PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C07K 7/00, 13/00, A61K 37/00	A1	WO 94/04562
		(43) International Publication Date: 3 March 1994 (03.03.94)

(21) International Application Number: PCT/US93/07643

(22) International Filing Date: 13 August 1993 (13.08.93)

(30) Priority data:
07/928,920 13 August 1992 (13.08.92) US

(71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; Fruit Street, Boston, MA 02114 (US).

(72) Inventors: FISHMAN, Mark, C. ; 43 Kenwood Avenue, Newton Centre, MA 02159 (US). FEDEROFF, Howard ; 125 Beechwood Drive, Mamaronneck, NY 10543 (US). ZUBER, Mauricio ; 4317 Wellington Drive, Fort Collins, CO 80526 (US). STRITTMATTER, Stephen ; 43 Belmont Street, Charlestown, MA 02129 (US). VALENZUELA, Dario ; 44 Harrison Street, Somerville, MA 02134 (US). NG, Shi-Chung ; 6 Anthony Lane, Lawrenceville, NJ 08648 (US). GRABCZYK, Edward ; 38 Bigelow Street #B, Cambridge, MA 02139 (US).

(74) Agents: CIMBALA, Michele, A. et al.; Sterne, Kessler, Goldstein & Fox, 1100 New York Avenue, N.W., Suite 600, Washington, DC 20005-3934 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MAMMALIAN GAP-43 COMPOSITIONS AND METHODS OF USE

(57) Abstract

Mammalian GAP-43 cDNA has been cloned. The nucleotide sequences and corresponding amino acid sequences for human GAP-43 as well as rat GAP-43 are disclosed. The substantially pure sequences of the present invention may be expressed in prokaryotic and eukaryotic hosts, and are of use in monitoring and regulating neuronal growth in animals including humans, for both therapeutic and diagnostic purposes. Novel membrane-targeting peptides are disclosed which are capable of directing any desired protein or peptide to the cell membrane of neuronal or non-neuronal cells. In addition, it is further disclosed that GAP-43 and peptide derivatives act as novel internal regulatory proteins and peptides of G proteins, such as G_o. In particular, the amino terminal domain of GAP-43 modulates neuronal growth through the regulation of G_o in the growth cone. These internal regulatory peptides also regulate intracellular receptor-dependent systems, by modulating the activation of a protein, such as a G protein, by a receptor, in both neuronal and non-neuronal cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NE**	Niger
BE	Belgium	**GN**	Guinea	**NL**	Netherlands
BF	Burkina Faso	**GR**	Greece	**NO**	Norway
BG	Bulgaria	**HU**	Hungary	**NZ**	New Zealand
BJ	Benin	**IE**	Ireland	**PL**	Poland
BR	Brazil	**IT**	Italy	**PT**	Portugal
BY	Belarus	**JP**	Japan	**RO**	Romania
CA	Canada	**KP**	Democratic People's Republic of Korea	**RU**	Russian Federation
CF	Central African Republic			**SD**	Sudan
CG	Congo	**KR**	Republic of Korea	**SE**	Sweden
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovak Republic
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LU**	Luxembourg	**TD**	Chad
CS	Czechoslovakia	**LV**	Latvia	**TG**	Togo
CZ	Czech Republic	**MC**	Monaco	**UA**	Ukraine
DE	Germany	**MG**	Madagascar	**US**	United States of America
DK	Denmark	**ML**	Mali	**UZ**	Uzbekistan
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

TITLE OF THE INVENTION

MAMMALIAN GAP-43 COMPOSITIONS AND METHODS OF USE

Cross-Reference to Related Applications

This is a continuation-in-part application of 07/683,455, filed April 10, 1991, which is a continuation-in-part application of Serial Number 546,453, filed July 2, 1990, which is a continuation-in-part application of Serial Number 456,635, filed December 22, 1989, which is a continuation-in-part application of application Serial Number 401,408, filed September 1, 1989, abandoned, which is a continuation-in-part of application Serial Number 305,239, filed February 2, 1989, abandoned, which is a continuation-in-part of application Serial Number 288,604, filed December 22, 1988, abandoned, which is a continuation-in-part of application Serial Number 189,223, filed May 2, 1988, abandoned.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the fields of molecular genetics and neurology. More particularly, the invention relates to the cDNA sequence and corresponding amino acid sequence of mammalian GAP-43, a neuronal growth-related protein. The present invention is further related to methods of regulating expression of GAP-43, thereby regulating axonal growth, and to methods of producing GAP-43 in prokaryotic or eukaryotic hosts cells or

organisms. More particularly, the invention is related to a novel membrane-targeting peptide derived from GAP-43, which is capable of regulating membrane binding and growth cone enrichment of GAP-43, and is also capable of directing any desired protein or polypeptide to the membrane of neuronal or non-neuronal cells. The invention further relates to the discovery that GAP-43 and biologically active peptides derived therefrom function as Internal Regulatory Proteins (IRPs), which can act intracellularly to modulate cell function. The present invention also is related to the clinical *in vivo* and *in vitro* diagnostic and therapeutic applications of GAP-43 and its regulatory and membrane-targeting elements in, *inter alia*, neurological indications in animals including humans.

Description of the Background Art

GAP-43 is one of the proteins that specifically characterizes growing axons (Skene, *Cell 37*:697 (1984); Meiri, *PNAS USA 83*:3537 (1986)). Axonally transported proteins are a small subset of total cellular proteins, and only a few of these vary such that their levels may be envisioned as directly mediating axonal growth (Skene *et al.*, *J. Cell. Biol. 89*:86 (1981); Benowitz *et al.*, *J. Neuroscience 3*:2153 (1983); Skene, *Cell 37*:697 (1984); Meiri, *PNAS USA 83*:3537 (1986)). Although direct evidence that any of these molecules mediate structural changes is lacking, GAP-43 is particularly attractive as a candidate since it is primarily a growth cone constituent, where it is bound to the internal surface of growth cone membrane and serves as a substrate for protein kinase C. (Aloyo, *J. Neurochem. 41*:649 (1983); Akers *et al.*, *Brain Res. 334*:147 (1985)). Furthermore, its level of gene expression correlates well with axonal growth, both in cell culture and *in vivo* (Basi, *Cell 49*:785 (1987); Karns, *Science 236*:597 (1987); Neve, *Molec. Brain Res. 2*:177 (1987)).

