66 as bait, we employed a yeast two-hybrid screening on a human brain cDNA library. We identified several candidates that interact with Nogo-66, one of which is necdin.

Necdin was identified as a nuclear protein from differentiated embryonal carcinoma p19 cells and belongs to the MAGE (melanoma antigen) protein family, which contain the MAGE homology domain (Maruyama et al., 1991; Barker and Salehi, 2002). It is expressed in postmitotic neurons throughout the central and peripheral nervous system (Maruyama et al., 1991; Aizawa et al., 1992; Uetsuki et al.,

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1996). Necdin has been reported to suppress cell proliferation, promote neuronal differentiation, and inhibit death of several cell lines and primary neurons (Hayashi et al., 1995; Taniura et al., 1998; Kobayashi et al., 2002; Takazaki et al., 2002; Kuwako et al., 2005; Kuwajima et al., 2006). Moreover, necdin-deficient mice display several phenotypes such as postnatal lethality, impaired neuronal development, and abnormal behaviors, which are consistent with some symptoms of neurobehavioral disorder in Prader–Willi syndrome (Gerard et al., 1999; Muscatelli et al., 2000; Kuwako et al., 2005).

In the present study, we identified a novel interaction between Nogo-66 and necdin by yeast two-hybrid screening. The interaction between Nogo-A and necdin was validated by Nogo-A co-immunoprecipitation with necdin in homogenates of mouse neural tissues and cultured cortical neurons. The co-localization between necdin and Nogo-A was demonstrated in cortical neurons and dorsal root ganglia neurons. Ectopic expression of Nogo-A in HEK293 and PC12 cells led to retention of necdin in the cytoplasm. Moreover, over-expression of Nogo-A was found to inhibit necdin-accelerated neurite outgrowth in PC12 cells and cortical neurons. Our results suggest a novel role of the intracellular Nogo-A play in regulation of neurite outgrowth.

Results

Necdin is identified as a Nogo 66-associated protein in a yeast two-hybrid screening

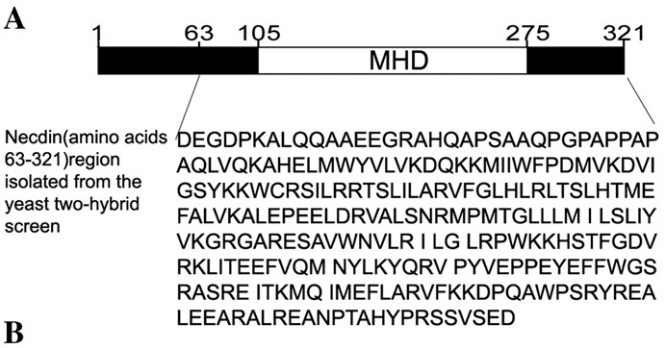
To identify new binding proteins of Nogo-A, a yeast two-hybrid screening of a brain cDNA library was performed with Nogo-66 as bait. In the screen, 52 potential positive clones were isolated based on both Leucine growth assay and β-galactosidase filter-lift assay. After eliminating repeated colonies through Alu I enzyme digestion, false positives were further eliminated by testing against LexA-lamin. Finally, the 8 positive clones were subjected to sequencing. Among them, 3 clones were identified harboring a cDNA fragment corresponding to full-length and the region of necdin(D63–D321), which contain the entire MAGE homology domain (MHD, L105–I275), as shown in Figs. 1A, B.

We next used co-imunoprecipitation to further confirm the interaction between necdin and Nogo-66 in yeast. Necdin was co-transformed into yeast strain with Nogo-66 or blank vector, and fusion proteins extracted from transformants were used to immunoprecipitate with anti-LexA antibody. HA-necdin was found to co-immunoprecipitate with LexA-Nogo-66 but not with LexA (Fig. 1C). This result confirmed that necdin can associate with Nogo-66 in yeast.

