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NUCLEOTIDE AND PROTEIN SEQUENCES OF NOGO GENES AND METHODS BASED THEREON

This application claims priority to United States provisional application no. 60/107,446, filed November 6, 1998, which is incorporated by reference herein in its entirety.

1. INTRODUCTION

The present invention relates to the gene, *Nogo*, and in particular to Nogo, its encoded protein products, as well as derivatives and analogs thereof. Production of Nogo proteins, derivatives, and antibodies is also provided. The invention further relates to therapeutic compositions and methods of diagnosis and therapy.

2. BACKGROUND OF THE INVENTION

In the central nervous system (CNS) of higher vertebrates, regeneration of axons after injury is almost absent and structural plasticity is limited. Growth inhibitors associated with CNS myelin are likely to play an important role. This is evidenced by a monoclonal antibody (mAb), IN-1, that neutralizes a potent neurite growth inhibitory myelin protein, thereby promoting long-distance axonal regeneration and enhancing compensatory plasticity following spinal cord or brain lesions in adult rats.

A number of *in vitro* and *in vivo* observations have revealed a new aspect of neurite growth regulation which is the presence of repulsive and inhibitory signals and factors (Keynes and Cook, 1995, Curr. Opin. Neurosci. 5:75-82). Most of these signals seemed to be proteins or glycoproteins. A first breakthrough towards identification of the factors was the purification and cDNA cloning of a chick brain-derived growth cone collapse inducing molecule, Collapsin-1, now called Semaphorin 3A.

A second group of repulsive guidance cues recently purified and cloned is now designated as Ephrins. They are ligands for the Eph receptor tyrosine kinase family. Ephrin-A5 and Ephrin-A2 are expressed as gradients in the optic tectum of the chick embryo, and their ectopic expression or deletion causes guidance errors of ingrowing retinal axons. Like the Semaphorins, the Ephrin family has 15 to 20 members, each with a complex and dynamic expression in and outside of the nervous system. The functions of most of these molecules remain to be analyzed.

A third group of guidance cues which can repulse growing axons and is expressed in the developing nervous system are the Netrins. Netrin has been purified as a floor plate

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derived chemoattractant for commissural axons in early spinal cords, like its C. elegans ortholog unc-6. Netrin-1 turned out to have repulsive effects for certain types of neurons depending on the type of receptor present on the target neuronal growth cones (Tessier-Lavigne et al., 1996, Science 274:1123-33).

Previously, a potent neurite growth inhibitory activity associated with adult CNS oligodendrocytes and myelin was reported by Canoni and Schwab (1988, J. Cell Biol. 106:1281-1288). A major constituent is a high molecular weight membrane protein (NI-250, with a smaller component, NI-35, in rat) which was recently purified, and which is related to the subject of the present invention, and is bound by the neutralizing mAb, IN-1 (Canoni and Schwab, 1988, J. Cell Biol. 106:1281-1288; U.S. Patent Nos. 5,684,133; 5,250,414; PCT Publication WO 93/00427).

Myelin-associated neurite growth inhibitors play a crucial role in preventing regeneration of lesioned CNS axons. When oligodendrocyte development and myelin formation is blocked in chicken or rats, the regeneration permissive period following CNS lesions is prolonged. Indeed, myelin formation coincides in time with the end of the developmental period where the CNS shows high structural plasticity and a high potential for regeneration.

NI-250 and NI-35 are likely to be major components of the myelin-associated growth inhibition as evidenced by *in vivo* application of IN-1 to spinal cord lesioned adult rats which induced regeneration of corticospinal axons over long distances and allowed motor and behavior functional recovery especially with regard to locomotion. Similar experiments on the optic nerve and the cholinergic septo-hippocampal pathway also demonstrated the *in vivo* relevance of the IN-1 recognized antigen, NI-35/250 (Schnell and Schwab, 1990, Nature 343:269-272; Bregman et al., 1995, Nature 378:498-501).

Unlesioned fiber systems also respond to the neutralization of neurite growth inhibitors by IN-1. Recent experiments have conclusively shown that following a selective corticospinal tract lesion (pyramidotomy), intact fibers sprout across the midline in the spinal cord and brainstem and establish a bilateral innervation pattern, accompanied by an almost full behavioral recovery of precision movements in the presence of IN-1 (Z'Graggen et al., 1998, J. Neuroscience 18(12):4744-4757).

Isolation of the gene that encodes the neurite growth inhibitory protein provides multiple opportunities for developing products useful in neuronal regeneration and in treatment of various neurological disorders, including CNS tumor.

Citation of a reference hereinabove shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to nucleotide sequences of *Nogo* genes (human, rat and bovine *Nogo* and *Nogo* homologs of other species), and amino acid sequences of their encoded proteins, as well as derivatives (*e.g.*, fragments) and analogs thereof. Nucleic acids hybridizable to or complementary to the foregoing nucleotide sequences are also provided. In a specific embodiment, the Nogo protein is a rat, bovine or human protein.

The invention also relates to a method of identifying genes which interact with *Nogo*.

Nogo is a gene provided by the present invention, identified by the method of the invention, that both encodes and interacts with neural growth regulatory proteins.

The invention also relates to *Nogo* derivatives and analogs of the invention which are functionally active, *i.e.*, they are capable of displaying one or more known functional activities associated with a naturally occurring Nogo protein. For example, a major inhibitory region between amino acids 542 to 722 have been identified. Such functional activities include, but are not limited to, neurite growth inhibition of neural cells, spreading and migration of fibroblasts, or any cell exhibiting neoplastic growth, the ability to interact with or compete for interaction with neural growth regulatory proteins, antigenicity which is the ability to bind (or compete with Nogo for binding) to an anti-Nogo antibody, immunogenicity which is the ability to generate antibody which binds to Nogo. These antibodies having the potential to induce neurite outgrowth or prevent dorsal root ganglia growth cone collapse by inhibiting the function of Nogo, and functional fragments or derivatives of Nogo, with the ability to inhibit neurite outgrowth.

The invention further relates to fragments (and derivatives and analogs thereof) of Nogo which comprise one or more domains of a Nogo protein such as the acidic and proline rich amino terminus (*e.g.*, at amino acids 31 to 58 of SEQ ID NO:2), the highly conserved carboxy terminus, and two hydrophobic stretches of 35 and 36 amino acids length in rat Nogo, also in the carboxy terminus (*e.g.*, at amino acids 988 to 1023, and at 1090 to 1125 of SEQ ID NO:2).

Antibodies to the various Nogo, and Nogo derivatives and analogs, are additionally provided. In particular, by way of example, two antibodies have been derived, the first antibody, termed AS 472, was derived using as immunogen a synthetic peptide corresponding to amino acids 623 to 640 of SEQ ID NO:2, and the second antibody, termed AS Bruna, was generated against the carboxy-terminus, amino acids 762 to 1163 of SEQ ID NO:2, of Nogo.

Methods of production of the Nogo proteins, derivatives and analogs, *e.g.*, by recombinant means, are also provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on Nogo proteins and nucleic acids. Therapeutic compounds of the invention include but are not limited to Nogo proteins and analogs and derivatives (including fragments) thereof; antibodies thereto; nucleic acids encoding the Nogo proteins, analogs, or derivatives; and Nogo ribozymes or Nogo antisense nucleic acids.

The present invention also relates to therapeutic and diagnostic methods and compositions based on Nogo proteins and nucleic acids and anti-Nogo antibodies. The invention provides for treatment of CNS and neural derived tumors by administering compounds that promote Nogo activity (*e.g.*, Nogo proteins and functionally active analogs and derivatives including fragments thereof; nucleic acids encoding the Nogo proteins, analogs, or derivatives, agonists of Nogo).

The invention also provides for treatment of diseases, disorders or damage which ultimately result in damage of the nervous system; such diseases, disorders or damage include, but are not limited to, central nervous system (CNS) trauma, (e.g. spinal cord or brain injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, multiple sclerosis, amyotrophic lateral sclerosis, and progressive supra-nuclear palsy); by administering compounds that interfere with Nogo activity (*e.g.*, a dominant negative Nogo derivative; antibodies to Nogo; anti-sense nucleic acids of *Nogo*; *Nogo* ribozymes or chemical groups that bind an active site of Nogo).

Animal models, diagnostic methods and screening methods for predisposition to disorders, and methods to identify and evaluate Nogo agonists and antagonists, are also provided by the invention.

3.1 DEFINITIONS

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product which is indicated by the name of the gene in the absence of any underscoring or italicizing. For example, "*Nogo*" shall mean the *Nogo* gene, whereas "Nogo" shall indicate the protein product of the *Nogo* gene.

4. DESCRIPTION OF THE FIGURES

Figure 1a-1b: (a) *Nogo* cDNA clones: CWP1-3 is a bovine cDNA clone isolated from the screening of a bovine spinal cord white matter cDNA library with degenerated oligonucleotides MSC5-8 (pooled) and MSC9. Complementary RNA derived from this clone was used for subsequent rat cDNA library screening. Oli3 and Oli18 are isolated

from an oligo d(T)-primed rat oligodendrocyte library. R1-3U21, RO18U1 and RO18U37-3 are isolated from a hexanucleotides-primed rat brain stem/spinal cord library (Stratagene). The positions of the 6 bovine NI220 (bNI220) peptide sequences are indicated on CWP1-3 and R13U21. Sequences at the junctions of different exons are marked on top of each clone. The question marks indicated on ROl8U1 identify the sequence on this clone which does not match sequences from any other *Nogo* clones. RO18U37-3 was sequenced-only from the 5'-end, and the unsequenced portion is represented by dots. (b) Schematics demonstrating the hypothetical mechanism for the generation of three *Nogo* transcripts. P1 and P2 represent the putative location of the alternative promoters. The minimum number of three exons is required for generating the three transcripts as shown schematically, although each exon could potentially be split into multiple exons.

Figure 2a-2b: (a) Nucleotide (SEQ ID NO:1) and amino acid sequences (SEQ ID NO:2) of *Nogo* transcript A (sequence generated by connecting RO18U37-3, Oli18, and R1-3U21 cDNA sequences). Oval box: presumed initiation codon; underlined with dots: acidic stretch; □: potential PKC sites; Δ: potential casein kinase II sites; thick underline: carboxy terminal hydrophobic regions and potential transmembrane domains; thin underline: potential N-glycosylation sites. (b) Peptide sequence comparison between sequenced, purified bovine NI220 (bNI220; SEQ ID NOS:3-8), and the corresponding bovine (SEQ ID NOS:9-14) and rat (SEQ ID NOS:15-20) sequences translated from rat and bovine cDNAs. Rat and bovine amino acid sequences, which do not match the bNI220 peptide sequences, are in lower case.

Figure 3a-3b: (a) Amino acid sequence comparison of the carboxy terminal 180 amino acid common regions of NSP (human; SEQ ID NO:21), S-REX (rat)(SEQ ID NO:22), CHS-REX (chicken; SEQ ID NO:23), NOGOBOV (bovine; SEQ ID NO:24), NOGORAT (rat; SEQ ID NO:25), a C. elegans EST clone (W06A7A; SEQ ID NO:26), and a D. melanogaster EST clone (US51048; SEQ ID NO:27). (b) Evolutionary conservation of the two hydrophobic regions. Percent similarities within and across species of the common hydrophobic regions are shown. Shaded letters: conserved amino acids.

Figure 4a-4c: (a) Northern hybridization of various tissues with the *Nogo* common probe. The common probe contains transcript A sequence between nucleotides 2583-4678. ON, optic nerve; SC, spinal cord; C, cerebral cortex; DRG, dorsal root ganglia; SN, sciatic nerve; PC12, PC12 cell line. (b) Northern hybridization of spinal cord and PC12 cells RNAs with an exon 1-specific probe (left panel) and of hindbrain (HB) and skeletal muscle

(M) RNAs with an exon 2 specific probe (right panel). (c) Northern hybridization with the Nogo common probe. K, kidney; B, cartilage (from breastbone); Sk, skin; M, skeletal muscle; Lu, lung; Li, liver; Sp, spleen. The three major transcripts are marked (4.6 kilobases (kb), 2.6 kb, and 1.7 kb). Δ: a diffuse but consistent band about 1.3 kb in size.

Figure 5a-5f: In situ hybridization of adult rat spinal cord and cerebellum sections. (a, d) Rows of oligodendrocytes (OL) in spinal cord and cerebellum white matter, respectively, can be seen labeled by the Nogo antisense "common" riboprobe. This is very similar to the signals detected when a consecutive spinal cord section was hybridized to an antisense plop riboprobe (b). (c) Neurons in grey matter (GM) were also labeled by the Nogo antisense "common" riboprobe. WM: white matter. Bright field and fluorescent view, respectively, of a cerebellum section double labeled with the Nogo antisense "common" riboprobe (e) and of an anti-GFAP antibody (f). Purkinje cells (double arrowheads) are strongly labeled with the Nogo probe, while astrocytes (arrowheads, black and white) are negative. A few cells in the granular cell layer (Gr) are also labeled with the *Nogo* probe, m: molecular layer. Scale bar: a, b, d-f: 50 p.m; c: 280 p.m.

Figure 6a-6i: In situ hybridization of optic nerves at different postnatal days (a,f: P0; b,g: P3; c,h: P7; d,e,i: P22) with either *Nogo* or *plp* (antisense or sense) probes. (a-d) Nogo antisense probe; (e) *Nogo* sense probe; (g-i) *plp* antisense probe; (f) *plp* sense probe. *Nogo* expression in oligodendrocyte precusors can be detected as early as P0, while plp expression was only beginning to be detectable in P3 optic nerves close to the chiasm (g).

Figure 7: AS Bruna and AS 472 both recognize a myelin protein of about 200 kD. Rat myelin extract and bovine q-pool were prepared according to Spillmann et al, 1998, J. Biol. Chem., 273(30):19283-19293. AS Bruna and AS 472 each recognized a 200 kD band as well as several lower bands in bovine myelin, which may be breakdown products of bNI220. AS Bruna stained a band 200 kD in rat myelin. I: AS Bruna; P: AS Bruna, preimmune serum; E: AS 472 affinity purified.

Figure 8a-8i: Immunohistochemistry on rat spinal cord and cerebellum using IN-1 (a-e), AS Bruna (d-f), and AS 472 (g-i), as indicated at the left of each panel. A strong myelin staining was observed in both tissues with all three antibodies when the frozen sections were fixed with ethanol/acetic acid (a, b, d, e, g, h). Treatment of the sections with methanol abolished the myelin staining except for oligodendrocyte cell bodies (arrows; c, f,

i). Arrowheads: Purkinje cells, WM, white matter; GM, grey matter, DR: dorsal root; Gr, granular cell layer; m, molecular layer. Scale bar: a, d, g: 415µm; b, c, e, f, h, i: 143µm.

Figure 9a-9d: Neutralizing activity of AS 472 and AS Bruna in different bioassays. (a) the NIH 3T3 fibroblasts were plated on cell culture dishes coated with q-pool and pre-treated with IN-1, AS Bruna, AS 472 or the corresponding pre-immune sera. Both polyclonal sera showed even a slightly better neutralization of the inhibitory substrate than IN-1. The pre-immune sera had no significant effect on the spreading of the NIH 3T3 cells. Addition of an excess of the peptide (P472) that was used to raise AS 472 competed the neutralizing activity whereas an unspecific peptide (Px) had no effect. (b) Pre-treatment of the inhibitory substrate with AS Bruna or AS 472 resulted in DRG neurite outgrowth comparable to what can be observed on a laminin substrate. Examples for neurite outgrowth from DRG on q-pool pre-treated with PBS (c; score = 0) and pretreated with AS Bruna (d; score = 4).

Figure 10a-10d: Injection of optic nerve explants with AS 472 results in ingrowth of axons. (a) Pairs of adult rat optic nerves were dissected, injected with AS 472 or preimmune serum and placed into chamber cultures such that one end of the nerves was in contact with dissociated P0 rat DRG neurons. (b) After two weeks *in vitro*, EM sections of the nerves were taken at 3.5 mm from the cut site (arrows in A) and systematically screened for intact axons (3 experiments). (c) Regenerated axon bundles (arrows) grow through degenerating AS 472 injected optic nerve. (d) Regenerating axons in contact with myelin. Magnification: c, 12,000x; d, 35,000x.