Absence of repair in the mature human central nervous system (CNS) is a formidable clinical problem. After acute ischemic or traumatic injury histopathological evidence of regeneration is minimal and neurological recovery usually absent or incomplete. On the other hand, neurons of the peripheral nervous system regenerate more predictably, as do CNS neurons of other species, such as goldfish or toad (Skene *et al.*, *J. Cell. Biol. 89*:86 (1981); Benowitz *et al.*, *J. Neuroscience 3*:2153 (1983)).

One explanation for the refractoriness of the mature mammalian CNS neuron might be an irreversible repression of molecules important to growth. In particular, GAP-43 has been suggested as critical to regeneration (Skene, *Cell 37*:697 (1984)). Evidence for this includes its enrichment in growth cones (Skene, *Science 233*:783 (1986); Meiri, *PNAS USA 83*:3537 (1986)) and its minimal expression in the adult as opposed to the perinatal CNS (Skene and Williard, *J. Cell. Biol. 89*:96 (1981); Karns, *Science 236*:597 (1987)). Moreover, GAP-43 increases to high levels after injury in neurons capable of regeneration, such as toad or goldfish optic nerve or mammalian peripheral nerve, but not after similar injury to mammalian CNS neurons (Skene *et al.*, *J. Cell. Biol. 89*:96 (1981)).

The present inventors have examined the role of GAP-43 in human CNS function and disease. Human GAP-43 cDNA has been cloned, and its developmental and adult distribution examined by assay of post-mortem tissue. In addition to high perinatal expression, the present inventors have discovered that GAP-43 expression persists in discrete regions of the adult, and unexpectedly, that acute ischemic injury is associated with heightened expression of GAP-43 even in areas where it is normally low.

SUMMARY OF THE INVENTION

Recognizing the potential importance of GAP-43 in mammalian CNS function and disease, the present inventors have succeeded in sequencing the mammalian GAP-43 gene by complimentary DNA (cDNA) cloning. The complete nucleotide sequence of the gene encoding rat GAP-43 and the amino acid sequence have been determined. cDNA for rat GAP-43 has been used as a probe to identify and clone cDNA for human GAP-43 from human brainstem and cerebellum libraries. The amino acid sequence for human GAP-43 also has been determined.

Thus, in one embodiment of the invention is provided substantially pure mammalian GAP-43 protein, or a functional derivative thereof. Also provided are rat and human GAP-43 proteins in substantially pure form, as well as the functional derivatives of these proteins. Specific embodiments of the invention comprise substantially pure rat and human GAP-43 proteins and polypeptides having amino acid sequences corresponding to those shown in Figures 2 and 5A, respectively, and their functional derivatives.

Another embodiment of the invention provides for cDNA comprising a nucleotide sequence as shown in or substantially similar to that shown in Figures 2 or 5A, or functional derivatives thereof. The cDNA of the invention may be incorporated into a suitable expression vector, such as a plasmid, and the vector may be used to transfect a prokaryotic or eukaryotic host cell, which may then express the cDNA under appropriate *in vivo*, *in vitro* or *in situ* conditions, all of which, together with the GAP-43 protein or polypeptide produced thereby, form additional embodiments of the invention.

Thus, yet another embodiment of the invention provides for a method of producing mammalian GAP-43 protein or polypeptide or a functional derivative thereof, comprising transfecting a prokaryotic or eukaryotic host cell with a vector comprising cDNA encoding mammalian GAP-43 protein or

polypeptide, culturing said host cell in a suitable medium and under conditions permitting expression of said mammalian GAP-43 protein or polypeptide, and separating said mammalian GAP-43 protein or polypeptide, or their functional derivatives, from said medium.

Employing the substantially pure GAP-43 antigens of the invention, the inventors have succeeded in generating antibodies against GAP-43, and such antibodies and their functional and chemical derivatives comprise additional embodiments of the present invention. The GAP-43 antibodies of the invention may be polyclonal or, preferably, monoclonal antibodies, and are suitable for a variety of preparative, diagnostic and therapeutic uses, which are to be understood as forming yet additional invention embodiments.

Further, the GAP-43 antigens and antibodies of the invention are well suited for appropriate labeling as, for example, with detectable or therapeutic labels, and for use with other active agents in compositions which may or may not be pharmaceutically acceptable, all of which may be determined as the particular preparative, diagnostic or therapeutic application may require. Such labeled GAP-43 antigens, antibodies and their functional and chemical derivatives, as well as such compositions, comprise embodiments of the present invention.

The GAP-43 antigens and, particularly, antibodies of the invention, together with their functional and chemical derivatives, may be employed in various diagnostic methods known to those of skill. Such methods, including but not limited to immunocytochemical and immunometric methods, form additional embodiments of the invention.

Accordingly, in one exemplary embodiment of the invention is provided a method of determining or detecting mammalian GAP-43 antigen or antibody in a sample, comprising contacting a sample suspected of containing GAP-43 antigen or antibody with detectably labeled GAP-43 antibody or antigen, respectively, incubating said sample with said antibody or antigen so

as to allow the formation of a GAP-43 antigen-antibody complex, separating the complex thus formed from uncomplexed antigen or antibody, and detecting the labeled complexed antibody or antigen. It will be appreciated that this embodiment of the invention, and others, may be carried out *in vivo*, *in vitro* or *in situ*, as may be desired.

When used in the preparative, diagnostic or therapeutic methods of the invention, the compounds and compositions of the invention may conveniently be included in a kit, and such kits form yet another embodiment of the present invention. There is thus provided, as a non-limiting example, a kit useful for the preparation, purification, isolation, determination or detection of GAP-43 antigen or antibody, or for therapeutic treatment with GAP-43 antigen or antibody, comprising carrier means being compartmentalized to receive in close confinement therein one or more container means, wherein one or more of said container means comprises preparatively, detectably or therapeutically labeled GAP-43 antigen or antibody, or their functional or chemical derivatives.

The present inventors also have evaluated GAP-43 expression in normal, as well as in damaged or diseased CNS tissue. It has been discovered that *in vivo* GAP-43 expression varies during development in neural tissue, and that regional variations in GAP-43 expression exist. Further, it has been discovered that GAP-43 expression undergoes significant changes as a result of damage to neural tissue.

Moreover, the inventors have discovered mechanisms by which mammalian GAP-43 expression may be enhanced or, if desired, inhibited. Particularly, it has been discovered that GAP-43 expression is enhanced by nerve growth factor, and that this is inhibited by certain steroids. Inasmuch as the ability to modulate GAP-43 expression may be of great therapeutic utility in treating mammals, and particularly humans, suffering from damage to, or from disease or dysfunction of, the central or peripheral nervous

system, the significance of these discoveries will be readily apparent. Further, by introducing into non-neural cells cDNA encoding GAP-43 or its functional derivative, the inventors have made the surprising discovery that even non-neural cells can form growth cone-like processes. Again, the potential therapeutic value of this discovery is profound.