To further map which region in necdin mediates the interaction with Nogo-66, various truncated mutants of necdin were constructed (Fig. 2A), and yeast two-hybrid filter assays were used to test the binding activity. Among N-terminal truncation mutants of necdin, both necdin (P83–D321) and necdin (P95–D321) bound with Nogo-66, whereas necdin (Q100–D321) showed little binding. On the other hand, necdin (P95–I275), a COOH-terminally truncated mutant, failed to bind with Nogo-66, but necdin (P95–W290) retained the binding ability (Fig. 2A). The binding affinity between Nogo-66 and necdin mutants was further quantified by liquid culture activity assay (Fig. 2B). Together, these results suggested that the central region of necdin (amino acids P95–W290), including the entire MHD (amino acids L105–I275), is indispensable for the association with Nogo-66.

Nogo-A interacts with necdin in neural tissues and cultured cortical neurons

To examine whether Nogo-A and necdin form a natural complex in mammalian tissues, we performed immunoprecipitation assays with homogenates from different tissues of adult mouse including brain, spinal cord, and spleen. Endogenous necdin was detected to exist in spleen although the level is lower than that in brain and spinal cord. Meanwhile, Nogo-A was not detected in spleen (Fig. 3A). The homogenates were immunoprecipitated with anti-necdin antibody and the precipitate was then immunoblotted with anti-necdin or anti-Nogo-A antibodies. As shown in Fig. 3B, Nogo-A could co-immunoprecipitate with necdin in brain and spinal cord but not in spleen, although necdin was expressed in all these tissues. Furthermore, the homogenates were immunoprecipitated with anti-Nogo-A antibody



B

Bait	Prey	Filter assay
pGilda-53	pB42AD-T	+
pGilda-nogo66	pB42AD	-
pGilda-nogo66	pB42AD-necdin	+
pGilda-nogo66	pB42AD-necdin(63-321)	+
pGilda-Lamin	pB42AD-necdin	-
pGilda	pB42AD	-

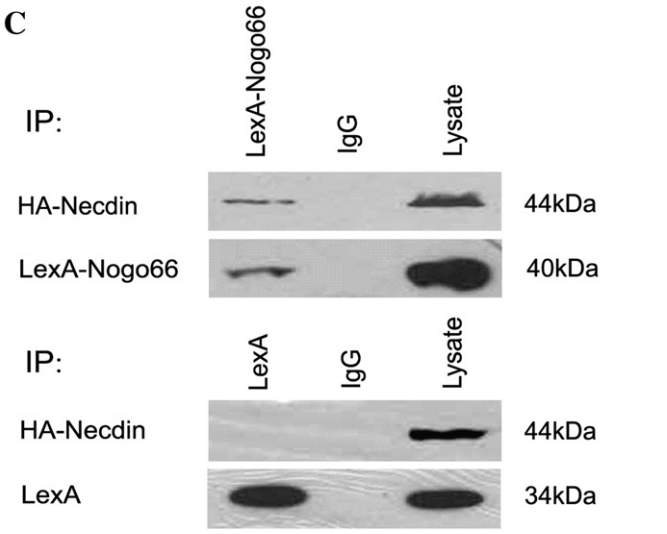


Fig. 1. Interaction between Nogo-66 and necdin in yeast. (A) Schematic presentation of the necdin protein. The human necdin segment (amino acids 63–321) containing the MHD was isolated from the two-hybrid screen. MHD, the melanoma antigen (MAGE) homology domain. MHD of human necdin protein contains amino acids (L105–I275). (B) Interaction of Nogo-66 in yeast by filter assay. The isolated segment and full-length necdin were co-transfected into yeast reporter strain EGY48 with the bait encoding Nogo-66 or pGilda vector, respectively. Filter assay was performed for β-galactosidase activity analysis. (C) Immunoprecipitation assay was used to detect association of necdin with Nogo-66 in yeast. The lysates from the EGY48 co-transformed pGilda-Nogo-66 (upper) or pGilda vector (down) with pB42AD-Necdin, which was constructed by inserting the full-length cDNA of human necdin into pB42AD plasmid, were immunoprecipitated by anti-LexA antibody and blotted by anti-HA antibody or anti-LexA antibody. IgG was serving as control, and lysates were used to detect expression of HA and LexA proteins.