Figure 11a-11c: Recombinant Nogo A expression in transfected COS cells. (a) Western blot showing immunoreactivity of AS Bruna to recombinant Nogo A (lane 2) and endogenous Nogo A from primary cultured rat oligodendrocytes (lane 3). The mobilities of these two proteins are virtually identical at about 200 kD on a 5% denaturing SDS gel. A control LacZ construct transfected sample (lane 1) showed no immunoreactivity with AS Bruna. The same blot was also probed with anti-myc antibody, 9E10, as indicated. The band that reacted with AS Bruna also reacted with the anti-myc tag antibody, 9E10 (lane 5), while the endogenous Nogo A did not (lane 6). The LacZ control transfection sample showed the expected band at about 118 kD (lane 4). COS cells transiently transfected with a Nogo A construct were double stained with AS Bruna (b) and IN-1 (c). Cells positively stained with AS Bruna were also positive with IN-1.

Figure 12: The nucleotide sequence (SEQ ID NO:28) of the bovine *Nogo* cDNA.

Figure 13: The amino acid sequence of rat Nogo A (SEQ ID NO:2) aligned with the theoretical amino acid sequence of human Nogo (SEQ ID NO:30). The human Nogo amino acid sequence was derived from aligning expressed sequence tags (EST) to the rat Nogo sequence and translating the aligned human ESTs using the rat Nogo as a guiding template.

Figure 14: Rat Nogo C nucleic acid (SEQ ID NO:31) sequence and its corresponding amino acid sequence (SEQ ID NO:32).

Figure 15a-15e: Nogo A is present on the oligodendrocyte plasma membrane, as demonstrated by immunocytochemistry and cell surface biotinylation of oligodendrocytes in culture.

Immunocytochemistry (a-d): Oligodendrocytes from optic nerves of P10 rats were dissociated and cultured for 2 days. Staining of live cells with mAb IN-1 (a) or AS 472 (c) showed immunoreactivity on oligodendrocyte cell bodies and processes. In the presence of the competing peptide P472, AS 472 showed only background labeling (all cell types) (d). Similar non-specific staining was seen when primary antibodies were omitted (b). . Evaluation: Number-coded dishes were randomly mixed and classified by 3 independent observers. 8/10 dishes were correctly classified AS 472-positive, mAb IN-1-positive or controls by all three observers.

Biotinylation (e): Rat P4 whole brain cultures enriched in oligodendrocytes were cell surface biotinylated with a membrane impermeable reagent after seven days in culture. Subsequently, cell homogenates were treated with streptavidin-Dynabeads. Precipitate (Ppt) and supernatant (sup) were blotted with AS472; they showed a distinct protein pattern: Cell surface Nogo A found in the precipitate showed a higher apparent molecular weight than intracellular Nogo A. This shift is probably due to glycosylation. The luminal ER protein BiP and the large majority of β-tubulin could only be found in the intracellular fraction.

Figure 16a-16j: Functional assays show the presence of Nogo A on the cell membrane of oligodendrocytes. Pre-incubation of optic nerve cultures with AS 472 (a, b) allowed the NIH 3T3 fibroblasts to spread over the highly branched oligodendrocytes which are outlined by immunofluorescent staining for GalC (01 antibody) (a). Arrows in the corresponding phase contrast image (b) indicate the NIH 3T3 fibroblasts spreading on top of the oligodendrocytes. (c, d): When AS 472 was added together with P472, the NIH 3T3

fibroblasts strictly avoided the territories of the GalC-positive oligodendrocytes (arrowheads) (Caroni and Schwab, 1988 Neuron 1:85-96). (e, f): In the presence of AS 472, P0 rat dissociated DRG neurons were able to extend neurites over the territory of highly branched oligodendrocytes (arrows in f). (g, h): The peptide P472 efficiently competed the neutralizing activity of AS 472: the neurites completely avoided the oligodendrocytes. AS 472 used in these experiments was generated against the rat 472 peptide sequence. (i, j): Quantification of these results (as described in methods) demonstrated the strong neutralizing activity of AS 472 in both types of assays. Scale bar: 40 μm.

Figure 17a-17e: Recombinant Nogo A is an inhibitory substrate and its inhibitory activity is neutralized by mAb IN-1. RecNogo A enriched extracts from a stable CHO-Nogo A cell line, or β-galactosidase, isolated in parallel from the stable CHO-LacZ cell line, were coated for the NIH 3T3 fibroblast spreading and DRG neurite outgrowth assays. (a) Silver gel of myc-his-tagged recLacZ (lane 1) and recNogo A (lane 2) shows the Nogo A band at 180 kD. The identity of the Nogo A band was confirmed by Western blot incubated with AS Bruna (lane 3) and an anti-myc antibody 9E10 (lane 4). (b) RecNogo A coated dishes were clearly inhibitory to the NIH 3T3 spreading. Pre-incubation with mAb IN-1 or AS Bruna resulted in a highly significant ($p<0.01$) neutralization of inhibitory activity. The control IgM mAb O1 and pre-immune serum were ineffective. CHO-LacZ extract had a partial inhibitory effect on the NIH 3T3 cells, probably due to endogenous CHO proteins. This inhibitory activity was not influenced by pre-incubation with antibodies.

(c) For DRG neurite outgrowth assays, the same protein material as in (b) was mixed with laminin and coated. RecNogo A had a very potent inhibitory effect on neurite outgrowth of dissociated DRG in a dose-dependent manner. The activity was neutralized by mAb IN-1 ($p<0.001$), but not by control mAb 01. Protein material isolated from CHO-LacZ cells was not inhibitory at any of the concentrations used, nor did incubation with antibodies have any effect on neurite outgrowth. Examples for scoring are shown in (d): 1 μg recNogo A, no or short neurites (arrows) score: 2, and in (e): 1 μg CHO-LacZ, long, branched neurites (arrowheads) score: 5-6. Statistical analysis was performed with two-tailed Student's t test. Scale bar: 280 μm.

Figure 18: Functional Analysis of Nogo Deletion Mutants. The following deletion constructs encoding fusion proteins containing fragments of Nogo or truncated portions of Nogo (as listed below) were generated as described in Section 6.2.7 hereinbelow.

Nogo-A: His-tag/T7-tag/vector/Nogo-A seq. aa1-1162

Nogo-B: His-tag/T7-tag/vector/Nogo-A seq. aa1-171 + 975-1162
Nogo-C: His-tag/T7-tag/Nogo-C N-terminus (11 aa) + Nogo-A seq. aa 975-1162
NiAext: His-tag/T7-tag/vector/Nogo-A seq. aa1-974/T7-tag
NiR: His-tag/T7-tag/vector/Nogo-A seq. aa1-171/vector
NiG: His-tag/T7-tag/Nogo-A seq. aa 172-974/His-tag
EST: His-tag/T7-tag/Nogo-A seq. aa 760-1162
NiG-D1: His-tag/T7-tag/Nogo-A seq. aa172-908/vector
NiG-D2: His-tag/T7-tag/Nogo-A seq. aa 172-866/His-tag
NiG-D3: His-tag/T7-tag/Nogo-A seq. aa 172-723/His-tag
NiG-D4: His-tag/T7-tag/Nogo-A seq. aa 172-646/vector
NiG-D5: His-tag/T7-tag/Nogo-A seq. aa 291-646/His-tag
NiG-D7: His-tag/T7-tag/Nogo-A seq. aa 172-234 + 292-974/His-tag
NiG-D8: His-tag/T7-tag/Nogo-A seq. aa 172-628
NiG-D9: His-tag/T7-tag/Nogo-A seq. aa 172-259 + 646-974/His-tag
NiG-D10: His-tag/T7-tag/Nogo-A seq. aa 291-974/His-tag
NiG-D14: His-tag/T7-tag/Nogo-A seq. aa 172-259
NiG-D15: His-tag/T7-tag/Nogo-A seq. aa 172-189 + 491-974/His-tag
NiG-D16: His-tag/T7-tag/Nogo-A seq. aa 172-189 + 619-974/His-tag
NiG-D17: His-tag/T7-tag/Nogo-A seq. aa 172-189 + 257-974/His-tag
NiG-D18: His-tag/T7-tag/Nogo-A seq. aa 172-189 + 261-974/His-tag
NiG-D20: His-tag/T7-tag/Nogo-A seq. aa 542-722/His-tag

The amino acid (aa) numbers are based on rat Nogo A amino acid sequence numbering (SEQ ID NO: 2) starting with the first methionine. The His-tag and T7-tag consist of 34 amino acids. The N- and C- terminal vector sequences are derived from the expression vector pET28.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to nucleotide sequences of *Nogo* genes, and amino acid sequences of their encoded proteins. The invention further relates to fragments and other derivatives, and analogs, of Nogo proteins. Nucleic acids encoding such fragments or derivatives are also within the scope of the invention. The invention provides *Nogo* genes and their encoded proteins of many different species. The *Nogo* genes of the invention include human, rat and bovine *Nogo* and related genes (homologs) in other species. The bovine subsequences disclosed in Spillman et al., 1998, J. Biol. Chem. 273:19283-19293, are not claimed as part of the present invention. In specific embodiments, the *Nogo* genes and proteins are from vertebrates, or more particularly, mammals. In a preferred

embodiment of the invention, the *Nogo* genes and proteins are of human origin. Production of the foregoing proteins and derivatives, *e.g.*, by recombinant methods, is provided.

The *Nogo* gene as provided by the present invention, encompasses nucleic acid molecules encoding three isoforms of Nogo; namely Nogo A, Nogo B and Nogo C. Reference to the gene "*Nogo*" shall include nucleic acid molecules encoding all three isoforms unless otherwise specified. Likewise, reference to Nogo protein shall include all three isoforms of Nogo unless otherwise specified. Nogo proteins of the invention can prevent regeneration of neurons in the spinal cord or brain (i.e. non-permissive substrate properties), inhibit dorsal root ganglia neurite outgrowth, induce dorsal root ganglia growth cone collapse, block NIH 3T3 cell spreading, block PC12 neurite outgrowth, etc.

The Nogo proteins, fragments and derivatives thereof are free of all central nervous system myelin material; in particular, they are free of all central nervous system myelin material with which the Nogo protein is naturally associated. Such material may include other CNS myelin proteins, lipids, and carbohydrates. The Nogo proteins, fragments and derivatives thereof of the invention are also preferably free of the reagents used in purification from biological specimens, such as detergents.

In a specific embodiment, the invention provides recombinant Nogo proteins, fragments and derivatives thereof as prepared by methods known in the art, such as expressing the *Nogo* gene in a genetically engineered cell.

The invention also relates to Nogo derivatives and analogs of the invention which are functionally active, *i.e.*, they are capable of displaying one or more known functional activities associated with a full-length (wild-type) Nogo protein. Such functional activities include but are not limited to the ability to interact (or compete for binding) with neural growth regulatory proteins, antigenicity [ability to bind (or compete with Nogo for binding) to an anti-Nogo antibody], immunogenicity (ability to generate antibody which binds to Nogo), preventing regeneration of neurons in the spinal cord or brain, conferring to a substrate the property of restricing growth, spreading, and migration of neural cells, and neoplastic cells, inhibiting dorsal root ganglia neurite outgrowth, inducing dorsal root ganglia growth cone collapse, blocking NIH 3T3 cell spreading *in vitro*, blocking PC12 neurite outgrowth, restricting neural plasticity, etc.

The invention further relates to fragments (and derivatives and analogs thereof) of Nogo which comprise one or more domains of the Nogo protein.

Antibodies to Nogo, its derivatives and analogs, are additionally provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on Nogo proteins and nucleic acids and anti-Nogo antibodies. The invention provides for treatment of disorders of growth regulated cells or organs by

administering compounds that promote Nogo activity (*e.g.*, Nogo proteins and functionally active analogs and derivatives (including fragments) thereof; nucleic acids encoding the Nogo proteins, analogs, or derivatives, agonists of Nogo).

The invention also provides methods of treatment of damage or disorder of the nervous system by administering compounds that antagonize, or inhibit, Nogo function (*e.g.*, antibodies, Nogo antisense nucleic acids, Nogo antagonist derivatives).

Animal models, diagnostic methods and screening methods for predisposition to disorders are also provided by the invention.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1 ISOLATION OF *NOGO* GENES

The invention relates to the nucleotide sequences of *Nogo* genes or nucleic acids. In one embodiment, *Nogo* nucleic acids comprise the rat cDNA sequence of Figure 2a (SEQ ID NO:1) identified as Nogo A as depicted in Figure 1b, or the coding regions thereof, or nucleotide sequences encoding a Nogo protein of 1163 amino acids in length or any functional fragment or derivative thereof (*e.g.*, a protein having the sequence of SEQ ID NO:2, as shown in Figure 2a).

In another embodiment, *Nogo* nucleic acids comprise the nucleotide sequence encoding Nogo B, whereas the Nogo B protein is equivalent to the amino terminal 172 amino acids fused to the carboxy terminal 188 amino acids of Nogo A, resulting in a truncated 360 amino acid protein. The transcripts for Nogo B arise as a result of alternative splicing which removes the intervening nucleotide coding sequence.

In yet another embodiment of the present invention, *Nogo* nucleic acids comprise the nucleotide sequences encoding Nogo C, whereas the Nogo C protein contains 11 amino acids at its amino terminus which are not present in Nogo A, and the carboxy terminal 188 amino acids of Nogo A and B. The Nogo C protein has 199 amino acids. The transcript encoding Nogo C is the result of transcription from an alternative Nogo promoter.

In yet another specific embodiment, the present invention provides bovine Nogo nucleic acid sequences (SEQ ID NO:28).

In yet another specific embodiment, the instant invention provides the nucleotide sequences encoding human Nogo, and fragments of human Nogo proteins, including the human equivalents to rat Nogo A, Nogo B and Nogo C. The human Nogo nucleic acid sequence is elucidated using the rat Nogo A transcript as a template and splicing together human expressed sequence tags (EST) to reveal a continuous nucleotide sequence. The rat and bovine amino acid sequences of Nogo also provided information on the proper

translational reading frame such that an amino acid sequence of human Nogo is deduced. The instant invention also provides amino acid sequences of fragments of the human *Nogo* gene.

The invention also provides purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a hybridizable portion) of a *Nogo* sequence; in other embodiments, the nucleic acids consist of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, 20 nucleotides, 500 nucleotides, 700 nucleotides, or 800 nucleotides of a *Nogo* sequence, or a full-length *Nogo* coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200 or 500 nucleotides in length. Nucleic acids can be single or double stranded. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a *Nogo* gene.

In a specific embodiment, a nucleic acid which is hybridizable to a *Nogo* nucleic acid (*e.g.*, having sequence SEQ ID NO:2; Figure 2a), or to a nucleic acid encoding a Nogo derivative, under conditions of low stringency is provided. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations as demonstrated in the example in Section 6.1.1).

In another specific embodiment, a nucleic acid which is hybridizable to a *Nogo* nucleic acid under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized

for 48 h at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

In another specific embodiment, a nucleic acid, which is hybridizable to a *Nogo* nucleic acid under conditions of moderate stringency is provided. For example, but not limited to, procedures using such conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 h at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which may be used are well-known in the art. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.1% SDS. Such stringency conditions are suitable for isolating nucleic acid molecules comprising *Nogo* gene sequences in another species, e.g., using the rat or bovine Nogo cDNA clones as probe to isolate the human Nogo cDNA.

A number of human expressed sequence tags (ESTs) reported in published nucleic acid sequence databases display a high degree of sequence identity when compared to segments of the *Nogo* gene sequences of the invention. The following preliminary list of human ESTs were identified and are listed by their Genbank accession numbers: AA158636 (SEQ ID NO:35), AA333267 (SEQ ID NO:36), AA081783 (SEQ ID NO:37), AA167765 (SEQ ID NO:38), AA322918 (SEQ ID NO:39), AA092565 (SEQ ID NO:40), AA081525 (SEQ ID NO:41), and AA081840 (SEQ ID NO:42) using ENTREZ Nucleotide Query. Prior to the present invention, none of the above-identified ESTs had been characterized with respect to the amino acid sequences these ESTs may encode *in vivo*. Nothing was known about the function of the proteins comprising the predicted amino acid sequences of the human ESTs. Furthermore, an EST, such as AA158636, aligning with the 5' end of rat Nogo cDNA and another EST, such as AA081840, aligning with the 3' end of rat cDNA, are not overlapping and would not be perceived to be part of the same human cDNA sequence.