Accordingly, in another aspect, the present invention comprises methods for evaluating or determining GAP-43 activity and expression in diseased or damaged CNS tissue, as well as in normal CNS tissue. The present invention further comprises methods of treating mammals, including humans, suffering from damaged, diseased or dysfunctioning central or peripheral nervous tissue, and methods of modulating structural remodeling in normal CNS tissue in mammals including humans.

Thus, in one embodiment, the invention comprises a method of inducing expression of GAP-43 in cells, comprising exposing said cells *in vivo*, *in vitro* or *in situ* to an effective amount of nerve growth factor. When cells are thus exposed *in vitro*, it will be possible in another embodiment to introduce such cells into or in close apposition to the location of damaged, diseased or dysfunctioning central or peripheral neural cells with therapeutic effect.

In another embodiment, GAP-43 expression may be induced or enhanced by introducing into non-neural or neural cells cDNA encoding GAP-43. This may be accomplished *in vivo, in vitro* or *in situ* by a variety of means, including transfection, transduction and direct microinjection, all of which form intended non-limiting embodiments of the invention. Alternatively, the cDNA of the invention may be introduced by means of a retroviral or viral vector, or may be attached to any number of cell surface receptor ligands and conveyed with such ligands into the cell. All of these methods, as well as the compositions and vectors comprising GAP-43 cDNA

and its functional and chemical derivatives, form additional embodiments of the present invention.

Similarly, yet additional embodiments of the present invention comprise methods of modulating structural remodeling, methods of modulating synaptic plasticity, and methods of modulating the microenvironment of cells, including neuronal and non-neuronal cells, comprising exposing said cells to an effective amount of one or more substances selected from the group consisting of nerve growth factor, steroid and their functional derivatives.

It has also been discovered that GAP-43 surprisingly contains a ten amino acid amino-terminus exon, and that this peptide is responsible for directing GAP-43 to the cell membrane, and especially to the growth cone regions of neuronal cells. It has further been discovered that this ten amino acid membrane-targeting peptide, and its functional derivatives, are capable of directing a desired protein or peptide to the cell membrane, when attached at or near the amino-terminus of such protein or peptide. This surprising discovery applies to proteins and peptides which are normally cytosolic, and not normally membrane-associated.

Thus, an additional embodiment of the present invention comprises a membrane-targeting peptide, or a functional derivative thereof, capable of directing any desired protein or peptide to the cell membrane of neuronal or non-neuronal cells. The membrane-targeting peptide of the invention, or the desired protein or peptide to which it is attached, may be diagnostically or therapeutically labeled. Methods of diagnostic or therapeutic *in vivo, in vitro* or *in situ* treatment of neuronal or non-neuronal cells of animals, including humans, using the membrane-targeting peptide form additional embodiments of the present invention.

In yet an additional embodiment, the present invention provides for genomic GAP-43, which has been isolated, and its intron-exon boundaries and transcriptional start sites mapped. Further, it has surprisingly been discovered

that the GAP-43 promoter is quite unusual in its structure, and may be useful for the expression of other structural genes.

In another embodiment, the present invention is directed to the surprising discovery that GAP-43 acts intracellularly to modify the binding capacity of other cell proteins, including that of G_o. The present invention thus provides for an important new class of internal regulatory proteins ("IRP"), of which GAP-43 is representative, comparable in effect and utility to external cell receptors. Further, it has been discovered that synthetic peptides comprising the amino terminus amino acids of GAP-43 also exhibit biological activity as internal regulatory proteins, and compositions comprising such proteins and their use constitute additional embodiments of the invention.

In another embodiment the invention is directed to the discovery that these IRP may be used to modulate structural remodeling in a neural cell. In other embodiments, the modulation of structural remodeling may result in either inhibition or stimulation of neural growth depending upon the particular G protein system involved.

In addition, the invention further relates to the discovery that modified GP-43, peptides in which cys^3 and cys^4 have been palmitoylated or substituted with amino acids such as THR, ASP or GLU, also modulate the binding of G proteins. These peptides may also be used to modulate structural remodeling in a neural cell.

Finally, the invention relates to a method for augmenting the activation of a desired protein by, a receptor by administering an IRP. In a particular embodiment, the protein is a G protein. In another embodiment, the activation of the G protein occurs in a neural cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Hybrid-selected translation of GAP-43 cDNA. The EcoRI insert, GAP43-2, was used to select mRNA by the procedure of Ricciardi *et al., PNAS 76*:4927 (1979). In brief, 0.5 mg of the GAP43-2 insert, or equivalent amounts of nonspecific DNA, the bacterial plasmid pSP65, were spotted onto nitrocellulose and hybridized with 17.5 mg of newborn rat brain polyadenylated [poly(A)$^+$] RNA in a solution with 65% formamide, 400 mM NaCl, 10 mM 1,4-piperazine diethanesulfonic acid (Pipes) pH 6.4 at 42°C for 16 hours. After being washed in standard saline citrate (SSC) (X1), 0.5% SDS at 65°C, the filter was boiled, and the RNA was precipitated with ethanol and translated with rabbit reticulocyte lysate, and the proteins were labeled with [^{35}S]methionine (Pelham *et al., Eur. J. Biochem. 67*:247 (1976)). Translation products, or products immunoprecipitated with the antibody to GAP-43, were separated on a 12% SDS-polyacrylamide gel. (A) In vitro translation products with (i) no exogenous RNA, (ii) pSP65-selected RNA, (iii and iv) GAP43-2-selected RNA, and (v) poly(A)$^+$ newborn brain RNA (newborn). (B) Immunoprecipitations by antibody to GAP-43 of the translation products of (A), as described for (A) except for the fourth lane, which shows immunoprecipitation of the translation product after having preabsorbed the GAP-43 antibody with GAP-43 protein, prepared as in Snipes *et al., Soc. Neurosci. Abstr. 12*:500 (1986).

