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Rational design, solution conformation and identification of functional residues of the soluble and structured Nogo-54, which mimics Nogo-66 in inhibiting the CNS neurite outgrowth

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ABSTRACT

The interaction between Nogo-66 and its receptor NgR represents a promising target for designing drugs to treat CNS axonal injury which often leads to permanent disability. Unfortunately, the isolated Nogo-66 is highly insoluble while its truncated form Nogo-40 is soluble but unstructured, thus retarding further characterization and application. Here, we rationally design another soluble form Nogo-54. CD and NMR characterization reveals that Nogo-54 is structured, and importantly, is able to mimic Nogo-66 in inhibiting neurite outgrowth. Strikingly, mutating its C-terminal four residues (Lys50, Glu51, Arg53, and Arg54) leads to a mutant Nogo-54m which has no dramatic structural change but whose inhibitory activity is completely abolished. This strongly suggests that the four charged residues contribute significantly to the inhibitory action of Nogo-66. Furthermore, our study also provides a soluble and structured mimic as well as a possible antagonist for Nogo-66 which may hold promising potential for various medical applications.

Patients with central nervous system (CNS) injuries such as spinal cord injury, traumatic brain injury, and stroke often result in permanent disability due to the inability of CNS neurons to regenerate axons after injury. Recent discoveries indicate that the failure of CNS regeneration largely results from the presence of inhibitory molecules of axon outgrowth in adult CNS myelin [1–3]. So far three myelin inhibitory proteins have been identified, namely, Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). Strikingly, all three molecules appear to initiate their inhibitory action via binding to the Nogo-66 receptor (NgR). Of the three myelin-associated molecules, the CNS enriched Nogo belonging to the reticulon protein family is composed of three splicing variants, namely, the 1192- residue Nogo-A, 373-residue Nogo-B, and 199-residue Nogo-C. Despite their size difference, all three Nogo variants contain a conserved extracellular domain called Nogo-66 with ~66 residues that is capable of inhibiting neurite growth and inducing growth cone collapse. This Nogo-66 inhibitory domain has been shown to be anchored on the oligodendrocyte surface and to bind NgR with a very high affinity [1–4]. Therefore, intervention in the Nogo–NgR binding provides an unprecedented opportunity for developing therapeutic agents to prompt adult CNS axonal regeneration. Such agents could also be used more broadly to repair damaged CNS axons resulting from neurodegenerative diseases such as multiple sclerosis [1–6].

Unfortunately, the high insolubility of the Nogo-66 recombinant protein has largely retarded the effort to investigate its structure–activity relationship and to further design antagonists. Currently its truncated form Nogo-40 has been demonstrated to be a competitive NgR inhibitor which is able to attenuate the inhibitory effects of myelin on neurite outgrowth and promote functional recovery and long-range axonal regeneration in an animal model of spinal cord injury [1]. However, being highly unstructured may significantly reduce the antagonistic activity of Nogo-40 [7]. Therefore, it is of fundamental and medical interests to design both structured and soluble Nogo-66 mimics as well as antagonists.

Very surprisingly, we recently found that all buffer-insoluble proteins we have including Nogo-66 can be easily solubilized in salt-free water to a high concentration [8–12]. This discovery therefore allowed our determination of the first structure and

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dynamics of Nogo-60 in aqueous solution [8]. Based on the obtained structured, we succeeded in rationally designing a buffer-soluble form designated as Nogo-54 by deleting the last six unstructured hydrophobic residues. Moreover, we have conducted detailed NMR and activity analysis of Nogo-54. The results indicate that Nogo-54 still has a helical conformation highly similar to that of the corresponding region of Nogo-60. More importantly, Nogo-54 is able to mimic Nogo-66 in inhibiting neurite outgrowth. Based on these results, we further designed a mutant Nogo-54m by replacing the C-terminal four charged residues (Lys50, Glu51, Arg53 and Arg54) with Ala. Very strikingly, Nogo-54 m shows no dramatic structural change but its inhibitory activity on neurite outgrowth was completely abolished.

Materials and methods

Cloning, expression, and purification of Nogo-54 and Nogo-54m. The human Nogo-A cDNA (designated KIAA 0886) was obtained from the Kazusa DNA Research Institute (Kazusa-Kamatari, Kisarazu, Chiba, Japan). PCR reactions with the primers (N1: 5′-CGC GCG CGC GGA TCC AGG ATA TAC A AG GGT G TGA T-3′, N2:5′-CGC GCG CGC CTC GAG CTA GCG CCT GAG TTC CTT TAT-3′) were used to generate DNA fragment encoding the human Nogo-54, corresponding to the Nogo-A residues 1055–1108. The PCR fragment was subsequently cloned into the His-tag vector pET32a (Novagen). The vector carrying the gene encoding Nogo-54m with Lys50, Glu51, Arg53 and Arg54 mutated to Ala was generated by QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). DNA sequences were verified by automated DNA sequencing. The recombinant Nogo-54 and Nogo-54m proteins were expressed in *Escherichia coli* BL21 cells and subsequently purified by Ni^{2+} affinity column and HPLC on a reverse-phase C18 column (Vydac) as previously detailed [8]. The recombinant Nogo-40 used as a control in the activity assay was produced as previously described [8]. For NMR isotope labeling, recombinant proteins were prepared by growing the cells in the M9 medium with additions of $(^{15}NH_4)_2SO_4$ for ^{15}N labeling. The identities of Nogo-54 and Nogo-54m peptides were verified by MALDI-TOF mass spectrometry.