Based on the *Nogo* gene sequences of the present invention, it is believed that these human ESTs represent portions of the human *Nogo* gene that are expressed in the tissue from which the ESTs were obtained. Accordingly, the present invention encompasses nucleic acid molecules comprising two or more of the above-identified human ESTs. The

ESTs may be expressed in the same human tissue, or in different human tissues. Preferably, the nucleic acid molecules of the invention comprise the nucleotide sequences of at least two human ESTs which are not overlapping with respect to each other, or which do not overlap a third or more human EST.

Since the above-identified human ESTs are now identified as fragments of the human *Nogo* gene due to the cloning of bovine and rat *Nogo* nucleic acids, it is contemplated that the human ESTs have similar functions relative to the other *Nogo* nucleic acid molecules in various methods of the invention, such as but not limited to, for example, the expression of human Nogo polypeptides, hybridization assays, and inhibition of Nogo expression as antisense nucleic acid molecules, etc.

Moreover, the present invention provides and includes the predicted amino acid sequence of the human Nogo protein, and fragments thereof. As shown in Figure 13, the amino acid sequence of rat Nogo protein (Figure 2a; SEQ ID NO:2) is aligned with the predicted amino acid sequence of human Nogo protein (Figure 13; SEQ ID NO:30). Accordingly, the present invention encompasses human Nogo proteins comprising the predicted amino acid sequence of human Nogo, Figure 13 and SEQ ID NO:30, or a subsequence of the predicted amino acid sequence of human Nogo, consisting of at least 6 amino acid residues, or one or more of the following predicted amino acid sequences of human Nogo fragments: MEDLDQSPLVSSS (Human Nogo, corresponding to amino acids 1-13 with SEQ ID NO:43), KIMDLKEQPGNTISAG (Human Nogo, corresponding to amino acids 187-203 with SEQ ID NO:44), KEDEVVSSEKAKDSFNEKR (Human Nogo, corresponding to amino acids 340-358 with SEQ ID NO:45), QESLYPAAQLCPSFEESEATPSPVLPDIVMEAPLNSAVPSAGASVIQPSS (Human Nogo, corresponding to amino acids 570-619 with SEQ ID NO:46). Naturally occurring human Nogo and recombinant human Nogo, and fragments thereof having an amino acid sequence substantially similar to the above-described amino acid sequences and able to be bound by an antibody directed against a Nogo protein are within the scope of the invention.

The present invention further provides nucleic acid molecules that encodes a human Nogo protein having an amino acid sequence substantially similar to the amino acid sequence as shown in Figure 13 (Figure 13; SEQ ID NO:30). In specific embodiments, nucleic acid molecules encoding fragments of human Nogo protein having an amino acid sequence substantially similar to the amino acid sequence as shown in Figure 13 (SEQ ID NO:30) are also contemplated with the proviso that such nucleic acid molecules do not comprise the nucleotide sequence of the above-identified human ESTs.

An amino acid sequence is deemed to be substantially similar to the predicted amino acid sequence of human Nogo protein when more than 50%, 55%, 60%, 65%, 70%, 75%,

80%, 85%, 90%, 95%, or 97% of the amino acid residues in the two molecules are identical when a computer algorithm is used in which the alignment is done by a computer homology program known in the art, for example a BLAST computer searching (Altschul et al., 1994, Nature Genet. 6:119-129) is used.

By way of example and not limitation, useful computer homology programs include the following: Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) (Altschul et al., 1990, J. of Molec. Biol., 215:403-410, "The BLAST Algorithm; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402) a heuristic search algorithm tailored to searching for sequence similarity which ascribes significance using the statistical methods of Karlin and Altschul 1990, Proc. Nat'l Acad. Sci. USA, 87:2264-68; 1993, Proc. Nat'l Acad. Sci. USA 90:5873-77. Five specific BLAST programs perform the following tasks:

1) The BLASTP program compares an amino acid query sequence against a protein sequence database.

2) The BLASTN program compares a nucleotide query sequence against a nucleotide sequence database.

3) The BLASTX program compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

4) The TBLASTN program compares a protein query sequence against a nucleotide sequence database translated in all six reading frames (both strands).

5) The TBLASTX program compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

As will be understood by those skilled in the art, the TBLASTN program is particularly useful to identify nucleic acids with a desired percent identity and the BLASTP program is particularly useful to identify amino acid sequences with a desired percent identity.

Smith-Waterman (database: European Bioinformatics Institute wwwz.ebi.ac.uk/bic_sw/) (Smith-Waterman, 1981, J. of Molec. Biol., 147:195-197) is a mathematically rigorous algorithm for sequence alignments.

FASTA (see Pearson et al., 1988, Proc. Nat'l Acad. Sci. USA, 85:2444-2448) is a heuristic approximation to the Smith-Waterman algorithm. For a general discussion of the procedure and benefits of the BLAST, Smith-Waterman and FASTA algorithms, see Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.

The uses of the predicted amino acid sequences of human Nogo, or the nucleotide sequences of human ESTs, including degenerate sequences encoding the predicted amino acid sequence of human Nogo, for isolating or identifying the human *Nogo* gene, fragments,

naturally occurring mutants and variants thereof, is within the scope of the invention. Such uses which will be known to one of skill in the art include but are not limited to using the information to prepare nucleic acid probes for DNA library screening, DNA amplification, genetic screening of the human population, and to prepare synthetic peptides for making antibodies. Detailed description of some of such uses are provided herein in later sections.

Nucleic acids encoding derivatives and analogs of Nogo proteins, and *Nogo* antisense nucleic acids are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or portion of a Nogo protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the Nogo protein and not the other contiguous portions of the Nogo protein as a continuous sequence. In this context, a portion means one or more amino acids.

Fragments of *Nogo* nucleic acids comprising regions conserved between (with homology to) other *Nogo* nucleic acids, of the same or different species, are also provided. Nucleic acids encoding one or more Nogo domains are provided in Figure 2a, for example, the conserved carboxy terminal domain of rat Nogo, which has about 180 amino acids, and is encoded by the last 540 nucleotides of the coding sequence prior to the stop codon. The nucleotide and amino acid sequences of two hydrophobic domains within the conserved carboxy terminus domain, *i.e.*, from amino acids 988 to 1023, and from amino acids 1090 to 1125, in rat Nogo A, are also provided. The nucleotide and amino acid sequences of the amino terminal acidic domain of rat Nogo A, from residues 31 to 58, are also provided.

To perform functional analysis of various regions of Nogo, a series of deletions in the *Nogo* gene has been generated and cloned into an expression vector by recombinant DNA techniques and expressed as a fusion protein. Nucleic acids that encode a fragment of a Nogo protein are provided, e.g., nucleic acids that encode amino acid residues 1-171, 172-974, 259-542, 542-722, 172-259, 722-974, or 975-1162 of SEQ ID NO: 2, or combinations thereof; and nucleic acids that encode amino acid residues 1-131, 132-939, 206-501, 501-680, 132-206, 680-939, and 940-1127 of SEQ ID NO: 30, or combinations thereof. Some of the deletion constructs comprises truncated portions of Nogo and additional nucleotide sequences encoding a hexahistidine tag and/or a T7-tag. Nucleic acids encoding truncated Nogo proteins that lacks amino acid residues 172-259, amino acid residues 974-1162, or amino acid residues 172-259 and 974-1162, of SEQ ID NO:2 but otherwise comprises the remainder of SEQ ID NO: 2; or amino acid residues 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:30 but otherwise comprises the remainder of SEQ ID NO: 30, are provided. The structure of exemplary deletion constructs are shown in Figure 18. The deletion constructs produce fragments or

truncated portion(s) of Nogo when introduced into a cell. The biological activities of these mutants were tested in various functional assays as described in Table 2 in Section 6.2.7.

Specific embodiments for the cloning of a *Nogo* gene, presented as a particular example but not by way of limitation, follows:

For expression cloning (a technique commonly known in the art), an expression library is constructed by methods known in the art. For example, mRNA (*e.g.*, human) is isolated, cDNA is made and ligated into an expression vector (*e.g.*, a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed Nogo product. In one embodiment, anti-Nogo antibodies can be used for selection.

In another embodiment, polymerase chain reaction (PCR) is used to amplify the desired sequence in a genomic or cDNA library, prior to selection. Oligonucleotide primers representing known *Nogo* sequences can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the Nogo conserved segments of strong homology between *Nogo* of different species. The synthetic oligonucleotides may be utilized as primers to amplify by PCR sequences from a source (RNA or DNA), preferably a cDNA library, of potential interest. PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). The DNA being amplified can include mRNA or cDNA or genomic DNA from any eukaryotic species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known *Nogo* nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions are preferred.

After successful amplification of a segment of a *Nogo* homolog, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete cDNA or genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*. In this fashion, additional genes encoding Nogo proteins and Nogo analogs may be identified.

The above-methods are not meant to limit the following general description of methods by which clones of *Nogo* may be obtained.

Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the *Nogo* gene. The nucleic acid sequences encoding Nogo can be isolated from vertebrate, mammalian, human, porcine, murine, bovine, feline, avian, equine, canine, as

well as additional primate sources, insects, etc. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired gene may be accomplished in a number of ways. For example, if an amount of a portion of a *Nogo* (of any species) gene or its specific RNA, or a fragment thereof (see Section 6.1.1), is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection can be carried out on the basis of the properties of the gene.

Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isolectric focusing behavior, proteolytic digestion maps, post-translational modifications, acidic or basic properties or antigenic properties as known for Nogo. Antibodies to Nogo are available, such as IN-1 and IN-2 (U.S. Patent 5,684,133), AS Bruna and AS 472. Preparation of AS Bruna and AS 472 are described in Section 6.1.7. The Nogo protein may

be identified by binding of labeled antibody to the putatively Nogo synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure or by western blotting of purified or whole cell extracts.

The *Nogo* gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified *Nogo* DNA of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor; see *infra*) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against Nogo protein. A radiolabeled *Nogo* cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the *Nogo* DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the *Nogo* genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the Nogo protein. For example, RNA for cDNA cloning of the *Nogo* gene can be isolated from cells which express *Nogo*. Other methods are possible and within the scope of the invention.

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). In a specific example, Nogo is cloned into pcDNA3 with epitope tags for simplified protein expression analysis (Section 6.1.10).

The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and *Nogo* gene may be modified by homopolymeric tailing.

Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated *Nogo* gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The Nogo sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native Nogo proteins, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other Nogo derivatives or analogs, as described in Sections 6.2.1 and 6.2.2 *infra* for Nogo derivatives and analogs.

5.2 EXPRESSION OF THE *NOGO* GENES

The nucleotide sequence coding for a Nogo protein or a functionally active analog or fragment or other derivative thereof (see Figures 1b and 2a; Sections 6.2.1 and 6.2.2), can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native *Nogo* gene and/or its flanking regions. The coding sequence can also be tagged with a sequence that codes for a well described antigen or biological molecule that has known binding properties to a binding partner (*e.g.* myc epitope tag, histidine tag, T7 epitope tag etc., see Section 6.2.6 and Figure 11a-11c). This additional sequence can then be exploited to purify the Nogo protein, protein fragment, or derivative using the interaction of the binding group with its corresponding partner, which is attached to a solid matrix.

A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector

system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the human *Nogo* gene is expressed, or a sequence encoding a functionally active portion of human *Nogo*, as a specific example, either Nogo A, Nogo B or Nogo C is expressed (Figure 1b). In yet another embodiment, a fragment of Nogo comprising a domain of the Nogo protein is expressed.

As used herein, a cell is "transformed" with a nucleic acid, when such cell contains a nucleic acid not natively present in the cell, after introduction of the nucleic acid into the cell or its ancestor, e.g., by transfection, electroporation, transduction, etc.

Nucleotide sequences encoding fragments of human Nogo A comprising an amino acid sequence selected from the group consisting of amino acid residues 1-131, 132-939, 206-501, 501-680, 132-206, 680-939, and 940-1127 of SEQ ID NO: 30 are also provided. Nucleotide sequences that encodes truncated portions of human Nogo A are also provided; the truncated proteins lack amino acid residues 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:30 but otherwise comprises the remainder of SEQ ID NO: 30.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Nogo protein or peptide fragment may be regulated by a second nucleic acid sequence so that the Nogo protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Nogo protein may be controlled by any promoter/enhancer element known in the art. An exemplary embodiment is to use one of Nogo's natural promoters, either P1 or P2, discussed in Section 6.2.1. A non-native promoter may also be used. Promoters which may be used to control *Nogo* expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower

mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a Nogo-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a *Nogo* coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the Nogo protein product from the subclone in the correct reading frame.

Expression vectors containing *Nogo* gene inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a *Nogo* gene inserted in an expression vector can be detected by nucleic acid hybridization

using probes comprising sequences that are homologous to an inserted *Nogo* gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a *Nogo* gene in the vector. For example, if the *Nogo* gene is inserted within the marker gene sequence of the vector, recombinants containing the *Nogo* insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the Nogo product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the Nogo protein in *in vitro* assay systems, *e.g.*, binding with anti-Nogo antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered Nogo protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

In other specific embodiments, the Nogo protein, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate

nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.3 IDENTIFICATION AND PURIFICATION OF THE *NOGO* GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of Nogo, preferably human Nogo, and fragments and derivatives thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" Nogo material as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) Nogo A protein, *e.g.*, non-permissive substrate properties, dorsal root ganglia growth cone collapse, NIH 3T3 spreading inhibition, neurite outgrowth inhibition, binding to a Nogo substrate or Nogo binding partner, antigenicity (binding to an anti-Nogo antibody), immunogenicity, etc.

In specific embodiments, the invention provides fragments of a Nogo protein consisting of at least 6 amino acids, 10 amino acids, 17 amino acids, 50 amino acids, 100 amino acids or of at least 220 amino acids. In other embodiments, the proteins comprise or consist essentially of the highly conserved Nogo carboxy terminal domain (carboxy terminal 188 amino acids of Nogo A). Fragments, or proteins comprising fragments, lacking the conserved carboxy terminal domain, or the hydrophobic carboxy terminal stretches, or the amino terminal acidic domain, or the amino terminal poly-proline region or any combination thereof, of a Nogo protein are also provided. Nucleic acids encoding the foregoing are provided.

Once a recombinant which expresses the *Nogo* gene sequence is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

Once the Nogo protein is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay including dorsal root ganglia growth cone collapse, NIH 3T3 spreading inhibition, inhibition of neurite regeneration in optic nerves (see Sections 6.2.4-6.2.5).

Alternatively, once a Nogo protein produced by a recombinant is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller, M., et al., 1984, Nature 310:105-111).

In another alternate embodiment, native Nogo C, can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a specific embodiment of the present invention, such Nogo proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in Figure 2a (SEQ ID NO:2), bovine in Figure 12 (SEQ ID NO:29), or human in Figure 13 (SEQ ID NO:30), as well as fragments and other derivatives (such as but not limited to those depicted in Figure 18), and analogs thereof, including proteins homologous thereto. Preferably, the Nogo proteins of the invention are free of all CNS myelin material with which it is normally associated.

5.4 STRUCTURE OF THE *NOGO* GENE AND PROTEIN

The structure of the *Nogo* gene and protein can be analyzed by various methods known in the art and several of these methods are described in the following subsections.

5.4.1 GENETIC ANALYSIS

The cloned DNA or cDNA corresponding to the *Nogo* gene can be analyzed by methods including but not limited to Southern hybridization (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), Northern hybridization (see *e.g.*, Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, T., 1982, Molecular Cloning, A Laboratory, Cold Spring Harbor, New York), and DNA sequence analysis. Polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh et al., 1989, Science 243:217-220) followed by Southern hybridization with a *Nogo*-specific probe can allow the detection of the *Nogo* gene in DNA from various cell types. Methods of amplification other than PCR are commonly known and can also be employed. In one embodiment, Southern hybridization can be used to determine the genetic linkage of *Nogo*. Northern hybridization analysis can be used to

determine the expression of the *Nogo* gene. Various cell types, at various states of development or activity can be tested for *Nogo* expression. The stringency of the hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific Nogo probe used. Modifications of these methods and other methods commonly known in the art can be used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of the *Nogo* gene. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, F., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequenator (*e.g.*, Applied Biosystems, Foster City, CA).

5.4.2 PROTEIN ANALYSIS

The amino acid sequence of the Nogo protein can be derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer.

The Nogo protein sequence can be further characterized by a hydrophilicity analysis (Hopp, T. and Woods, K., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the Nogo protein and the corresponding regions of the gene sequence which encode such regions.