Figure 2. Nucleotide sequence and predicted amino acid sequence of GAP-43. The cDNA library was generated with RNA from dorsal root ganglia from embryonic day 17-18 rats. Total cellular RNA was isolated by the method of Chirgwin *et al., Biochemistry 18*:5294 (1979), and poly(A)$^+$ RNA was selected with oligo-dT cellulose. Double-stranded cDNA was generated by the ribonuclease H method described by Gubler *et al., Gene 25*:263 (1983), ligated to EcoRI linkers, and ligated into the EcoRI site of the

lambda phage cloning vectors, lgt10 and lgt11. The longest clone identified, GAP43-2, and two phage with smaller inserts, were identified from about 5 x 10^4 plaques in the lgt11 library after induction with isopropyl b-D-thiogalactopyranoside (IPTG), by using the rabbit antibody to GAP-43, followed by alkaline phosphatase-conjugated antibody to rabbit immunoglobulin G (Promega Biotec). The cDNA inserts were subcloned into the EcoRI sites of M13 mp18. Initial DNA sequence analysis of the two shorter clones revealed that they were included within the longest. The insert, GAP43-2, was sequenced by using the series of overlapping restriction fragments shown below the sequence by the dideoxynucleotide chain-termination method (Sanger *et al., PNAS 74*:5463 (1977)). The 3′ end of this fragment is the EcoRI site common to the three independent lgt11 isolates, which is thought to be an EcoRI site that occurs naturally in the GAP-43 gene. Since none of the clones contained an insert with a polyadenylation sequence, it is likely the EcoRI sites within the cDNA were unsuccessfully methylated during the library construction. The predicted protein sequence for GAP-43 is shown above the DNA sequence. The first methionine in italics was chosen as the start of the coding region for the reasons described hereinafter. It is unlikely that the only other methionine, shown here as amino acid 5, could alternatively serve as the initiation codon. The amino acid residues that were identified by direct protein sequencing from the arginine (R) at amino acid 7 to the isoleucine (I) at amino acid 20 are overlined. The first cycle of sequencing at which the amino acid could be determined with certainty was this arginine. The next amino acid could not be determined with certainty. The inability to sequence the unfragmented protein suggests that the amino terminus may be blocked. E, EcoRI; M, MspI; V, PvuII; H, HaeIII; P, PstI; S, Sau3A. The arrow between nucleotides 100 and 101 indicates the boundary between the first and second exons; the arrow between nucleotides 664 and 665 indicates the start of the third exon.

Figure 3. Regulation of GAP-43 expression in PC12 cells. PC12 cells were passaged in RPMI medium containing 10% horse serum and 5% fetal bovine serum. Forty hours after plating the cells, the medium was changed to include the different additives. After 4 days, the cells were photographed (A), then RNA was isolated from each cell culture. RNA (10 mg per sample) was denatured and run on a 1.2% agarose-formaldehyde gel, transferred to a GeneScreen nylon filter, bound to the filter by ultraviolet cross-linking, and probed with ^{32}P-labeled GAP43-2 (B). The final wash was SSC (x0.2), 0.1% SDS at 65°C. The additives included were (i) none, (ii) 50 ng of NGF per milliliter, (iii) 10^{-3}M dibutyryl cAMP, and (iv) 50 ng of NGF per milliliter and 10^{-3}M dibutyryl cAMP. The last lane is 10 mg of RNA from newborn brain run as a positive control for the blotting and hybridization procedure.

Figure 4. Developmental regulation and tissue specificity of GAP-43 gene expression. Total cellular RNA was isolated from the designated rat tissues by a modification of the procedure of Chirgwin *et al., Biochemistry 18*:5294 (1979). Each RNA (10 mg) was denatured, underwent electrophoresis in a 1.2% agarose-formaldehyde gel, and was transferred to nitrocellulose. The filter was hybridized overnight at 42°C with the EcoRI insert from lgt11 GAP43-2 labeled with deoxycytidine 5'-[a-^{32}P]triphosphate by nick translation. The final wash was done in SSC (x0.2), 0.1% SDS at 65°C. RNA samples: (i) embryonic day 13 (E13) heart (H), (ii) E13 liver (L), (iii) E13 brain (B), (iv) E13 dorsal root ganglion (DRG), (v) to (viii) embryonic day 17 heart, liver, brain, and dorsal root ganglion; (ix) to (xii) newborn heart, liver, brain, and dorsal root ganglion; (xiii) to (xvi) adult heart, liver, brain and dorsal root ganglion. The positions of the 18S and 28S ribosomal RNA are shown at the right. Below is hybridization of the same filter with a cDNA probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Piechacyk *et al., Cell 42*:589 (1985)).

Figure 5. (A) Nucleotide sequence and deduced amino acid sequence of human GAP-43 cDNA. E: EcoRI; H: HaeIII; M: MspI. The coding region is denoted by thick bar. The scale is 100 bp. Arrows show the overlapping restriction fragments that were sequenced. (B) Alignment of human, rat GAP-43 and mouse P-57 amino acid sequences. Vertical bars indicate identity, and colons show conservative substitutions. The amino acids are represented by IUPAC-IUB CBN one-letter symbols. The rat sequence is that of Figure 2, and the mouse sequence is from Cimler *et al., J. Biol. Chem.* *262*:12158 (1987). (C) Stem-loop structures in the 3'-untranslated region predicted by fold program of Zuker *et al.*, *Nucl. Acids Res.* *9*:133 (1981).

Figure 6. Northern blot showing the regional restriction of GAP-43 expression with maturation. Ten mg total RNA from 8-day-old, 16-year-old, and 64-year-old brain regions were loaded in each lane and the blot was probed with human GAP-43 probe Cla as described in Example II. The positions of 18S and 28S rRNA bands are indicated.

Figure 7. Northern blot showing that GAP-43 expression increases in the wake of an ischemic event. (A) Ten mg of RNA from different brain regions of a patient with a stroke in Area 17 (visual cortex). Expression in A17 has increased to levels comparable to the highest in the brain (A11). (B) Ten mg of RNA from Area 3,1,2,5 from three patients, all run and blotted on the same blot with an unrelated band excised between lanes 2 and 3. Lanes 1 and 2 were histologically normal, whereas 3 included a small stroke, and shows an increase in GAP-43 expression.