Circular dichroism (CD) and NMR experiments. CD samples with protein concentrations of 50 μM were prepared by dissolving the lyophilized Nogo-54 and Nogo-54m in 5 mM phosphate buffer, with pH value adjusted to 5.4. CD experiments were performed on a Jasco J-810 spectropolarimeter equipped with a thermal controller [8]. The far-UV CD spectra were collected using 1-mm pathlength cuvettes with a 0.1-nm spectral resolution. Data from five independent scans were added and averaged.

NMR samples were prepared by dissolving the lyophilized Nogo-54 and Nogo-54m in 10 mM phosphate buffer, pH 5.4, with an addition of 50 μL of D_2O for NMR spin-lock. All NMR experiments were acquired on an 800 MHz Bruker Avance spectrometer equipped with pulse field gradient units at 278 K as described previously [8,13]. The NMR spectra acquired included two-dimensional ^{1}H–^{15}N HSQC, three-dimensional ^{15}N-edited HSQC-TOCSY, HSQC-NOESY. NOE connectivities were derived from a ^{15}N HSQC-NOESY spectrum. NMR data were processed with NMRPipe [14] and analyzed with NMRView [15]. Structural graphs were prepared using MolMol software [16].

Neurite outgrowth assay. Inhibitory activities of Nogo-54 and Nogo-54m on neurite outgrowth were measured as previously described [17]. Briefly, diethyl ether-cleaned glass coverslips were coated overnight at 4 ℃ with poly-L-lysine (100 μg/ml in distilled water) and then washed three times with distilled water. Purified neurons from 6- to 8-day-old mice cerebella were seeded onto the coverslips (1 × 105 cells/mL) and allowed one hour to adhere. Subsequently His-tag, Nogo-40, Nogo-54, and Nogo-54m were added in the cell culture wells at different concentrations. After cultured for 24 h, the cells were fixed for one hour by adding in 4% paraformaldehyde and stained with Coomassie brilliant blue (0.2%) for 30 min at room temperature. Coomassie brilliant blue-stained neurons were analyzed by evaluating the length of individual neurites. Only neurites that did not contact other cells or neurites and had a length of at least one cell diameter were measured. The neurite length was compared with that in the presence of His-tag. For each experimental condition, at least three independent experiments were performed in duplicate and more than 120 neurites (30 neurites/coverslip) were measured. SPSS software was used to determine the significance of differences, with $p < 0.05$ and $p < 0.01$ being considered to be significant and highly significant, respectively.

Results

Design, NMR conformation, and inhibitory activity of Nogo-54

The extracellular Nogo-66 domain was found to be highly insoluble in buffer when it was isolated from the membrane and expressed as a recombinant protein [7]. Nevertheless, our recent discovery that buffer insoluble proteins could be solubilized in pure water led to the successful determination of the Nogo-66 structure in pure water by use of NMR spectroscopy [8]. A close examination of the Nogo-60 structure reveals that the last six residues (Leu55-Phe56-Leu57-Val58-Asp59-Asp60) adopt no regular secondary structure but constitute a large hydrophobic patch, which might be responsible for its high insolubility in buffer. Therefore, we rationally designed a truncated form Nogo-54 with the six residues deleted. Indeed, Nogo-54 became buffer-soluble and preliminary CD analysis showed that it still had a similar helical conformation in both buffer and pure water.

We further conducted a detailed NMR characterization on Nogo-54. A pair of ^{15}N-edited heteronuclear HSQC-TOCSY and HSQC-NOESY NMR spectra was collected on a ^{15}N-labeled Nogo-54 sample at a protein concentration of ~500 μM. Analysis of the NMR data led to the sequential assignment of Nogo-54 as labeled in its HSQC spectrum (Fig. 1a). Moreover, as NMR chemical shift deviations (CSD) from those expected for random coils ($\Delta\delta = \delta_{obs} - \delta_{coil}$) are very sensitive indicators of protein secondary structures [18–19], we calculated Hα chemical shift deviations for both Nogo-60 and Nogo-54 (Fig. 1b). Interestingly, except for the C-terminal residues Glu51-Leu52-Arg53-Arg54, the majority of the Nogo-54 residues have negative Hα CSD values highly similar to those of Nogo-60, suggesting that Nogo-54 has a helical conformation very similar to that of the corresponding Nogo-60 region [8]. Furthermore, as seen in Fig. 1c, an extensive manifestation of characteristic NOEs including $d_{NN}(i, i+1)$, $d_{NN}(i, i+2)$, $d_{\alpha N}(i, i+3)$, and $d_{\alpha N}(i, i+4)$ strongly indicated that Nogo-54 assumed a well-formed α-helical conformation [20]. In particular, the high similarity of the NOEs patterns of Nogo-54 and Nogo-60 [8] revealed that Nogo-54 also assumed a well-formed helical conformation constituted by three helical segments as observed in the Nogo-60 structure [8]. Taken together, the CSD values and NOE pattern of Nogo-54 provide residue-specific evidence that the deletion of the last six residues induced no major change in the Nogo-54 structure.

To assess whether Nogo-54 still retained the inhibitory activity on neurite outgrowth, we have measured its effect on neurite outgrowth of the mice cerebella neurons, with inclusion of His-tag, Nogo-40 as controls. While His-tag, Nogo-40 showed no statistical difference from that of the blank even at a high dose, Nogo-54 demonstrated a dose-dependent inhibitory effect on neurite out-