Secondary, structural analysis (Chou, P. and Fasman, G., 1974, Biochemistry 13:222) can also be done, to identify regions of Nogo that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, open reading frame prediction and plotting, as well as determination of sequence homologies, can also be accomplished using computer software programs available in the art.

Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, A., 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, *in* Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

5.5 GENERATION OF ANTIBODIES TO NOGO PROTEINS AND DERIVATIVES THEREOF

According to the invention, the Nogo protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. Antibodies directed to a recombinant fragment of rat and bovine Nogo are produced (Section 6.1.7), these antibodies also cross react with other species epitopes. In another embodiment, fragments of a Nogo protein identified as hydrophilic are used as immunogens for antibody production.

Various procedures known in the art may be used for the production of polyclonal antibodies to a Nogo protein or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a Nogo protein encoded by a sequence of SEQ ID NO:2 in Figure 2a, SEQ ID NO:29 in Figure 12, SEQ ID NO:32 in Figure 14, or SEQ ID NO:30 in Figure 13, (rat Nogo A, bovine Nogo, rat Nogo C, or human Nogo respectively) or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native Nogo protein, or a synthetic version, or derivative (*e.g.*, fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a Nogo protein sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric

antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for Nogo together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce Nogo-specific single chain antibodies. Techniques described for the construction of Fab expression libraries can also be utilized (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for Nogo proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a Nogo protein, one may assay generated hybridomas for a product which binds to a Nogo fragment containing such domain. For selection of an antibody that specifically binds a first Nogo homolog but which does not specifically bind a different Nogo homolog, one can select on the basis of positive binding to the first Nogo homolog and a lack of binding to the second Nogo homolog.

Antibodies specific to a domain of a Nogo protein are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the Nogo protein sequences of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

Anti-Nogo antibodies and fragments thereof containing the binding domain are Therapeutics.

5.6 NOGO PROTEINS, DERIVATIVES AND ANALOGS

The invention further relates to Nogo proteins, and derivatives (including but not limited to fragments) and analogs of Nogo proteins. Nucleic acids encoding Nogo protein

derivatives and protein analogs are also provided. In one embodiment, the Nogo proteins are encoded by the Nogo nucleic acids described in Section 5.1 *supra*. In particular aspects, Nogo A, Nogo B, or Nogo C proteins and derivatives, or analogs are of animals, *e.g.*, mouse, rat, pig, cow, dog, monkey, human, fly, or frogs are within the scope of the invention.

The production and use of derivatives and analogs related to Nogo are also within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, wild-type Nogo protein. As one example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of Nogo activity, etc. Derivatives or analogs that retain, or alternatively lack or inhibit, a desired Nogo property of interest (*e.g.*, binding to a Nogo binding partner, can be used as inducers, or inhibitors, respectively, of such property and its physiological correlates. A specific embodiment relates to a Nogo fragment that can be bound by an anti-Nogo antibody. Derivatives or analogs of Nogo can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in Sections 6.1.10 to 6.1.12.

In order to map the active region(s) of Nogo, a series of Nogo deletion mutants have been prepared by recombinant DNA techniques as described in Section 6.2.7. The portions of Nogo which are present in the deletion mutants are shown in Figure 18. In a specific embodiment, the invention provides fragments of Nogo e.g., fragments comprising (or alternatively consisting of) Nogo A (SEQ ID NO: 2) amino acid numbers 1-171, 172-974, 259-542, 542-722, 722-974, 172-259, or 975-1162, or combinations of the foregoing. Truncated mutants of Nogo lacking amino acid numbers 172-259 and/or 975-1162 of SEQ ID NO:2 are also provided, as these regions appear to be non-essential and can be removed from Nogo without affecting biological activity. The corresponding fragments of human Nogo A comprising (or alternatively consisting of) amino acid numbers 1-131, 132-939, 206-501, 501-680, 132-206, 680-939, or 940-1127 of SEQ ID NO: 30 are also provided. Truncated mutants of human Nogo A are also provided which lack amino acid numbers 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:30.

In a specific embodiment, the fragments are free of all CNS myelin material and/or display inhibitory activity of Nogo. Fusion proteins comprising one or more of the above fragments fused to a non-Nogo sequence are also provided.

Nogo gene derivatives can be made by altering *Nogo* sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the

degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *Nogo* gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of *Nogo* genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the Nogo derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a Nogo protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be conservatively substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a Nogo protein consisting of at least 10 (continuous) amino acids of the Nogo protein is provided. In other embodiments, the fragment consists of at least 17 or 50 amino acids of the Nogo protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. Derivatives or analogs of Nogo include but are not limited to those molecules comprising regions that are substantially homologous to Nogo or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, for example BLAST computer searching (Altschul et al., 1994, Nature Genet. 6:119-129)) or whose encoding nucleic acid is capable of hybridizing to a coding Nogo sequence, under stringent, moderately stringent, or nonstringent conditions.

Molecules comprising Nogo fragments are also provided, e.g., containing hydrocarbon linkages to other moieties including labels or bioactive moieties.

The Nogo derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned *Nogo* gene sequence can be modified by

any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of Nogo, care should be taken to ensure that the modified gene remains within the same translational reading frame as Nogo, uninterrupted by translational stop signals, in the gene region where the desired Nogo activity is encoded.

Additionally, the Nogo-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the Nogo sequence may also be made at the protein level. Included within the scope of the invention are Nogo protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of Nogo can be chemically synthesized. For example, a peptide corresponding to a portion of a Nogo protein which comprises the desired domain or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Nogo sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ϵ-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-

methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the Nogo derivative is a chimeric, or fusion, protein comprising a Nogo protein or fragment thereof (preferably consisting of at least a domain or motif of the Nogo protein, or at least 10 amino acids of the Nogo protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a Nogo-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of *Nogo* fused to any heterologous protein-encoding sequences may be constructed. Such heterologous protein-encoding sequences include, for example, the hexahistidine tag, and the T7 tag. A specific embodiment relates to a chimeric protein comprising a fragment of Nogo of at least six amino acids.

In another specific embodiment, the Nogo derivative is a molecule comprising a region of homology with a Nogo protein.

In a preferred embodiment, the Nogo derivatives (e.g., fragments) are proteins that are non-naturally occurring.

Other specific embodiments of derivatives and analogs are described in the subsection below and examples sections *infra*.

5.6.1 DERIVATIVES OF NOGO CONTAINING ONE OR MORE DOMAINS OF THE PROTEIN

In a specific embodiment, the invention relates to Nogo derivatives and analogs, in particular Nogo fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of a Nogo protein, including but not limited to the conserved carboxy terminal and hydrophobic domains or the amino terminal acidic or poly proline rich domains, functional (*e.g.*, binding) fragments of any of the foregoing, or any combination of the foregoing.

A specific embodiment relates to molecules comprising specific fragments of Nogo that are those fragments in the respective Nogo protein most homologous to specific fragments of a rat or bovine Nogo protein. A fragment comprising a domain of a Nogo

homolog can be identified by protein analysis methods as described in Sections 6.1.2, 6.1.8, 6.1.9, 6.1.10, 6.1.11, or 6.1.12.

In another specific embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a Nogo protein but that also lacks one or more domains (or functional portion thereof) of a Nogo protein. In another embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a Nogo protein, and that has one or more mutant (*e.g.*, due to deletion or point mutation(s)) domains of a Nogo protein (*e.g.*, such that the mutant domain has decreased function).

5.7 ASSAYS OF NOGO PROTEINS, DERIVATIVES AND ANALOGS

The functional activity of Nogo proteins, derivatives and analogs can be assayed by various methods. The description of functional assays in the following sections are not meant to be limiting, and may include other assays known to one skilled in the art.

5.7.1 ASSAYS OF NOGO IN VITRO NEURITE GROWTH INHIBITION

In a specific embodiment, Nogo proteins, derivatives and analogs can be assayed for inhibition of NIH 3T3 spreading or inhibition of PC12 neurite outgrowth using *in vitro* tissue culture (Section 6.1.10).

In an alternative embodiment, Nogo proteins, derivatives and analogs can be used to assay for explanted chick dorsal root ganglia growth cone collapse induced by the presence of Nogo. Similarly, Nogo function can be assayed for inhibition of neurite outgrowth of explanted chick dorsal root ganglia (Spillman et al., 1998 J. Biol. Chem. 273:19283-19293).

5.7.2 ASSAYS OF NOGO IN VIVO FUNCTIONAL PROPERTIES

In one example, antagonists of Nogo proteins, derivatives and analogs can be used for *in vivo* assays of function using an animal model for corticospinal tract (CST) regeneration over long distances and behavior recovery.

In a preferred embodiment, a rodent corticospinal tract is damaged by surgical resection or spinal cord contusion, and antagonists of Nogo are administered to the animal. Neural plasticity, regeneration and functional recovery, as compared to untreated control animals or control antibody treated animals, are monitored for structural plasticity or regeneration by anatomical techniques, mainly by labeling of defined neural tracts. Functional recovery is measured by locomotion and by electrophysiology skill tests

executed by the rodent (*e.g.* sticky paper test, food pellet reaching task, etc.) (Thallmair et al., 1998 Nat. Neuroscience 1(2):124-131).

5.7.3 NOGO LIGAND BINDING INHIBITION AND ASSAYS THEREOF

In one embodiment, where one is assaying for the ability to bind or compete with wild-type Nogo for binding to anti-Nogo antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where a Nogo-binding protein is identified, the binding can be assayed, *e.g.*, by means well-known in the art. In another embodiment, physiological correlates of Nogo binding to its substrates can be assayed.

Other methods will be known to the skilled artisan and are within the scope of the invention.

5.8 THERAPEUTIC USES

The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such "Therapeutics" include but are not limited to: Nogo proteins and analogs and derivatives (including fragments) thereof (*e.g.*, as described hereinabove); antibodies thereto (as described hereinabove); nucleic acids encoding the Nogo proteins, analogs, or derivatives (*e.g.*, as described hereinabove); *Nogo* antisense nucleic acids, and Nogo agonists and antagonists. Disorders involving deregulated cellular growth, *e.g.* CNS tumors, are treated or prevented by administration of a Therapeutic that promotes Nogo function. Disorders in which neurite growth, regeneration, or maintenance are deficient or desired are treated by administration of a Therapeutic that antagonizes (inhibits) Nogo function. The above is described in detail in the subsections below.

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human Nogo protein, derivative, or analog, or nucleic acid, or an antibody to a human Nogo protein, is therapeutically or prophylactically administered to a human patient.

5.8.1 TREATMENT AND PREVENTION OF DISORDERS INVOLVING DEREGULATED CELLULAR GROWTH

Diseases and disorders involving deregulated cellular growth are treated or prevented by administration of a Therapeutic that promotes (*i.e.*, increases or supplies) Nogo function. Examples of such a Therapeutic include but are not limited to Nogo proteins, derivatives, or fragments that are functionally active, particularly that are active in inhibition of neurite extension or cellular growth inhibition (*e.g.*, as demonstrated in *in vitro* assays or in animal models), and nucleic acids encoding a Nogo protein or functionally active derivative or fragment thereof (*e.g.*, for use in gene therapy). Preferably, the Nogo proteins, derivatives or fragments thereof are free of all CNS myelin material with which it is naturally associated. Other Therapeutics that can be used, *e.g.*, Nogo agonists, can be identified using *in vitro* assays or animal models, examples of which are described *infra*.

In specific embodiments, Therapeutics that promote Nogo function are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an absence or decreased (relative to normal or desired) level of Nogo protein or function, for example, in patients where Nogo protein is lacking, genetically defective, biologically inactive or underactive, or underexpressed; or (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of Nogo agonist administration. The absence or decreased level in Nogo protein or function can be readily detected, *e.g.*, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed Nogo RNA or protein. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize Nogo protein (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect Nogo expression by detecting and/or visualizing Nogo mRNA (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.), etc.

Diseases and disorders involving deregulated cellular growth that can be treated or prevented include but are not limited to proliferative disorders, malignant tumors, nervous system tumors, etc. Examples of these are detailed below.

5.8.1.1 NEOPLASTIC GROWTH

Neoplastic growth and related disorders that can be treated or prevented by administration of a Therapeutic that promotes Nogo function include but are not limited to those listed in Table 1 (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia):

TABLE 1
NEOPLASTIC GROWTH AND RELATED DISORDERS

Solid tumors
sarcomas and carcinomas
glioma, glioblastoma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
neuroblastoma
retinoblastoma

In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative disorders, are treated or prevented in the central nervous system, spinal cord or any neural tissues.

5.8.1.2 PREMALIGNANT CONDITIONS

The Therapeutics of the invention that promote Nogo activity can also be administered to treat premalignant conditions and to prevent progression to a neoplastic or malignant state, including but not limited to those disorders listed in Table 1. Such prophylactic or therapeutic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79.) Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical

metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic that promotes Nogo function. As mentioned *supra*, such characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface protein, etc. (see also *id.*, at pp. 84-90 for characteristics associated with a transformed or malignant phenotype).

In other embodiments, a patient which exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a Therapeutic: neurofibromatosis of Von Recklinghausen or retinoblastoma; *see* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

In another specific embodiment, a Therapeutic of the invention is administered to a human patient to prevent progression to kidney, cartilage (of the breast bone), skin, skeletal muscle, lung, or spleen of cancer, melanoma, or sarcoma.

5.8.1.3 HYPERPROLIFERATIVE AND DYSPROLIFERATIVE DISORDERS

In another embodiment of the invention, a Therapeutic that promotes Nogo activity is used to treat or prevent hyperproliferative or benign dysproliferative disorders. Specific embodiments are directed to treatment or prevention of cirrhosis of the liver (a condition in which scarring has overtaken normal liver regeneration processes), treatment of keloid (hypertrophic scar) formation (disfiguring of the skin in which the scarring process interferes with normal renewal), psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate determination), benign tumors, fibrocystic conditions, and tissue hypertrophy (*e.g.*, prostatic hyperplasia).

5.8.1.4 GENE THERAPY

In a specific embodiment, nucleic acids comprising a sequence encoding a Nogo protein or functional derivative thereof, are administered to promote Nogo function, by way of gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting Nogo function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the Therapeutic comprises a Nogo nucleic acid that is part of an expression vector that expresses a Nogo protein or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the Nogo coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the Nogo coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the Nogo nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or

transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector that contains the Nogo nucleic acid is used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The Nogo nucleic acid to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to the central nervous system. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the respiratory epithelia, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene

therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; and Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234.

In addition to Adenoviruses, Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300.

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic

stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a Nogo nucleic acid is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Additional methods that can be adapted for use to deliver a nucleic acid encoding a Nogo protein or functional derivative.

5.8.2 TREATMENT AND PREVENTION OF DISORDERS IN WHICH NOGO BLOCKS REGENERATION

Diseases and disorders in which neurite extension, growth or regeneration are desired are treated by administration of a Therapeutic that antagonizes (inhibits) Nogo function. The diseases, disorders or damage which ultimately result in damage of the nervous system include, but are not limited to, central nervous system (CNS) trauma, (e.g. spinal cord or brain injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, multiple sclerosis, amyotrophic lateral sclerosis, and progressive supra-nuclear palsy); by administering compounds that interfere with Nogo activity (*e.g.*, a dominant negative Nogo derivative; antibodies to Nogo; anti-sense nucleic acids that encode *Nogo*; Nogo ribozymes or chemical groups that bind an active site of Nogo).

Therapeutics that can be used include but are not limited to *Nogo* antisense nucleic acids, and Nogo nucleic acids that are dysfunctional (*e.g.*, due to a heterologous (non-*Nogo* sequence) insertion within the *Nogo* coding sequence) that are used to "knockout" endogenous *Nogo* function by homologous recombination (see, *e.g.*, Capecchi, 1989, Science 244:1288-1292). Anti-Nogo antibodies (and fragments and derivatives thereof containing the binding region thereof) can be used as an antagonist of Nogo. In a specific

embodiment of the invention, a nucleic acid containing a portion of a *Nogo* gene in which *Nogo* sequences flank (are both 5′ and 3′ to) a different gene sequence, is used, as a Nogo antagonist, to promote *Nogo* inactivation by homologous recombination (see also Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438). Other Therapeutics that inhibit Nogo function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of Nogo to another protein, or inhibit any known Nogo function, as preferably assayed *in vitro* or in cell culture, although genetic assays may also be employed. Preferably, suitable *in vitro* or *in vivo* assays, are utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected tissue.