Figure 8. *In situ* hybridization reveals increased GAP-43 expression in regions adjacent to infarcts. (A1) Higher magnification of infarcted region in B showing diffuse infiltration of tissue by lipid-laden macrophages and reactive astrocytes. There are no remaining neurons in this region (x160). (A2) Normal adjacent cortex with abundant histopathologically intact neurons (x300). (B) Lower magnification view of the visual cortex with an organizing

ischemic infarct (10 to 14 days old) involving one gyrus (arrowheads) and intact cortex in the adjacent gyrus (arrows) (x12). (C) In normal visual cortex GAP-43 expression is restricted to a few scattered neurons by darkfield examination (arrowheads). (D) In the infarcted cortex there is no specific binding of the antisense GAP-43 probe. The large bright foci are from areas of coagulative necrosis which also label nonspecifically with the sense probe (G). In contrast, in the adjacent intact cortex numerous neurons express GAP-43 (E-brightfield and F-darkfield labeled with antisense probe). (H) A control brightfield and (I) darkfield labeled with the sense probe showing absence of specific GAP-43 binding (C-I x160).

Figure 9. Enhanced GAP-43 expression in neurons of cerebellar cortex several days following a bout of severe hypotension and hypoxia. All sections were processed simultaneously. (A) Normal adult cerebellar cortex showing absence of detectable GAP-43 labeling. (B) Post-ischemic cerebellar cortex showing markedly increased expression of GAP-43 in the Purkinje cell and outer granule cell layers. (C) A section adjacent to B hybridized with the sense strand probe as control (all x315).

Figure 10. Effect of GAP-43 on process formation in CHO cell lines. Empty bars represent cells with processes in 4 CDM8-transfected lines and solid bars represent 4 cell lines expressing GAP-43. Cell lines were obtained as described in Example V. The percentage of cells with processes was assayed by plating CHO cells onto poly-D-lysine-coated coverslips. Cells with processes longer than 20 microns were scored as positive. To ensure comparability, all assays were performed within the time window that extended from 30 to 45 minutes after plating. An important component of this assay was the time window selected, since, after longer plating times, or as cells reached confluence, processes were much less evident. As many cells as possible were counted during this time window, and all cells examined were included. The number of cells counted for the different lines was: 1A, 406;

1B, 408; 2A, 287; 2B, 303; 5E, 234; 4, 333; 12, 156; 14, 161. The proportion of cells with processes in GAP-43 expressing cell lines was significantly greater than in controls ($p < 0.001$).

Figure 11. Schematic representation of experiments demonstrating that the amino-terminus exon is responsible for directing the GAP-43 protein to the cell membrane, and that it directs membrane targeting of chloramphenicol acetyl transferase. The left column ("CONSTRUCTION") indicates the gene construction used for transfection of COS, NIH 3T3, CHO or PC12 cells. The right column ("MEMBRANE") indicates whether the expressed protein or fragment was membrane-associated (+) or not (-), as assayed by sub-cellular fractionation followed by Western blotting, direct immunofluorescence, or both. The intact GAP-43 gene (GAP) was significantly membrane-associated, as were GAP constructions lacking substantial portions of exon 2 (GAP(-intern.)) or the carboxy-terminus region of the GAP-43 gene (GAPtag). However, when the nucleotides encoding the first four amino acids of GAP-43 were deleted (GAP(- 1-4)), the expressed protein fragment was not membrane-associated.

Point mutations were introduced into the sequences encoding the cysteines at positions three (C3) or four (C4) of the first exon, to result in expression of alanine in the resulting protein. Mutation of either C3 (GAP $*C_3$) or C4 (GAP $*C_4$) resulted in reduced membrane levels (+/-) as compared to intact GAP-43. Reduction was especially marked when C4 was altered. Mutation of both C3 and C4 (GAP $*C_{3,4}$) eliminated membrane association altogether.

Transient expression of the gene encoding chloramphenicol acetyl transferase (CAT) produced no membrane-associated protein, as expected for this normally cytosolic enzyme. When the nucleotide sequence encoding the ten amino acids of the first GAP-43 exon was ligated to the amino-terminus

end of the CAT gene (GAP(1-10)CAT), the expressed protein was membrane-associated.

Figure 12. Western blot showing that normal GAP-43 has both a membrane and a cytosolic component (M = membrane; C = cytosolic) when transfected into CHO cells. Mutation of the nucleotide sequence encoding the third or fourth cysteines (C-3 or C-4) of the first GAP-43 exon interfered with the membrane-binding component, while mutation of both cysteines (C-3,4) abolished membrane binding completely. Control cells (CON) had no GAP-43; Brain membranes (BR) had GAP-43 of the same molecular weight as that from the transfected gene.

Figure 13. Map of the rat GAP-43 gene.

A. A linear depiction of the GAP-43 gene in the 5′ to 3′ orientation. Representations of the phage inserts that were used for mapping are shown. The three exons are depicted as vertical bars. The sites shown are for restriction endonucleases BamHI (B), KpnI (K), and SacI (S).

B. Intron-exon boundaries and 3′ polyadenylation site. The exons and adjoining regions were sequenced and intron-exon boundaries were determined by comparison to the cDNA sequence as described herein, taking the best fit to consensus splice sites (Mount, *Nucl. Acids Res. 10*:459-472 (1982)). The major polyadenylation site was determined by RNAse protection. The putative polyadenylation signal and a tandem pair of a consensus motif often found immediately 3′ of utilized polyadenylation signals (McLauchlan *et al., Nucl. Acids Res. 13*:1347-1368 (1985)) are underlined.

Figure 14. Sequence of the GAP-43 promoter region. Nucleotide position +1 denotes the A of the initiating ATG codon of the GAP-43 protein. This sequence includes the variably sized first exon which ends at +30. Major transcriptional start sites are denoted by arrows. Purine residues have been underscored by asterisks. The consensus Pit-1 binding site is overlined.

Figure 15. Mobility shift in restriction fragments induced by H-DNA. A schematic map of the GAP-43 promoter region from -518 to +85 showing locations of restriction sites and the major homopurine-homopyrimidine regions (thickened and labeled I, II, and III). Below are representations of the GAP-43 promoter fragments liberated by digestion of plasmid bs1.5RIX4 with the following enzymes: 1) SspI, 603 bp; 2) XbaI/SspI, 560bp; 3) SmaI/SspI, 490bp; 4) SspI/NheI, 409bp; 5) SspI/NsiI, 284bp (contains region I), 319bp (contains regions II and III; 6) SspI/AccI, 314bp (region I), 289bp (regions II and III); 7) XbaI/NheI, 360bp; and 8) SmaI/NheI, 295bp.

Figure 16. Partial protein sequence for p34 and p38.

Figure 17. DRG Growth Cones. DRG growth cones on laminin-coated glass after 30 min. incubation (a) with F12 medium only, (b) with 0.3 mg/ml of chick E10 brain-derived crude collapsing activity, and (c) with the same concentration of collapsing activity as in after PTX-pretreatment for 2 hours.

Figure 18. DRG Growth Cone Collapse Induced By Brain Membrane Extracts And Mastoparan Is Blocked By PTX. (a) Chick E7 DRG explants were treated with no PTX (closed square), 100 ng/ml PTX (closed circle), or 200 ng/ml PTX (closed triangle), and then CHAPS-solubilized embryonic brain extracts were added. The percentage of collapsed growth cones is shown as a function of protein concentration in the extract added. (b) The collapse of control (closed square) or PTX-pretreated (100 ng/ml; closed circle) DRG growth cones was measured in the presence of the indicated concentrations of MP. Data are shown as the average and S.E.M. of three to six independent assays.

Figure 19. DRG Growth Cone Collapse Induced by Myelin Is Blocked By Pertussis Toxin. The percentage of collapsed growth cones of E7 DRG was scored in the absence (left pair) and the presence (right pair) of 100 ng/ml PTX. The collapse assay was performed as in Figure 21(a), except that the

incubation time with crude collapsing activity is 1 hour instead of 30 min. (hatched bar). In the control, F12 medium only was added to DRG explants (open bar). Data are shown as the average and S.E.M. of three to six independent assays. Pertussis toxin itself does not affect the percentage of collapsed growth cones. Thus, in the absence of collapsing activity, the percentages of fan-shaped growth cones are 73.8 + 6.5 % (n=6) without pertussis toxin and 70.0 + 5.1 % (n=5) in the presence of PTX (no significant difference, $p<0.05$).

Figure 20. Pertussis Toxin Partially Blocks Retinal Growth Cone Collapse Induced By Brain Membrane Extracts. The effects of CHAPS-solubilized membranes from chick embryonic brain are shown on retinal growth cones in the control (left pair) and after PTX-pretreatment (right pair). The temporal half of chick E7 retinae were isolated and cut into small fragments. Each fragment was explanted onto laminin-coated plastic chamber slides. The collapse assay using brain membrane extracts was performed as in Figure 18a (hatched bar). In the control, only F12 medium was added to the retinal explants (open bar). For PTX-pretreatment, retinal explants were incubated with F12 medium containing PTX (100 ng/ml) for 2 hour prior to the addition of the membrane extracts. Data are shown as the average and S.E.M. of three to six independent assays.

Figure 21. Modulation of (35S)GTPγS binding to G_o by GAP-43 and GAP-43 peptides. Specific (35S)GTPγS binding to G_o is stimulated to 310+40% (n=7) of control in the presence of 1 uM GAP-43 (A, left). The same concentration of GAP-43 without G_o binds no detectable (35S)GTPγS. Variation of the GAP-43 concentration demonstrates an EC50 of 150 nM for this effect (A, right). With different preparations of GAP-43, the range of EC50 was 150-800 nM, perhaps reflecting partial inactivation of GAP-43 during purification. The addition of 1 mM peptide containing the first 24 amino acids from GAP-43,

MLCCMRRTKQVEKNDEDQKIEQDG

stimulates binding to the same level as with 1 uM GAP-43 (250%), and the addition of both GAP-43 and 1-24 peptide does not cause further stimulation (B, left). This peptide does not bind (35S)GTPγS in the absence of Go. Three other GAP-43 peptides,

GAP(35-53)=ATKIQASFRGHITRKKLKD,

GAP(53-69)=DEKKGDAPAAEAEAKEK, and

GAP(210-226)=ARQDEGKEDPEADQEHA,

have no effect on (35S)GTPγS binding to G_o at 1 mM. The stimulation of binding by the 1-24 peptide is saturable with an EC50 of 20 uM (B, right). A peptide consisting of the first 10 amino acids of GAP-43, MLCCMRRTKQ, inhibits (35S)GTPγS binding to G_o by 80 % at 1 mM (C, left). A peptide with threonine in place of cysteine at positions 3 and 4 has no effect on (35S)GTPγS binding to Go. The IC50 for the 1-10 peptide is 20 uM (C, right). Panel D shows (35S)GTPγS binding to G_o as a function of GAP-43 concentration in the presence or absence of 50 uM 1-10 peptide. Note that GAP-43 reverses inhibition by the peptide, and that the EC50 for GAP-43 is shifted from 600 nM to 12 uM by this concentration of peptide. This is consistent with direct competition between the 1-10 peptide and GAP-43.

Methods. (35S)GTPγS (New England Nuclear, 2000 Ci/mmol) binding was determined as described previously (Huff, *et al., J. Biol. Chem. 260*: 10864-10871 (1985); Northrup, *et al., J. Biol. Chem. 257*: 11416-11423 (1982)) by incubation of G_o (1 nM, 10 ng) and (35S)GTPγS (2 nM) in 100 ul of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 6 mM MgCl2, 0.1% Lubrol PX, 200 ug/ml bovine serum albumin, 1 mM dithiothreitol for 60 min at 30°C, filtration through nitrocellulose, and scintillation counting of bound radioactivity. The concentration of (35S)GTPγS in these assays is approximately one tenth of the apparent KD (Huff, *et al., J. Biol. Chem. 260*:

10864-10871 (1985); Northrup, *et al., J. Biol. Chem. 257*: 11416-11423 (1982)), so that changes in both apparent KD and Bmax alter the level of binding. Total radioactivity bound was less than 10% of that added in all cases. Non-specific binding was determined in the presence of 10 uM unlabeled GTPγS and subtracted from total binding to determine specific binding. All assays were performed in duplicate. An average assay tube contained 800,000 cpm and yielded 30,000 cpm total binding and 1500 cpm non-specific binding. The addition of GAP-43 increased specific binding without effect on non-specific binding. GAP-43 activated two different samples of G_o and one of alphao, all prepared in Lubrol PX. However, the extent of the activation varied among the preparations. Activation by GAP-43 appears to be sensitive to the detergent used in the purification of G_o and the composition of the binding buffer. GAP-43 was purified by a modification of the method of Zwiers *et al. (J. Neurochem. 44*:1083-1090 (1985)), and was greater than 97% pure by Coomassie blue staining of SDS-PAGE gels. The protein was dialyzed extensively against the assay buffer and added to the GTPγS-G_o incubations in the concentrations indicated above. Peptides were synthesized at the Howard Hughes Medical Institute laboratory (Massachusetts General Hospital, Boston, MA), purified by reverse phase HPLC, and structure was confirmed by amino acid sequencing. The peptides were dissolved in assay buffer and added to the GTPγS-G_o incubation at the indicated concentrations.