In specific embodiments, Therapeutics that inhibit Nogo function are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an increased (relative to normal or desired) level of Nogo protein or function, for example, in patients where Nogo protein is overactive or overexpressed; or (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of Nogo antagonist administration. The increased levels in Nogo protein or function can be readily detected, *e.g.*, by quantifying protein and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed Nogo RNA or protein. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize Nogo protein (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect Nogo expression by detecting and/or visualizing respectively Nogo mRNA (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.), etc.

5.8.2.1 ANTISENSE REGULATION OF *NOGO* EXPRESSION

In a specific embodiment, Nogo function is inhibited by use of *Nogo* antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding Nogo or a portion thereof. A *Nogo* "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a portion of a *Nogo* RNA (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of a *Nogo* mRNA. Such antisense nucleic acids have utility as Therapeutics that inhibit Nogo function, and can be used in the treatment or prevention of disorders as described *supra*.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In a specific embodiment, the *Nogo* antisense nucleic acids provided by the instant invention can be used to promote regeneration of neurons of the central nervous system in particular, including regeneration of the corticospinal tract, plasticity during recovery, regrowth of neurons and healing of damage associated with traumatic injuries, strokes, and neurodegenerative diseases.

The invention further provides pharmaceutical compositions comprising an effective amount of the *Nogo* antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention is directed to methods for inhibiting the expression of a *Nogo* nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising an *Nogo* antisense nucleic acid of the invention.

Nogo antisense nucleic acids and their uses are described in detail below.

5.8.2.1.1 *NOGO* ANTISENSE NUCLEIC ACIDS

The *Nogo* antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 oligonucleotides). In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a *Nogo* antisense oligonucleotide is provided, preferably of single-stranded DNA. In a most preferred aspect, such an oligonucleotide comprises a sequence antisense to the sequence near one of the two promoter sequences of the *Nogo* gene, or a sequence encoding carboxy-terminal portion of the *Nogo* gene. It may

be desirable to selectively inhibit the expression one of the Nogo isoforms. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The Nogo antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

In a specific embodiment, the *Nogo* antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see, *e.g.*, PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). In another embodiment, the oligonucleotide is a 2′-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the *Nogo* antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the *Nogo* antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells.

Expression of the sequence encoding the Nogo antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *Nogo* gene, preferably a human *Nogo* gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded Nogo antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a *Nogo* RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.8.2.1.2 THERAPEUTIC USE OF *NOGO* ANTISENSE NUCLEIC ACIDS

The *Nogo* antisense nucleic acids can be used to treat (or prevent) disorders of a cell type that expresses, or preferably overexpresses, *Nogo*. In a specific embodiment, such a disorder is a growth proliferative disorder. In a preferred embodiment, a single-stranded DNA antisense Nogo oligonucleotide is used.

Cell types which express or overexpress *Nogo* RNA can be identified by various methods known in the art. Such methods include but are not limited to hybridization with a *Nogo*-specific nucleic acid (*e.g.* by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into Nogo, immunoassay, etc. In a preferred aspect, primary tissue from a patient can be assayed for Nogo expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of a *Nogo* antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a disease or disorder which is of a type that expresses or overexpresses *Nogo* RNA or protein.

The amount of *Nogo* antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising *Nogo* antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the *Nogo* antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265:16337-16342).

5.9 DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific Therapeutic is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a Therapeutic, and the effect

of such Therapeutic upon the tissue sample is observed. For example, a Therapeutic that is an inhibitor of Nogo function can be assayed by measuring neurite regrowth or functional recovery of motor control in the patient.

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.10 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the Therapeutic comprises a nucleic acid are described above; additional appropriate formulations and routes of administration can be selected from among those described hereinbelow.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (*see* Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); *see also* Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is

known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a

hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.11 DIAGNOSIS AND SCREENING

Nogo proteins, analogues, derivatives, and subsequences thereof, *Nogo* nucleic acids (and sequences complementary thereto), anti-Nogo antibodies, have uses in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting Nogo expression, or monitor the treatment thereof. In particular, such an immunoassay is carried out by a method comprising contacting a sample derived from a patient with an anti-Nogo antibody

under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, can be used to detect aberrant Nogo localization or aberrant (*e.g.*, low or absent) levels of Nogo. In a specific embodiment, antibody to Nogo can be used to assay in a patient tissue or serum sample for the presence of Nogo where an aberrant level of Nogo is an indication of a diseased condition. By "aberrant levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as immunohistochemistry, pathology, western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, immunohistochemistry assays, protein A immunoassays, to name but a few.

Nogo genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. *Nogo* nucleic acid sequences, or subsequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in Nogo expression and/or activity as described *supra*. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to *Nogo* DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

In specific embodiments, diseases and disorders involving cellular growth and development disorders can be diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting decreased levels of Nogo protein, Nogo RNA, or Nogo functional activity as demonstrated growth inhibition, or by detecting mutations in Nogo RNA, DNA or protein (*e.g.*, translocations in Nogo nucleic acids, truncations in the Nogo gene or protein, changes in nucleotide or amino acid sequence relative to wild-type Nogo) that cause decreased expression or activity of Nogo. Such diseases and disorders include but are not limited to those described in Section 3 and Section 5.8.1.1. By way of example, levels of Nogo protein can be detected by immunoassay, levels of Nogo RNA can be detected by hybridization assays (*e.g.*, Northern blots, dot blots), Nogo binding to cellular growth inhibitor protein receptors can be done by

binding assays commonly known in the art, translocations and point mutations in Nogo nucleic acids can be detected by Southern blotting, RFLP analysis, PCR using primers that preferably generate a fragment spanning at least most of the Nogo gene, sequencing of the Nogo genomic DNA or cDNA obtained from the patient, etc.

Kits for diagnostic use are also provided, that comprise in one or more containers an anti-Nogo antibody, and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-Nogo antibody can be labeled (with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). A kit is also provided that comprises in one or more containers a nucleic acid probe capable of hybridizing to *Nogo* RNA. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides) that are capable of priming amplification [*e.g.*, by polymerase chain reaction (see *e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Qβ replicase, cyclic probe reaction, or other methods known in the art] under appropriate reaction conditions of at least a portion of a *Nogo* nucleic acid. A kit can optionally further comprise in a container a predetermined amount of a purified Nogo protein or nucleic acid, *e.g.*, for use as a standard or control.

5.12 SCREENING FOR NOGO AGONISTS AND ANTAGONISTS

Nogo nucleic acids, proteins, and derivatives also have uses in screening assays to detect molecules that specifically bind to Nogo nucleic acids, proteins, or derivatives and thus have potential use as agonists or antagonists of Nogo, in particular, molecules that thus affect cellular growth regulation. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as neural growth promoters for drug development. The invention thus provides assays to detect molecules that specifically bind to Nogo nucleic acids, proteins, or derivatives. For example, recombinant cells expressing *Nogo* nucleic acids can be used to recombinantly produce Nogo proteins in these assays, to screen for molecules that bind to a Nogo protein. Molecules (*e.g.*, putative binding partners of Nogo) are contacted with the Nogo protein (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to the Nogo protein are identified. Similar methods can be used to screen for molecules that bind to Nogo derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

By way of example, diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to Nogo. Many

libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718); Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

in vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a Nogo protein (or nucleic acid or derivative) immobilized on a solid phase

and harvesting those library members that bind to the protein (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a Nogo protein or derivative.

5.13 ANIMAL MODELS

The invention also provides animal models, including but not limited to models in mice, hamsters, sheep, pigs, cattle, and preferably non-human mammals.

In one embodiment, animal models for diseases and disorders involving neurite extension, growth and regeneration are provided. Such an animal can be initially produced by promoting homologous recombination between a Nogo gene in its chromosome and an exogenous Nogo gene that has been rendered biologically inactive (preferably by insertion of a heterologous sequence, *e.g.*, an antibiotic resistance gene). In a preferred aspect, this homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing the insertionally inactivated Nogo gene, such that homologous recombination occurs, followed by injecting the ES cells into a blastocyst, and implanting the blastocyst into a foster mother, followed by the birth of the chimeric animal ("knockout animal") in which a Nogo gene has been inactivated (see Capecchi, 1989, Science 244:1288-1292). The chimeric animal can be bred to produce additional knockout animals. Such animals can be mice, hamsters, sheep, pigs, cattle, etc., and are preferably non-human mammals. In a specific embodiment, a knockout mouse is produced.

Such knockout animals are expected to develop or be predisposed to developing diseases or disorders involving the central nervous system and thus can have use as animal models of such diseases and disorders, *e.g.*, to screen for or test molecules (*e.g.*, potential nervous system disorder therapeutics) for the ability to inhibit tumors of nerve tissue and thus treat or prevent such diseases or disorders.

The present invention is not to be limited in scope by the microorganism deposited or the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying Figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

6. EXAMPLE: CHARACTERIZATION OF THE NUCLEOTIDE AND PROTEIN PRODUCT OF THE NOGO GENE

The examples described herein demonstrate that the cloned gene, *Nogo*, encodes a protein that is a potent neural cell growth inhibitor and is also recognized by the monoclonal antibodies described in Schwab et al., U.S. Patent No. 5,684,133.

6.1 MATERIALS AND METHODS

The following sections describe materials and methods used in the present invention. One of ordinary skill in the art will recognize that these materials and methods are merely illustrative of the presently claimed invention and modifications are envisioned by the present inventors. Such modifications are intended to fall within the scope of the appended claims.

6.1.1 PURIFICATION OF BOVINE NOGO FROM MYELIN

All purification steps were carried out at 4°C and inhibitory substrate activity of the obtained fractions was routinely determined by the NIH 3T3 spreading and PC12 neurite outgrowth assays (Section 6.1.10). Bovine spinal cord tissue was carefully cleaned by stripping off the meninges and cut into small pieces. The myelin was then extracted in extraction buffer (60 mM CHAPS, 100 mM Tris-Cl, pH 8.0, 10 mM EDTA buffer, pH 8.0, 2.5 mM iodacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml peptstatin A).

To obtain spinal cord extract, the tissue was homogenized directly in CHAPS extraction buffer in a ratio of (1:1; w:v). The homogenate was centrifuged twice at 100,000 X g (Kontron type: K50.13, fixed angle) for 1 hour at 4°C. The clear supernatant (extract) was immediately applied to a Q-Sepharose column (2.6 X 11.5 cm), equlibrated in buffer A (20 mM Tris-Cl, pH 8.0, 0.5% (w/v) CHAPS). Bound proteins were eluted with a five-bed volume linear gradient from 0 to 1 M NaCl in buffer A (100 ml gradient in 50 minutes). Active fractions containing bovine NI220 eluted around 0.4 M NaCl and were pooled (q-pool 1) for subsequent applications on Superdex 200 (2.6 X 60 cm) column, equilibrated in buffer B (150 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5% (w/v) CHAPS).

Active fractions, after gel filtration (s-pool 1), were separated by 6% SDS-PAGE under reducing conditions and low constant power (2 watts/gel) for a total of 2500 Volt-hours. Bands and gel regions were identified after Coomoassie Blue staining (0.1% w/v R250 in 50% methanol and 10% acetic acid), cut out, and extracted in 800 μl of gel elution

buffer (0.5% (w/v) CHAPS, 20 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0, 2.5 mM iodacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml peptstatin A) for at least 48 hours at 4°C.

6.1.2 MICROSEQUENCING OF PURIFIED NOGO

The IN-1 neutralizable active gel-eluted material of several gels was re-run on a 10% SDS-polyacrylamide gel under reducing conditions, and stained with 0.1%(w/v) Coomassie Blue R250 in 50% methanol and 10% acetic acid. The 220 KDa band was cut out, and endoproteinase Lys-C digestion (1:50 molar ratio) was performed directly in the gel. The sample was acidified and applied to a reverse phase high performance liquid chromatography column, peptides were separated with a linear gradient (0-100%) of 0.04% trifluoroacetic acid and 80% acetonitrile, and fractions containing single peptide species were subjected to automated Edman degradation.

6.1.3 ELECTROPHORESIS OF PURIFIED NOGO

High resolution SDS-PAGE was carried out using 6%(w/v) SDS-polyacrylamide gels (10 X 24 X 0.01 cm) under reducing conditions (100 mM dithiothreitol). Transfer onto Immobilon-P membranes (Millipore) was performed in 20 mM Tris base, 192 mM glycine, pH 8.3, 0.037%(w/v) SDS, 20% methanol with a semi-dry transfer apparatus (Bio-Rad, Trans Blot SD). Transfer time was 2 h at 0.8 mA/cm^2. Blocking reagent (1 hour at room temperature) was 3% gelatin in PBS (phosphate-buffered saline, pH 7.2, 8 g NaCl, 0.2 g of kH_2PO_4, 2.8 g of $Na_2HPO_4\,12H_2O$, and 0.2 g of KCl, dissolved in 1 liter of water) and the washing solution contained 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.4% Tween (3 X 10 minutes at room temperature). Incubation time for the first antibody (for dilution with 1% gelatin in PBS) was usually overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2000) was incubated for 1 hour at room temperature. The ECL chemiluminescence system was used for detection (Amersham Pharmacia Biotech)

6.1.4 cDNA LIBRARY PROBING

White matter was freshly dissected from bovine spinal cord, and poly(A)$^+$ RNA was extracted using the FastTrack kit (Invitrogen). Construction of cDNA libraries was performed using the Uni-ZAP kit (Stratagene) following the manufacturer's instructions. The complexity of the libraries was greater than $4x10^6$ plaque forming units in total, and the average size of the inserts was approximately 1.8 kilobases.

Degenerate oligonucleotides MSC5-8 (MSC5: TCIGTIGGYAAIACIGCIGGYAARTC (SEQ ID NO:47); MSC6: TCIGTIGGIAGIACIGCIGGYAAYTC (SEQ ID NO:48); MSC7: TCIGTIGGYAAIACIGCIGGIAGRTC (SEQ ID NO:49); MSC8: TCIGTIGGIAGIACIGCIGGIAGRTC (SEQ ID NO:50)) were designed from the bNI220 peptide 1 sequence, and MSC9 (GARATHGCIGAIATHCARGAYGGIGA (SEQ ID NO:51)) was designed from bNI220 peptide 2 sequence. Oligonucleotides were synthesized by MWG Biotech (Munchenstein, Switzerland) and labeled with the DIG DNA 3'-end labeling kit. Riboprobes were synthesized using the DIG RNA labeling kit (Boehringer Mannheim).

Probe hybridization and washing conditions were as described by the manufacturer (MSC5-8 and MSC9 were used at a hybridization and washing temperature of 57°C). Probe detection was performed using the CDP-star system (Boehringer Mannheim). CDNA library handling and screening was done according to the protocols for lambda ZAP cDNA libraries (Stratagene). Genescreen (DuPont) nylon membranes were used for plaque lifts.

6.1.5 DNA SEQUENCING

Both strands of CWP1-3, Oli18, Oli3, and R1-3U21 were sequenced with the Perkin Elmer AB1377 system by Microsynth (Balgach, Switzerland). DNA sequences were analyzed by the DNASIS program (Hitachi). Database searches were performed with the BLAST program (NCBI).

6.1.6 RNA ANALYSIS

Total RNA and poly(A)$^+$ RNA were extracted from tissues using the RNAgent (Promega) or FastTrack kit (Invitrogen), respectively. RNAs were separated by electrophoresis on 1% formaldehyde gels and transferred to Genescreen membranes. Blots were hybridized with antisense riboprobes, which were generated with the DIG RNA labeling kit (Boehringer Mannheim), from the relevant plasmids. Blot hybridization, washing, and CDP star-detection conditions were as described by the manufacturer. The 'common' probe, EST111410 (TIGR, ATCC, Rockville, MD, USA) contains transcript A sequence between nucleotides 2535-4678, the exon 1 specific probe contains transcript A sequence between nucleotides 65-769, and the exon 2 specific-probe contains transcript A sequence between nucleotides 815-3183.

6.1.7 ANTISERA PRODUCTION

Antiserum 472 (AS 472) was generated by Research Genetics, Inc. (Huntsville AL, USA) against the synthetic peptide P472, SYDSIKLEPENPPPYEEA (bovine sequence; SEQ ID NO:33), which corresponds to rat Nogo amino acid sequence at 623 to 640 of SEQ ID NO:2, with three mismatches.