Figure 22. Homology between GAP-43 amino terminus and the cytoplasmic tail of G-linked receptors. The first 10 amino acids of GAP-43 were compared to the sequence of the G-linked receptor family. Homology between the first 7 amino acids of GAP-43 and the amino terminal segment of the cytoplasmic tail of a number of G-linked receptors is shown. Residues which conform to the consensus sequence hydrophobic LEU CYS CYS X basic-basic are shaded.

The cysteines illustrated in the receptors are located approximately 13 amino acids distal to the last transmembrane region, correspond to cys341 of the B2-adrenergic receptor, and are aligned as described in Higashijima, *et al.* (*J. Biol. Chem. 263*: 6491-6494 (1988)). The sequences shown are from human (Figure 5) and fish (LaBate, *et al.*, *Neuron 3*:299-310 (1989)) GAP-43, human beta1-adrenergic receptor (B1-adrenergic, Frielle, *et al., Proc. Natl. Acad. Sci., U.S.A. 84*:7920-7924 (1987)), human beta2 adrenergic receptor (B2-adrenergic, Koblika, *et al., Proc. Natl Acad. Sci. U.S.A. 84*:46-50 (1987)), human rhodopsin (rhodopsin, Nathans, *et al., Proc. Natl. Acad. Sci. U.S.A. 81*:4851-4855 (1984)), human blue-opsin (blue-opsin, Nathans, *et al., Science 232*:193-202 (1986)), human alpha1 adrenergic receptor (A1-adrenergic, Cotecchia, *et al., Proc. Natl. Acad. Sci. U.S.A. 85*:7159-7163 (1988)), human platelet alpha2 adrenergic receptor (pA2-adrenergic, Koblika, *et al., Science 238*:650-656 (1987)), human muscarinic receptor subtypes 1 through 5 (Mus-ACh-1 to -5, (Higashijima, *et al., J. Biol. Chem. 263*:6491-6494 (1988); Perulta, *et al., EMBO J. 6*:3923-3929 (1987)), human serotonin receptor subtype 1A (5HT-1A, Koblika, *et al., Nature 329*:75-79 (1987)), and rat serotonin receptor subtypes 1C and 2 (5HT-1C, (Julius, *et al., Science 241*:473-479 (1988)), and 5HT-2, (Pritchett, *et al., EMBO J. 7*:4135-4140 (1988)).

Figure 23. Dose-dependent reduction by calmodulin of GAP-43-stimulated GTPγS binding to G_o. Vertical axis for both panels is GTPγS binding expressed as a percentage of control. Left Panel, C, calmodulin; G, GAP-43. Addition of GAP-43 to G_o (Lane 2) increased GTPγS binding to over 200% as compared to G_o alone (Lane 1). Addition of calmodulin reduced this binding to control levels (Lane 5). No GTPγS binding was seen in the absence of G_o, using GAP-43 alone (Lane 3) or GAP-43 plus calmodulin (Lane 4). Right panel, calmodulin concentration, μM,

demonstrating the dose-dependency of the effect of calmodulin on GTPγS binding to G_o.

Figure 24. Filopodial formation by non-neuronal cells transfected with GAP-43. (A) Stably transfected CHO cells were examined for filopodial formation after 15 minute incubation on poly-L-lysine coated glass. Two control cells from lines expressing CDM8 (a) or CAT (b) exhibit no filopodia, but several GAP-43-expressing cells (c-e) do form filopodia under the same conditions. The percentage of CHO cells with filopodia in each population is quantitated in Figure 30; the cells shown here are representative of positive and negative cells. The scale bar is 8 μ. (B) COS cells expressing (CAT (■) or GAP-43 (▲) were analyzed for filopodial formation as in Experimental Procedures. Note that at the shortest times examined, more GAP-43 cells have filopodia, and that few cells have filopodia after 30 minutes. The zero time point represents cells that were fixed before plating. This is one of four experiments with similar results.

Figure 25. Decreased spreading of non-neuronal cells transfected with GAP-43. (A) COS cells expressing CAT (■) or GAP-43 (●) were plated for the indicated times on poly-L-lysine coated glass, and then analyzed for the percentage of cells spread. Note that more spread cells are detected in the control CAT transfection. This is one of four experiments with similar results. (B) Four control A431 cell lines (a,b,c,d) exhibit a more flattened, spread phenotype as compared to four GAP-43-expressing A431 cell lines (e,f,g,h). The cells were fixed 2 hours after plating. The scale bar is 50 μ. (C) Control or GAP-43-expressing A431 cell lines were assayed for cell spreading or laminin-coated glass. The data are averaged from 6 control and 7 GAP-43 cell lines, and the standard error is shown. (D) Separate A431 cell lines transfected with a GAP-43 expression vector (a,b,c,d,e), or with control plasmid (f,g) were analyzed for GAP-43 expression by immunoblotting. Note that control cells do not express GAP-43, but that the transfected cells do so.

The level of GAP-43 expression in these cell lines per mg total protein is 10-50% of that in neonatal brain. (E) The adhesion of (^{35}S)methionine labelled GAP-43 and control A431 lines was determined as in Experimental Procedures. The percent of radioactivity adherent to plates at 45 minutes is plotted with standard errors for 6 control and 6 GAP-43 cell lines.

Figure 26. The first 10 amino acids of GAP-43 are necessary and sufficient to induce cell shape changes. COS cells expressing the indicated proteins were analyzed for filopodia (A) and spreading (B). Note that GAP-43, GAP-43 with a deletion from 40-189, GAP-43(1-40)/CAT fusion and GAP-43(1-10)/CAT are active in both assays, but that GAP-43 with a deletion form 2-5 and GAP-43(1-6)/CAT are indistinguishable from the CAT control. The values shown are means ± SEM for 3-7 separate transfections. (C) The COS cell filopodia results for CAT, GAP-43, GAP-43 with a deletion from 40-189, and the GAP-43(1-40)/CAT were confirmed in stably transfected CHO clonal cell lines. The control data includes 3 cell lines transfected with the *neo* gene alone and three lines expressing CAT as well.

Figure 27. Basic residues at position 6 and 9 are required for GAP-43 modulation of cell shape. COS cells expressing the indicated CAT, GAP-43 or GAP-43(1-10)/CAT proteins were analyzed for filopodia (A) and for spreading (B). Note that GAP-43, GAP-43 with arg^{7} changed to gly, GAP-43(1-10)/CAT, and the fusion protein with arg^{7} changed to glycine are active in both assays. In contrast, mutants with arg^{6} or lys^{9} changed to glycine exhibit levels of filopodia and spreading that are the same as CAT controls. The values shown are means ± SEM for 3-7 separate transfections.