Antiserum Bruna (AS Bruna) was generated against a fragment of recombinant Nogo protein, expressed in *E. coli* as a fusion protein. Specifically, the carboxy-terminus of the rat Nogo A nucleotide sequence encoding amino acids 762 to 1,163 of SEQ ID NO:2 (expressed in *E. coli* using the Novagen pET system) was used to generate AS Bruna anti-Nogo antisera.

6.1.8 ELECTROPHORESIS AND WESTERN BLOTTING

SDS-PAGE and Western blotting was performed by standard methods well known to those skilled in the art. Antibodies were diluted as follows: AS Bruna 1: 7,500; AS 472 1:2,000; anti-myc (9E10) 1:5,000 (Invitrogen); anti-BiP 2 µg/ml (Stressgen); mAb IN-1 hybridoma supernatant was used undiluted. Secondary antibodies were: HRP conjugated anti-rabbit (Pierce; 1:20,000); anti-mouse IgM (1:50,000); and alkaline phosphatase conjugated anti-mouse (Milan Analytica AG, La Roche, Switzerland; 1:5,000).

6.1.9 IMMUNOHISTOCHEMISTRY

Adult rat spinal cord or cerebellum was rapidly dissected, embedded in OTC compound and frozen at -40°C. Twenty post mortem sections were cut and fixed in ethanol/acetic acid at 40°C. Imunostaining was performed as described by Rubin et al., 1994, J. Neurocytol. 23:209-217, except that the quenching step was omitted. Alternatively, tissue sections were fixed by methanol (2 minutes at -20°C), and immunostaining was carried out as per Rubin et al., *supra*. Primary antibodies used were (Antibody: (dilution)): Hybridoma supernatant of IN-1: (undiluted); AS Bruna: (1:5000); or affinity purified AS 472: (1:50).

6.1.10 NIH 3T3 FIBROBLAST SPREADING ASSAY

NIH 3T3 fibroblasts were plated onto culture dishes pre-coated with 5 µg/well (=1 cm^2) q-pool. Q-pool is the pooled active fractions of bovine spinal cord extract separated on a Q sepharose column. IN-1 was used as undiluted culture supernatant (1-10 µg/ml), AS Bruna and pre-immune serum were diluted 1:1000 in PBS, and AS 472 and pre-immune serum were diluted 1:500 in PBS. To compensate for activity variations in different q-pool preparations the number of inhibited, round cells plated on the q-pool was normalized to

100% and to 0% plated on buffer control (Spillman et al., 1998, J. Biol. Chem. 273:19283-93).

6.1.11 DRG NEURITE OUTGROWTH ASSAY

Dorsal root ganglia (DRG)s were dissected from E16 embryonic chicken in Hank's balanced salt solution (HBSS), divided into two parts and plated on dishes pre-coated with q-pool in 100 µl F12 medium with 10% (FCS) and 1% methocel. Neurite outgrowth from individual DRGs was scored after 24 hours incubation at 37°C in a semi-quantitative way using a scale of 0 (no outgrowth) to 4 (maximum outgrowth).

6.1.12 DRG/OPTIC NERVE CO-CULTURE ASSAY

Optic nerves were dissected from adult rats, irradiated with 5500 Grays and injected either with AS 472 or the corresponding pre-immune serum (1:10 dilution). Pairs of nerves were cultured in 3-chamber cultures such that one end of each nerve reached through a silicon grease/teflon ring barrier into the middle chamber, where dissociated cultured primary DRGs neurons from P0 rats were placed. After two weeks in culture, the nerves were fixed by standard techniques known in the art and embedded for electron microscopy (EM), and ultrathin sections were taken at a distance of about 3.5 mm from the DRG-exposed stump. Sections were systematically analyzed for the presence of regrowing axons using a Zeiss EM 902.

6.1.13 NOGO A EXPRESSION IN COS CELLS

The Nogo A open reading frame was subcloned into the pcDNA3.1mychis vector (Invitrogen) using standard cloning techniques known in the art. The resulting plasmid (Nogo-myc19) gave rise to a recombinant protein containing the Nogo A sequence fused to the myc-his tag (21 amino acids). Nogo-myc19 (2µg DNA per 35 mm dish) or control plasmid (pcDNAmychisLacZ) were transfected into COS cells using superfect (Qiagen) according to the manufacturer's protocol. Transfected cells were harvested 36-48 hours after transfection. Based on immunofluorescent staining with anti-myc antibody and enzymatic β-galactosidase color reaction, the average transfection rate was estimated to be about 20%. Transfected COS cells were fixed with 95% ethanol /5 % acetic acid (4°C, 25 minutes), blocked in PBS/ 10% FCS, and incubated with AS Bruna (1:200) or IN-1 (1:2) for 2 hours in PBS/ 1% FCS at room temperature. Cells were washed with PBS and reacted with fluorescent secondary antibodies (goat anti-rabbit-FITC for AS Bruna and goat anti-mouse-TRITC for IN-1 detection, Jackson Immuno Research Lab. Inc., West Grove, PA).

6.1.14 OLIGODENDROCYTE CULTURES

Oligodendrocytes isolated from new-born rat brain were plated on 75 cm^2 poly-lysine flasks (Sigma, St. Louis, MO) and cultured for 10-12 days in DMEM supplemented with 5% FCS. Enriched, mixed populations of oligodendrocytes and their progenitors were released from the astrocyte monolayer by shaking overnight at 210 rpm in an orbital shaker. The cells were plated at a density of 1-2x10^6 cells on poly-lysine coated 35 cm^2 dishes. Progenitors were allowed to differentiate in chemically defined medium (CDM) for 3-4 days.

6.1.15 CELL SURFACE BIOTINYLATION

P4 rat whole brain cultures were prepared as described in van der Haar, et al. (1998, J. Neurosci. Res. 51:371-81). At day 7 *in vitro* they were biotinylated with the cell-impermeable EZ-LINK-Sulfo-NHS-LC-Biotin (Pierce) as described except that all steps were carried out at 15°C and cells lysed in 1 ml of lysis buffer (0.05M NaH_2PO_4 pH8.0, 0.15M NaCl, 0.5% CHAPS (Sigma), 2.5mM iodacctamidc, 1mM phenylmethylsulfonyl fluoride, 0.1μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Biotinylated proteins were immuno-precipitated with Dynabeads M-280 Streptavidin (Dynal) subjected to SDS-PAGE and transferred to nitrocellulose membranes that were probed with AS472, α-BiP and α-β-tubulin. The membranes were stripped with Re-Blot Western Blot Recycling Kit (Chemicon).

6.1.16 IMMUNOCYTOCHEMISTRY

Optic nerve oligodendrocytes were prepared as described in Schwab and Caroni (1988, J. Neurosci. 8:2381-2393). Two day-old cultures were incubated with AS 472 (1:200) or mAb IN-1 (1:3) in medium for 25 minutes at room temperature (rt). Cultures were washed, fixed with 4% paraformaldehyde/ 5% sucrose in PBS, and blocked in 0.1M maleic acid/ 2% blocking reagent (Boehringer Mannheim) for 1 hour. Secondary alkaline phosphatatase conjugated antibodies (Milan Analytica) were used at 1:7,500 in 0.1M maleic acid/ 1% blocking reagent (1 hour, rt). Transfected COS cells were fixed with 95% ethanol /5 % acetic acid (4°C, 25 minutes), blocked, and incubated with AS Bruna (1:200) or mAb IN-1 for 2 hours at rt. Cells were reacted with goat anti-rabbit-FITC, and goat anti-mouse TRITC (Jackson Immuno Research Lab).

6.1.17 OPTIC NERVE CHAMBER

Pairs of optic nerve were cultured in a 3-chamber culture system as described in Schwab et al. (1988, J. Neurosci. 8:2381-2393), injected with and exposed to either AS 472 or the corresponding pre-immune serum (1:10). Optic nerves were embedded for electron

microscopy (EM), and ultra-thin sections were taken at a distance of about 3.5 mm from the DRG-exposed stump. Sections were systematically analysed for the presence of regrowing axons using a Zeiss EM 902 microscope.

6.2 EXPERIMENTAL RESULTS

The following section discloses the experimental results obtained from the methods sections set forth in 6.1 and subsections.

6.2.1 ISOLATION OF NOGO CDNA

The bovine homologue of rat NI-250 was purified, bNI220, and peptides of the purified protein were generated by protease digestion. Multiple digoxygenin-labeled degenerate oligonucleotides were designed according to six different bNI220 peptide sequences. Several cDNA clones were isolated from the screening of a bovine white matter library using these oligonucleotides. The insert of the longest clone (CWPI-3, Figure la) was used to synthesize probes for subsequent screening of rat cDNA libraries. Selected clones from such screenings are shown in Figure la. DNA sequence analysis of these cDNA clones suggested that three different transcripts originate from one gene, and this gene was designated Nogo. The different transcripts likely result from both alternative promoter usage and alternative splicing (Nogo A, Nogo B and Nogo C; Figure 1b). DNA sequences were compiled from the clones shown in Figure 1A to create the transcript A, the DNA sequence of which is shown in Figure 2a.

Conceptual translation of the three transcripts gives rise to protein products designated Nogo A (1163 amino acids), Nogo B (360 amino acids) and Nogo C (199 amino acids). Since Nogo A contains all six peptide sequences obtained from purified bNI220 (Figure 2b), it is likely equivalent to the purified protein, rat NI-250. Nogo A, B, and C have a common carboxy terminus of 188 amino acids (the common domain), and Nogo A and B share an amino terminus of 172 amino acids. Nogo A is longer than Nogo B by 803 amino acids due to alternative splicing.

None of the Nogo isoforms possess a hydrophobic stretch of amino acids at the N-terminus, which could be used as a conventional signal peptide. However, proteins have been described which lack a conventional signal peptide but are still transferred through membranes, for example fibroblast growth factor (Florkiewicz et al., 1995, J. Cell. Physiology 162:388-399), ciliary neurotrophic factor (Sendtner et al., 1994, J. Neurobiology 25:1436-1353) and interleukin-1 (Rubartelli et al., 1990, EMBO J. 9:1503-1510). Membrane proteins such as commissureless (Tear et al., 1996, Neuron 16:501-514) also lack a conventional signal peptide yet are inserted into the membrane.

Although there is no in-frame stop codon in the putative 5'-untranslated region which would unequivocally define the start codon, the following evidence suggests that the methionine indicated in Figure 2a is the start codon for Nogo A and Nogo B: (1) The sequence around this presumed start codon conforms well with the consensus sequence for translation start sites (GCCGCC A/G CCATGG; SEQ ID NO:39); (2) Extensive efforts were made to search for more upstream sequences by both library screening and 5'-RACE. None of these searches have resulted in the identification of more upstream sequences; and (3) Eukaryotic recombinant Nogo A expressed from the above mentioned methionine has an apparent molecular weight of about 200 kD, as estimated by SDS-PAGE, which is indistinguishable from endogenous Nogo A from rat oligodendrocytes (Figure 11a).

6.2.2 NOGO SEQUENCE ANALYSIS

Nogo A contains seven potential N-glycosylation sites, however biochemical evidence indicates Nogo A does not have a major polysaccharide component. Nogo A also has nineteen recognition sites for PKC, and seven recognition sites for casein kinase II (Figure 2a). All three Nogos have two common carboxy terminal hydrophobic domains of 35 and 36 amino acids, respectively. Either or both of them may be used as trans- or intra-membrane domains, which is consistent with the characterization of bNI220 as an integral membrane protein. Nogo A (as well as Nogo B and C) does not contain any motifs of known cell adhesion molecules, extracellular matrix proteins, or other guidance molecules.

Nogo sequences were used to search different databases for homologous genes, the carboxy terminals common domain of the three Nogo products is similar (62.5%) to an identified human gene, *nsp* (*cl13* and *s-rex* in rat, and *chs-rex* in chicken) (Figure 3). An EST from C. *elegans* and a *Drosophila melanogaster* EST also have significant similarity (16.6% and 13.6%, respectively) to both *Nogo* and *nsp* at this same region. The 180 amino acid carboxy terminal domains of both Nogos and Nsps are highly conserved across mammalian species (98.3% and 97.3%, respectively), which suggests that they may perform similar and essential functions. Outside of this region, the similarity for a given protein among species is also high (73% between rat and bovine Nogo A; 76.2% between NSP-A and S-rexb; 50% between Chs-rexb and NSP-A or S-rexb). The similarity between NSPs and Nogos are, however, limited to their carboxy terminal, hydrophobic, common domain (Figure 3a), and to the acidic nature of the proteins outside of this conserved region. The NSPs (NSP-A, -B and -C) have been previously described as neuro-endocrine specific products with unknown functions. In situ hybridization and immunohistology showed a neuronal localization of NSPs in the nervous system. Another human gene, nsp-like-1,

which also has a carboxy terminal hydrophobic region with 50% similarity to both *Nsp* and *Nogo* was recently identified.

6.2.3 NOGO TISSUE EXPRESSION

The *Nogo* expression pattern was examined by Northern blotting and in situ hybridization. When a "common" probe (Section 6.1.6) was used, three major Nogo transcripts (Designated: A, 4.6 kb; B, 2.6 kb; and C, 1.7 kb) were detected in the optic nerve, the spinal cord, and the cerebral cortex (Figure 4a). In dorsal root ganglia, only the two larger transcripts were detected. A 2.6 kb, major transcript was detected in PC12 cell, whereas a 4.6 kb band can be detected only after long exposures (Figure 4a). In sciatic nerve, lower levels of transcripts were detected with the 2.6 kb band being the major transcript. When spinal cord and PC 12 cell poly $(A)^+$ RNA were hybridized to an exon 1 specific probe, only the 4.6 kb and the 2.6 kb transcripts were detected; when the hindbrain and skeletal muscle poly$(A)^+$ RNAs were hybridized to an exon 2-specific probe, only the 4.6 kb transcript in hindbrain was detected (Figure 4b). These results verify the transcript map shown in Figure lB. Northern blotting results, however, also demonstrated that *Nogo* expression is not restricted to the nervous system (Figure 4c); *Nogo* transcripts were also detected in skeletal muscle (1.7 kb), kidney (2.6 kb and 1.7 kb), cartilage (from the breastbone, 1.7 kb), skin (1.7 kb), lung (2.6 kb), and spleen (2.6 kb). Except for the skeletal muscle, which expresses Nogo C transcripts at a high level, the level of *Nogo* transcripts outside of the nervous system is lower than that of the nervous system. So far, the 4.6 kb *Nogo* A transcripts seem to be uniquely transcribed in the nervous system.

In situ hybridizations on adult rat CNS tissue sections using the common probe showed moderate labeling of rows of cell bodies in the white matter of various parts of the brain and the spinal cord. This arrangement is typical of interfascicular oligodendrocytes (Figure 5a, d). In addition to oligodendrocytes, several types of neurons also express Nogo transcripts at high levels (Figure 5c, e). In cerebellum, double staining of sections with an anti-GFAP antibody and in situ hybridization clearly showed a strong labeling of the Purkinje cells by the Nogo probe, whereas astrocytes were not labeled (Figure 5e, f). In developing optic nerves, *Nogo* transcripts were detected as early as postnatal day 0 (P0), i.e., several days before the mRNAs of the major myelin proteins proteolipid protein (PLP) and myelin basic protein (MBP) can be detected (Figure 6). This timing is consistent with the appearance of the first galactocerebroside-positive oligodendrocytes and the expression of a neurite growth inhibitory activity, which can be neutralized by IN-1.

Antisera were generated against a synthetic peptide based on a bovine Nogo A specific sequence (AS 472), and against a 45 kD recombinant, partial rat Nogo A (AS

Bruna) (Section 6.1.7). AS 472 and AS Bruna each, recognized a protein of about 200 kD in bovine myelin, and AS Bruna further recognized a 200 kD rat myelin protein on Western blots (Figure 7). Sections of adult rat spinal cord and cerebellum were stained with AS 472, AS Bruna, and IN-1. When the sections were fixed with ethanol/acetic acid (a procedure shown earlier to be required for preservation and accessibility of IN-1 antigens) strong staining of white matter/myelin was seen with all three antibodies (Figure 8). Staining of oligodendrocyte cell bodies was particularly distinct with AS Bruna. Treatment of the fresh frozen sections with methanol instead of ethanol/acetic acid abolished the myelin stain except for oligodendrocyte cell bodies.

AS Bruna and AS 472 also stained some types of neurons including motor neurons in spinal cord, and granular and molecular layers in the cerebellum. Purkinje cells stained strongly with AS 472 and AS Bruna, but there was no detectable staining with IN-1.

6.2.4 NOGO ANTIBODIES INHIBIT NOGO INDUCED GROWTH INHIBITION *IN VITRO*

Semi-purified bovine spinal cord NI-220 preparations (q-pool) can prevent NIH 3T3 fibroblast spreading and neurite outgrowth. In the presence of Nogo antisera, either AS Bruna, AS 472, or IN-1, the q-pool inhibitory activity was reduced, *i.e.*, NIH 3T3 fibroblasts underwent spreading and embryonic chicken dorsal root ganglia (DRG) extend neurites on dishes coated with q-pool (Figure 9). Specificity was demonstrated by addition of peptide P472, which was the peptide used to raise AS 472 (Section 6.1.7). P472 successfully blocked the inhibitory effect of AS 472, whereas, a control peptide had no effect on the inhibition.

Furthermore, the presence of Nogo A on the cell surface of oligodendrocytes was demonstrated immunocytochemically, functionally and biochemically using AS 472. When live, primary cultured oligodendrocytes were stained with either mAb IN-l or AS 472 a relatively weak (as compared to immunocytochemistry for galactocerebroside) but clear surface staining was observed on differentiated oligodendrocytes (Figure 15a, c). Addition of the competing peptide (P472) for AS 472 or omitting the primary antibody abolished the specific staining (Figure 15b, d). Cell surface biotinylation and subsequent precipitation with streptavidin further proved the presence of Nogo A on the plasma membrane of oligodendrocytes. In the precipitate, AS 472 detected a band running about 40 kD above the intracellular, probably non-processed and non-glycosylated AS 472 immuno-positive band. The ER protein BiP could not be detected in the biotinylated fraction (Figure 15e).

Nogo A as an oligodendrocyte cell surface molecule was also analyzed functionally. Co-culturing of oligodendrocytes and NIH 3T3 fibroblasts or oligodendrocytes and DRG neurons showed clearly the inhibitory properties of mature oligodendrocytes. These assays

demonstrate that NIH 3T3 fibroblasts and DRG neurites strongly avoided the territory of the oligodendrocytes, an effect that was neutralized by mAb IN-1. In the presence of AS 472, this inhibition was equally reduced (Figure 16a, b and e, f), while preincubation of AS 472 with P472 restored the oligodendrocyte-mediated inhibition (Figure 16c, d and g, h). Quantification revealed the highly significant neutralizing capacity of mAb IN-1 and AS 472 in both types of assays (Figure 16i and j).

RecNogo-A (Figure 17a) produced by a stably transfected CHO cell line was tested for its activity on NIH 3T3 fibroblast spreading and DRG neurite outgrowth. Recombinantly produced β-galactosidase isolated from a stable CHO cell line (CHO-LacZ) was enriched in parallel with recNogo-A and was used as a control for inhibitory activity of endogenous CHO proteins in both assays. In the NIH 3T3 fibroblast spreading assay, recNogo-A-containing CHO extract (Nogo-A: about 1-5% of total protein; fig. 9a) exhibited clear inhibitory effects on cell spreading at 10 $\mu g/cm^2$ (Figure 17b). This effect was dose-dependent: at 20 $\mu g/cm^2$ the inhibitory activity was higher, whereas 5 $\mu g/cm^2$ was not inhibitory (data not shown). The inhibitory activity could be neutralized to background levels by preincubation of the coated protein with mAb IN-1 or AS Bruna, whereas a control antibody against galactocerebroside (mAb O1) or AS Bruna preimmune serum had no effect (Figure 17b).

In addition to its strong effect on the NIH 3T3 fibroblast spreading, recNogo-A-containing CHO extract, but not CHO-LacZ extract had a potent inhibitory effect on neurite outgrowth from primary cultured neurons: Dissociated DRG neurons were inhibited by recNogo-A in a dose-dependent manner (Figure 17c). This inhibitory activity could be neutralized by mAb IN-1, but not by the control mAb O1 (Figure 17c-e). Recombinant protein isolated from CHO-LacZ was not inhibitory at 1 and 5 μg, and addition of mAbs O1 or IN-1 had no effect on neurite outgrowth.

6.2.5 NEURITE REGROWTH *IN VITRO*

The ability of new-born rat DRG neurites to regenerate and grow through adult CNS tissue was investigated. Pairs of optic nerves were dissected from adult rats and cultured in a special chamber culture system such that DRG neurites had access to one end of each nerve (Figure 10a). In each culture, one of the two nerves was injected with and exposed to pre-immune rabbit serum, the second nerve was injected with AS 472, which was also present in the side chamber around the nerve. Following two weeks *in vitro* in the presence of NGF, the cultures were fixed, disassembled, and embedded for electron microscopy (EM). EM sections were taken about 3.5 mm from the end of the nerve in contact with DRG neurons. Pre-immune serum injected nerves contained no or only a few axons (Figure

10b). The latter were exclusively found associated with basement membranes and astrocytes at the surface of the nerves. In contrast, the majority of the optic nerves injected with AS 472 contained considerable numbers of axons, often up to several hundred. Contact with myelin could frequently be seen (Figure 10c, d).

6.2.6 RECOMBINANT NOGO A RECOGNITION BY IN-1

When Nogo A was expressed as a carboxy terminal myc-his-tagged recombinant protein in transfected-COS cells, western blotting using both anti-myc antibody and AS Bruna demonstrated that the recombinant Nogo A has an apparent molecular weight of about 200 kD on denaturing SDS gels (Figure 11a). On the same blot, a band of similar mobility was detected by AS Bruna in rat primary culture oligodendrocytes, suggesting that recombinant Nogo A has a nearly identical molecular weight as the endogenous Nogo A from oligodendrocytes (Figure 1a). When transfected COS cells were stained by immunofluorescence with IN-1 and AS Bruna, IN-1 and AS Bruna recognized the same, transfected cells (Figure 11b, c). The majority of the immunoreactivity was localized intracellularly and was accessible only after permeabilization.

6.2.7 MAPPING THE NOGO ACTIVE REGION(S)

A series of deletion mutants of Nogo was generated in order to map the inhibitory domain(s) or region(s) of Nogo. The deletion constructs of the *Nogo* gene were generated by using internal restriction sites, exonuclease III-mung bean digestions and polymerase chain reactions. A description of the mutants is provided in Figure 18 and its Brief Description. The majority of constructs have an N-terminal T7-tag for identification with anti-T7 monoclonal antibodies; and an N- or C-terminal hexahistidine tag ("His-tag") for purification using immobilised-Co(II)-affinity chromatography. The Nogo deletion mutants, named NiG-D1, NiG-D2, up to NiG-D20, were all tested using the NIH 3T3 fibroblast-spreading assay to determine inhibitory activity. Some mutants were tested in PC12 neurite outgrowth, dissociated rat DRG neurite outgrowth or retinal ganglion stripe assays. The results are shown in Table 2 below.

Table 2. Functional Activities of Nogo Deletion Mutants

	3T3	PC12	DRG	RGC
Nogo-A	++		+	+
Nogo-B	+			
Nogo-C	-			-
NiAext	++	+		+
EST	+/-			
NiR	+			
NiG	++			+
NiG-D1	+			
NiG-D2	+			
NiG-D3	++			
NiG-D4	+			
NiG-D5	-			
NiG-D7	+			
NiG-D8	+			
NiG-D9	+			
NiG-D10	+/-			
NiG-D14	-			
NiG-D15	+			
NiG-D16	+			
NiG-D17	+			
NiG-D18	+			
NiG-D20	++			

A positive result in the NIH 3T3 fibroblast assay (3T3) or PC12 assay is scored when fibroblasts or PC12 cells are inhibited from spreading on a plate coated with a preparation of Nogo obtained from a deletion mutant. A positive result in the embryonic chicken dorsal root ganglion neurite outgrowth assay (DRG) or ganglion growth cone collapse assay (RGC) indicates that neurite outgrowth is inhibited or that the growth cone is caused to collapse in the presence of a preparation of Nogo obtained from a deletion mutant.

The data indicate that a major inhibitory domain was identified in the Nogo-A specific region from amino acid numbers 172-974, particularly amino acid numbers 542-722. In addition, the N-terminal sequence of Nogo-A and Nogo-B (amino acid numbers 1-171) was also inhibitory for 3T3 spreading. Based on the results, regions of Nogo from amino acid numbers 172-259, and from numbers 975-1162 appear to be non-essential and can be removed without loss of inhibitory activity.

7. EXAMPLE: HUMAN NOGO NUCLEIC ACIDS AND PROTEINS, DERIVATIVES AND FRAGMENTS

The instant invention provides the nucleotide sequences encoding human Nogo protein, and fragments of human Nogo proteins, including the human equivalents to rat Nogo A, Nogo B and part of Nogo C. The human Nogo amino acid sequence is depicted in Figure 13 and has been assigned SEQ ID NO:30.

The instant invention also provides nucleotide sequences of fragments of the human *Nogo* gene. The human Nogo nucleotide sequence can be determined using the rat Nogo A transcript as an aid to align and splice together human expressed sequence tags (EST) that are homologous to the rat or bovine cDNA sequence.

For example, the ESTs AA081783 and AA333267 (Section 5.1) overlap with each other and correspond to rat Nogo A (Figure 2a; SEQ ID NO:1) nucleic acid positions 765 to 1272. The ESTs AA322592, AA092565, and AA081525 (Section 5.1) also overlap with each other and the overlapping sequences correspond to rat Nogo nucleic acids 1642 to 2131. These two independent sets of overlapping ESTs cannot be aligned to give the human sequence without direct computer comparison to the rat or bovine Nogo nucleotide sequence of the present invention. For the initial computer alignment, *ENTREZ Nucleotide QUERY* is preferable. Other computer alignment programs are listed in Section 5.1, as alternative examples and are not meant to limit the scope of computer programs that can be used.

8. DISCUSSION

8.1 CLONING OF THE NEURITE GROWTH INHIBITOR NOGO

Nogo A has many properties that support it being the previously described rat NI-250, a major neurite outgrowth inhibitory protein of CNS myelin and the antigen of the IN-1. At the molecular level, Nogo A contains all six peptides originally obtained by sequencing of bNI-220, the most inhibitory component of bovine spinal cord myelin. At the expression level, oligodendrocytes are the major cell type in adult CNS for Nogo A expression, and the timing of *Nogo* expression in the optic nerve matches the previous description of a IN-1 neutralized myelin inhibitory activity for neurite growth. Moreover, Western blotting revealed the presence of Nogo A in active q-pool fractions, and white matter from various CNS regions was stained with AS Bruna as well as AS 472 (specific for Nogo A) in a pattern identical to that of IN-1. Both of these facts agree with the interpretation that Nogo A is NI-250.

Two antisera raised against Nogo A sequences, AS Bruna and AS 472, greatly decreased the inhibitory activity of a partially purified bovine spinal cord preparation

(q-pool). AS 472 also allowed ingrowth of large numbers of dorsal root ganglia axons over several millimeters into adult optic nerve explants, very similar to IN-1.

Although the calculated molecular weight of Nogo A is about 140 kD, it has an apparent molecular weight of about 200 kD on denaturing SDS gels, which is in the range of the previous estimation of about 250 kD. The aberrant mobility of Nogo A in SDS gels is likely caused by its acidic nature rather than post-translational modifications. Aberrant mobility of proteins on SDS-PAGE has been postulated for other highly acidic proteins such as the Growth-Associated Protein GAP-43, as well as NSP-A. Furthermore, bacterially expressed recombinant Nogo A has the same apparent molecular weight as that of the endogenous Nogo A expressed by rat oligodendrocytes. This argues against major modifications of Nogo A by mechanism such as glycosylation.

8.2 NOGO PREVENTS REGENERATION AND RESTRICTS PLASTICITY OF THE ADULT CNS

The expression of *Nogo* in rat optic nerve oligodendrocytes from P0 on agrees well with the earlier findings of a IN-1 neutralizable neurite growth inhibitory activity. Interestingly, this expression precedes that of the main myelin proteins, and compact myelin formation by several days. The appearance of Nogo, possibly in response to axonal signals, could prevent further axon growth in the corresponding fiber tracts (axon numbers peak at E20 in rat optic nerves). Nogo could also inhibit collateral formation and thereby stabilize the general structure of the differentiated CNS. In grey matter of different CNS regions, the content of myelin and IN-1 immunoreactivity correlates inversely with the level of GAP-43 and the plastic potential of the given regions. Indeed, IN-1 antibodies applied to the adult CNS allow sprouting and plasticity to occur in the brainstem and the spinal cord to an extent known previously only of the new-born CNS. The large functional recovery that parallels this plasticity indicates that sprouting axons are able to form functionally appropriate connections.

It has been demonstrated previously that the response of neurons to inhibitory Nogos differs between neurons of different ages. Presumably, this is due to a differential expression of receptors, which will hopefully soon be characterized. Like for the Netrins and many growth factors the existence of different Nogo receptors, which trigger different responses, remains a possibility. The fact that Nogos are also expressed in some types of neurons points to possible interactions between the same and/or different Nogo isoforms.

8.3 NOGO BELONGS TO A NEW FAMILY OF NEURITE REGULATORY MOLECULES

Sequence analysis of Nogo reveals no known motifs of cell surface or matrix proteins involved in axonal guidance (repulsive or attractive); *i.e.* no Immunoglobulin, fibronectin type III, or EGF-domains could be identified. Neither was there homology to described neural growth inhibitors, the Semaphorins, the Netrins, or the Ephrins.

Nogos form a novel family with a group of recently described proteins, the NSP/s-rex and the NSP-like 1 proteins, based on the similarity of their carboxy terminal 180 amino acids. Like in the case of *Nogo*, both alternative promoter usage (both *Nsp* and *Nsp-like 1* gene) and alternative splicing (*Nsp* only) are responsible for the production of alternative protein products with common carboxy termini containing two stretches of hydrophobic amino acids. As indicated by the name, the NSPs (neuroendocrine-specific proteins) are predominantly expressed in neurons and several endocrine cell types. They localize mainly intracellularly in association with the endoplasmic reticulum. The NSP-like 1 gene is expressed predominantly in brain and muscle. The functions of neither the NSP nor the Nsp-like 1 families are known. The fact that potential orthologs exist in both C. elegans and Drosophila melanogaster suggests that Nogo, with its nerve regeneration and sprouting inhibitory activity, may be a newly evolved and heretofore undescribed member of the NSP family.

8.4 NOGO IN NON-NEURONAL TISSUES

The *Nogo* C transcript is expressed in skeletal muscle at a level comparable to that of the nervous system. One conceivable function of muscle Nogo C is to repel motor axons and to restrict them to the motor endplate region. Low levels of *Nogo* expression can also be detected in other non-nervous tissues. The observed inhibition of fibroblast and astrocyte spreading by myelin extract and NI-250 alludes to the presence of receptors and response mechanisms for Nogo proteins in these cells. This suggests a possible general function of Nogos in contact inhibition of cell movement.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A Nogo protein that is free of all central nervous system myelin material with which it is natively associated.

2. The protein of claim 1 which is selected from the group consisting of Nogo A and Nogo B.

3. The protein of claim 2 which comprises the amino acid sequence of Figure 2a (SEQ ID NO: 2).

4. The protein of claim 2, the amino acid sequence of which consists of an amino acid sequence selected from the group consisting of residues 1-1163 depicted in Figure 2a (SEQ ID NO: 2), and residues 1-172 fused to 975-1163 depicted in Figure 2 (SEQ ID NO: 2).

5. A purified Nogo C protein.

6. The protein of claim 5, which comprises the amino acid sequence of SEQ ID NO: 32 depicted in Figure 14.

7. The protein of claim 5, the amino acid sequence of which consists of SEQ ID NO: 32 depicted in Figure 14.

8. The protein of claim 2 or 5 which is recombinantly produced.

9. The protein of claim 2 or 5 which has the sequence of a mammalian protein.

10. The protein of claim 1, 2 or 5 which has the sequence of a human protein.

11. A protein comprising an amino acid sequence that has at least one conservative amino acid substitution in the amino acid sequence depicted in Figure 2a (SEQ ID NO:2), the amino acid sequence depicted in Figure 13 (SEQ ID NO:29) or the amino

acid sequence depicted in Figure 14 (SEQ ID NO:32), and which is able to be bound by an antibody directed against a Nogo protein having an amino acid sequence selected from the group consisting of residues 1-1163 of SEQ ID NO: 2, residues 1-172 fused to 975-1163 of SEQ ID NO: 2, and residues 1-199 of SEQ ID NO: 32.

12. The protein of claim 1 which comprises an amino acid sequence that is displaying a greater than 50% homology to the amino acid sequence of SEQ ID NO:2, as determined by a BLAST computer algorithm.

13. The protein of claim 1 which is encoded by a first nucleic acid that is hybridizable to a second nucleic acid having the nucleotide sequence depicted in Figure 2a (SEQ ID NO:1) or the nucleotide sequence depicted in Figure 12 (SEQ ID NO:28).

14. A purified fragment of the protein of claim 1 which is able to be bound by an antibody directed against a Nogo protein, and wherein said purified fragment is free of all central nervous system myelin material.

15. A purified fragment of the protein of claim 5 or 11 which is able to be bound by an antibody directed against a Nogo protein.

16. A purified protein comprising a fragment of a Nogo protein comprising an amino acid sequence selected from the group consisting of residues 31-57 depicted in Figure 2a (SEQ ID NO:2), the residues 11-191 depicted in Figure 14 (SEQ ID NO:32), the residues 988-1023 depicted in Figure 2a (SEQ ID NO:2), and residues 1090-1125 depicted in Figure 2a (SEQ ID NO:2), residues 994-1174 depicted in Figure 13 (SEQ ID NO: 29), residues 977-1012 depicted in Figure 13 (SEQ ID NO: 29), and residues 1079-1114 depicted in Figure 13 (SEQ ID NO:29).

17. A purified unglycosylated protein which is selected from the group consisting of Nogo A, Nogo B and Nogo C.

18. A purified protein that is free of all central nervous system myelin material and that is encoded by a first nucleotide sequence that is hybridizable to a second nucleic acid, said second nucleic acid having a nucleotide sequence depicted in Figure 2a (SEQ ID NO:1) or a nucleotide sequence depicted in Figure 12 (SEQ ID NO:28), which

protein is able to be bound by an antibody directed against a second protein having an amino acid sequence depicted in Figure 2a (SEQ ID NO:2).

19. A chimeric protein comprising a fragment of the protein of claim 2, 5, or 11 which is able to be bound by an antibody directed against a Nogo protein, fused by a covalent bond to at least a portion of a second protein, which said second protein is different from said fragment of protein of claim 2, 5, or 11.

20. A purified molecule comprising a fragment of the protein of claim 1 which is able to be bound by an antibody directed against a Nogo protein, and wherein said purified molecule is free of all central nervous system myelin material.

21. A purified molecule comprising a fragment of the protein of claim 5, or 11 which is able to be bound by an antibody directed against a Nogo protein.

22. An isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:33.

23. An isolated nucleic acid comprising:

(a) a nucleotide sequence that encodes a polypeptide having an amino acid sequence selected the group consisting of residues 1-1163 depicted in Figure 2a (SEQ ID NO:2), residues 1-172 fused to 975-1163 depicted in Figure 2a (SEQ ID NO:2), and residues 1-199 depicted in Figure 14 (SEQ ID NO:32); or

(b) the complement of the nucleotide sequence of (a).

24. An isolated first nucleic acid capable of hybridizing to a second nucleic acid, said second nucleic acid having a nucleotide sequence complementary to a nucleotide sequence that encodes a polypeptide having an amino acid sequence selected from the group consisting of residues 1-1163 depicted in Figure 2a (SEQ ID NO:2), residues 1-172 fused to 975-1163 depicted in Figure 2a (SEQ ID NO:2), and residues 1-199 depicted in Figure 14 (SEQ ID NO:32); and encodes a naturally occurring protein that is able to be bound by an antibody to a protein having an amino acid sequence of SEQ ID NO: 2.

25. The isolated nucleic acid of claim 24 which encodes a naturally occurring human Nogo protein.

26. An isolated first nucleic acid that is hybridizable to a second nucleic acid, said second nucleic acid having a nucleotide sequence depicted in Figure 2a (SEQ ID NO:1) or a nucleotide sequence depicted in Figure 12 (SEQ ID NO:28), and wherein said first nucleic acid encodes a first protein that is able to be bound by an antibody directed against a second protein having an amino acid sequence depicted in Figure 2a (SEQ ID NO:2).

27. An isolated nucleic acid that encodes a naturally occurring protein that is able to be bound by an antibody to a protein having an amino acid sequence of SEQ ID NO: 2, and that has a greater than 70% nucleotide sequence homology to a nucleotide sequence that encodes a polypeptide having an amino acid sequence selected from the group consisting of residues 1-1163 of SEQ ID NO:2, residues 1-172 fused to 975-1163 of SEQ ID NO:2, and residues 1-199 of SEQ ID NO:32, as determined by a BLAST computer algorithm.

28. The nucleic acid of claim 27 in which the naturally occurring protein is a human protein.

29. An isolated nucleic acid comprising a nucleotide sequence encoding a fragment of a Nogo protein that displays one or more functional activities of the Nogo protein, wherein said Nogo protein is not a human Nogo protein, a Drosophila Nogo protein or a Nogo protein of Caenorhabditis elegans.

30. An isolated nucleic acid comprising a nucleotide sequence encoding a protein comprising an amino acid sequence that has a greater than 50% homology to the amino acid sequence of SEQ ID NO: 29, as determined by a BLAST computer algorithm.

31. An isolated nucleic acid that encodes at least 220 continuous amino acid residues of the amino acid sequence of SEQ ID NO:2.

32. An isolated nucleic acid that comprises the nucleotide sequences of at least two non-overlapping human expressed sequence tags selected from the group consisting of: AA158636 (SEQ ID NO:35), AA333267 (SEQ ID NO:36), AA081783 (SEQ ID NO:37), AA167765 (SEQ ID NO:38), AA322918 (SEQ ID NO:39), AA092565 (SEQ ID NO:40), AA081525 (SEQ ID NO:41), and AA081840 (SEQ ID NO:42).

33. A vector comprising the nucleic acid of any one of claims 22, 23, 24, and 25 operably linked to a non-native promoter.

34. An expression vector comprising the nucleic acid of any one of claims 22, 23, 24, and 25.

35. A recombinant cell transformed with the nucleic acid of any one of claims 22, 23, 24, and 25.

36. The recombinant cell of claim 35 which is a prokaryotic recombinant cell.

37. The recombinant cell of claim 35 which is a eukaryotic recombinant cell.

38. A method of producing a recombinant protein comprising culturing a recombinant cell transformed with the nucleic acid of claim 22, 23, 24, or 25 such that a protein encoded by the nucleic acid is expressed by the cell, and recovering the expressed protein.

39. The method of claim 38 wherein the recombinant cell is a prokaryotic recombinant cell.

40. The method of claim 38 wherein the recombinant cell is an eukaryotic recombinant cell.

41. Use of a therapeutically effective amount of a Nogo protein or a fragment thereof, that is free of all central nervous system myelin material with which the Nogo protein is natively associated, said fragment being active in inhibiting cell proliferation, in the preparation of a medicament for treating a subject with a neoplastic disease of the central nervous system.

42. The use of claim 41 in which the neoplastic disease is glioma, glioblastoma, medulloblastoma, craniopharyngioma, ependyoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendrglioma, menagioma, neuroblastoma, or retinoblastoma.

43. The use of claim 41 in which the subject is a human.

44. Use of a therapeutically effective amount of a ribozyme or an antisense Nogo nucleic acid that inhibits the production of Nogo, in the preparation of a medicament for treating a subject with damage to the central nervous system.

45. Use of a therapeutically effective amount of a ribozyme or an antisense Nogo nucleic acid that inhibits the production of Nogo, in the preparation of a medicament for inducing regeneration or sprouting of neurons in a subject.

46. Use of a therapeutically effective amount of a ribozyme or an antisense Nogo nucleic acid that inhibits the production of Nogo, in the preparation of a medicament for promoting structural plasticity of the central nervous system of a subject.

47. A recombinant non-human animal that is the product of a process comprising introducing a nucleic acid encoding at least a domain of a Nogo protein into the genome of the animal, or a progeny of said animal.

48. A recombinant non-human animal in which a *Nogo* gene has been inactivated or deleted.

49. The animal of claim 48 in which the *Nogo* gene has been inactivated by a method comprising introducing a nucleic acid into the animal or an ancestor thereof, which nucleic acid comprises a non-Nogo gene sequence flanked by Nogo gene sequences that promote homologous recombination.

50. A purified fragment of a Nogo protein comprising an amino acid sequence selected from the group consisting of amino acid residues 1-171, 172-974, 259-542, 542-722, 172-259, 722-974, and 975-1162 of SEQ ID NO: 2, that is free of all central nervous system myelin material.

51. A purified fragment of a Nogo protein that lacks amino acid residues 172-259, amino acid residues 974-1162, or amino acid residues 172-259 and 974-1162, of

SEQ ID NO:2 but otherwise comprises the remainder of SEQ ID NO: 2, and that is free of all central nervous system myelin material.

52. A purified fragment of a Nogo protein comprising an amino acid sequence selected from the group consisting of amino acid residues 1-131, 132-939, 206-501, 501-680, 132-206, 680-939, and 940-1127 of SEQ ID NO:29, that is free of all central nervous system myelin material.

53. A purified fragment of a Nogo protein that lacks amino acid residues 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:29 but otherwise comprises the remainder of SEQ ID NO:29, and that is free of all central nervous system myelin material.

54. A purified protein comprising a fragment of a Nogo protein, which protein (a) lacks amino acid residues 172-259, amino acid residues 974-1162, or amino acid residues 172-259 and 974-1162, of SEQ ID NO:2; and (b) displays the neurite growth inhibitory activity of said Nogo protein, and is free of all central nervous system myelin material.

55. A purified protein comprising a fragment of a Nogo protein, which protein (a) lacks amino acid residues 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:29; and (b) displays the neurite growth inhibitory activity of said Nogo protein, and is free of all central nervous system myelin material.

56. An isolated nucleic acid that encodes a protein comprising an amino acid sequence selected from the group consisting of amino acid residues 1-171, 172-974, 259-542, 542-722, 172-259, 722-974, and 975-1162 of SEQ ID NO: 2.

57. An isolated nucleic acid that encodes a protein that lacks amino acid residues 172-259, amino acid residues 974-1162, or amino acid residues 172-259 and 974-1162, of SEQ ID NO:2 but otherwise comprises the remainder of SEQ ID NO: 2.

58. An isolated nucleic acid that encodes a protein comprising an amino acid sequence selected from the group consisting of amino acid residues 1-131, 132-939, 206-501, 501-680, 132-206, 680-939, and 940-1127 of SEQ ID NO:29.

59. An isolated nucleic acid that encodes a protein that lacks amino acid residues 132-206, amino acid residues 939-1127, or amino acid residues 132-206 and 939-1127, of SEQ ID NO:29 but otherwise comprises the remainder of SEQ ID NO:29.

60. A vector comprising the nucleic acid of any one of claims 56, 57, 58 and 59.

61. A recombinant cell transformed with the nucleic acid of any one of claims 56, 57, 58 and 59.

62. A fusion protein comprising the fragment of claim 52, 53, or 55 fused to an amino acid sequence of a non-Nogo protein.

63. A method of obtaining polyclonal antibodies to a protein, wherein said protein consists of an amino acid sequence with at least 60% identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32, and SEQ ID NO:33 as determined by BLAST computer algorithm, and wherein said method comprises:

(a) immunizing an animal with said protein that is free of all central nervous system myelin material with which said protein is natively associated; and,

(b) recovering polyclonal antibodies from the animal.

64. A method of obtaining polyclonal antibodies to a protein, wherein said protein consists of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32 and SEQ ID NO:33, and wherein said method comprises:

(a) immunizing an animal with said protein or a derivative or a fragment thereof, said derivative or fragment consisting of at least 10 amino acids of said protein, that is free of all central nervous system myelin material with which said protein is natively associated; and,

(b) recovering polyclonal antibodies from the animal.

65. The method of claim 64, wherein said derivative or fragment consists of at least 17 amino acids of said protein.

66. The method of claim 64 wherein said derivative or fragment consists of an amino acid sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45 and SEQ ID NO:46.

67. The method of claim 65, wherein said derivative or fragment consists of 18 amino acids of said protein.

68. The method of claim 63 or 64, wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:2.

69. The method of claim 63 or 64 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:29.

70. The method of claim 63 or 64 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:32.

71. The method of claim 63 or 64 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:33.

72. The method of claim 64, wherein said derivative is a fusion protein, said fusion protein comprising (i) a first amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32, and SEQ ID NO:33 or a fragment of at least 10 amino acids of said first amino acid sequence; and (ii) a second amino acid sequence, different from said first amino acid sequence, that is covalently bound to said first amino acid sequence.

73. The method of claim 72, wherein said fragment is a fragment of at least 17 amino acids of said first amino acid sequence.

74. The method of claim 73, wherein said fragment consists of 18 amino acids of said first amino acid sequence.

75. The method of claim 72, wherein said first amino acid sequence is the amino acid sequence of amino acids 762-1163 of SEQ ID NO:2.

76. The method of claim 72, wherein said fragment consists of an amino acid sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45 and SEQ ID NO:46.

77. The method of claim 64, wherein said fragment is a synthetic peptide.

78. The method of claim 64, wherein said fragment consists of the amino acid sequence of amino acids 623-640 of SEQ ID NO:2.

79. An isolated antiserum sample comprising polyclonal antibodies produced according to the method of any one of claims 63-78.

80. A method of immunizing a non-human animal, said method comprising administering to said animal an immunogenic amount of a protein consisting of an amino acid sequence with at least 60% identity to an amino acid sequence selected from the

group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32, and SEQ ID NO:33 as determined by BLAST computer algorithm, wherein said protein is free of all central nervous system myelin material with which said protein is natively associated.

81. A method of immunizing a non-human animal, said method comprising administering to said animal an immunogenic amount of a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32 and SEQ ID NO:33, and wherein said protein is free of all central nervous system myelin material with which said protein is natively associated.

82. A method of immunizing a non-human animal, said method comprising administering to said animal an immunogenic amount of a derivative or a fragment of a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32 and SEQ ID NO:33, and wherein said derivative or fragment (i) is free of all central nervous system myelin material with which said protein is natively associated; and (ii) consists of at least 10 amino acids of said protein.

83. The method of claim 80, 81 or 82, wherein said animal is a mouse.

84. The method of claim 82, wherein said fragment consists of at least 17 amino acids of said protein.

85. The method of claim 84, wherein said fragment consists of 18 amino acids of said protein.

86. The method of claim 82, wherein said fragment consists of an amino acid sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45 and SEQ ID NO:46.

87. The method of claim 80, 81 or 82 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:2.

88. The method of claim 80, 81 or 82 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:29.

89. The method of claim 80, 81 or 82 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:32.

90. The method of claim 80, 81 or 82 wherein said amino acid sequence is the amino acid sequence of SEQ ID NO:33.

91. The method of claim 80, 81 or 82 wherein said derivative is a fusion protein, said fusion protein comprising (i) a first amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:29, SEQ ID NO:32, and SEQ ID NO:33 or a fragment of at least 10 amino acids of said first amino acid sequence; and (ii) a second amino acid sequence, different from said first amino acid sequence, that is covalently bound to said first amino acid sequence.

92. The method of claim 91, wherein said fragment is a fragment of at least 17 amino acids of said first amino acid sequence.

93. The method of claim 92, wherein said fragment consists of 18 amino acids of said first amino acid sequence.

94. The method of claim 91, wherein said fragment consists of an amino acid sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, and SEQ ID NO:46.

95. The method of claim 91, wherein said first amino acid sequence is the amino acid sequence of amino acids 762-1163 of SEQ ID NO:2.

96. The method of claim 82, wherein said fragment is a synthetic peptide.

97. The method of claim 82, wherein said fragment consists of the amino acid sequence of amino acids 623-640 of SEQ ID NO:2.

98. A pharmaceutical composition comprising a therapeutically effective amount of the protein of claim 1-13, 16, 17, 18, or 19.

99. A pharmaceutical composition comprising a therapeutically effective amount of the fragment of claim 14 or 15.

100. A pharmaceutical composition comprising a therapeutically effective amount of the molecule of claim 20 or 21.