Figure 28. Cysteine residues at position 3 and 4 are required for the morphoregulatory activity of GAP-43. COS cells expressing the indicated proteins were analyzed for filopodia (A) and spreading (B). Note that GAP-43, is active in both assays, but that GAP-43 with one or both cysteines at

position 3 and 4 changed to threonine is the same as the CAT control. The values shown are means ± SEM for 3-7 separate transfections.

Figure 29. A GAP-43/CAT fusion protein without cell shape-modulating activity is localized in membrane fractions. COS cells expressing CAT (a), GAP-43(1-40)/CAT (b), (1-10)/CAT (c) or GAP-43(1-6)/CAT (d) were separated into membrane (top) and soluble (bottom) fractions and analyzed for CAT immunoreactivity on blots of SDS-PAGE gels. Membrane and soluble fractions from 10^6 cells were loaded in each lane. Note that all three fusion proteins are detected in the membrane fraction but CAT is in the cytosolic fraction.

Figure 30. Basic residues at position 6, 7 and 9 of GAP-43 are not required for membrane binding. COS cells expressing GAP-43 (a), GAP-43 with arg^6 changed to gly (b), GAP-43 with arg^7 changed to gly (c), or GAP-43 with lys^9 changed to gly (d) were separated into membrane (m) and cytosolic (c) fractions and analyzed by immunoblot for GAP-43 immunoreactivity. Note that all four proteins are present primarily in the membrane fraction. Material from 10^6 cells was loaded in each lane.

Figure 31. The amino terminus of GAP-43: functional domains and comparison to the G protein activator region of receptor proteins. The first ten amino acids of GAP-43 contain a membrane targeting signal (residues 1-6, Figure 33 and a G protein activator region (residues 1-10). The G protein activator region is compared to that of the insulin-like growth factor II receptor (Okamoto *et al.*, *Cell* 62:709-717 (1990)), and the β2-adrenergic receptor (Okamoto *et al.*, *Cell* 67:723-730 (1991a)). The arrangement of basic residues identified for G protein activation identified by Nishimoto and colleagues (Okamoto *et al.*, *Cell* 62:709-717 (1990); Okamoto *et al.*, *Cell* 67:723-730 (1991a)) is shown at the bottom. The second basic in the BBxB, or BBxxB motif is boxed but not shaded because an IGF-II peptide with this residue altered is still active (Okamoto *et al.*, *Biochem. Biophys. Res. Comm.*

179:10-16 (1991b)). Similarly, GAP-43 with arg^7 changed to gly is still active in altering cell shape (Figure 31). The initiator methionine of GAP-43 is boxed to indicate the charge of the α amino group at that position.

Figure 32. Filopodial Formation in COS Cells Transiently Transfected With Expression Vectors Encoding CAT, GAP-43, or GAP-CAT Fusion Proteins.

Figure 33. CAT Immunoblot Analysis of COS Cell Transfections After Transfection With CAT and GAP-CAT Fusion Proteins. Lanes (a)-(d) are membrane fractions. Lanes (e)-(h) are from soluble fractions. Lanes (a) and (e) are from the same CAT transfection; (b) and (f) from GAP (1-40)-CAT; (e) and (g) from GAP (1-10); and (d) and (h) from GAP (1-6)-CAT.

Figure 34. GAP-43 Peptides (100 μM) Stimulate GTPγS Binding to G_o.

Figure 35. GTPγS Binding to G_o in the Presence of Various GAP-43 Peptides.

Figure 36. Neurite Outgrowth From Control and Briefly Permeabilized N1E-115 Cells in the Presence of the Indicated GAP-43 Peptides.

Figure 37. GAP-43 and Receptor Stimulate G_o Synergistically

(A) The purity of the rat brain GAP-43 preparation used in all experiments is demonstrated by Coomassie Blue staining of an SDS-PAGE gel. The mobility of M_r standards of 97, 66, 45, 31, 21.5, and 14 kDa is indicated at right.

(B) The GTPase activity of G_o reconstituted into phospholipid vesicles together with muscarinic m2 receptor was measured as a function of GAP-43 concentration added. GAP-43 increases GTPase in a saturable fashion to a greater extent in the presence (o) than in the absence (o) of the receptor agonist, carbachol (CCh). One of two experiments with similar results is shown.

Figure 38. GAP-43 Augments G Protein-Coupled Receptor Action in *X. Laevis* Oocytes.

(A) Immunoblot analysis for GAP-43 demonstrates no detectable protein in control oocytes (b, 240 μg protein), as compared to strong staining of a neonatal rat brain sample loaded with much less total protein (c, 20 μg protein). After injection of oocytes with a low concentration of GAP-43 (a, 240 μg protein), the level of GAP-43 is one-twelfth or less than that of the brain sample.

(B) The current response of an oocyte to acetylcholine is shown before and after injection of the dose of GAP-43 in A. Note that the inward current response in this cell is enhanced after GAP-43 injection. Five of five cells exhibited a similar two-fold augmentation of acetylcholine responsiveness. The peak current response after GAP-43 saturated the voltage clamp apparatus at 600 nA in this one example.

(C) The 5HT response of one oocyte is illustrated before and after injection of GAP-43 at the concentration shown in A. After GAP-43 injection, the response is two- to five-fold greater. The response increased by more than two-fold in 13 of 15 GAP-43 injected cells, and 1 of 18 buffer-injected control cells.

(D) Immunoblots for GAP-43 demonstrate that oocytes injected with a higher concentration of GAP-43 contain approximately the same concentration of GAP-43 as neonatal rat brain by GAP-43 immunoblots (a, 10 μg total oocyte protein; b, 20 μg oocyte protein; c, 10 μg brain protein; d, 20 μg brain protein).

(E) The response of three separate $5HT_{1c}$ mRNA-injected oocytes from the same frog was recorded 20-100 minutes after injection with buffer (an average control response), or the concentration of GAP-43 in D (2 μM final GAP-43 concentration; two typical cells). The 5HT response was markedly enhanced in all 11 GAP-43-injected cells examined in this paradigm.

Figure 39. GAP-43 Stimulates Ca^{++}-Activated Chloride Channel Opening.

(A) Injection of the lower dose of GAP-43 (0.2 μM final concentration) produced no inward current in 5 of 5 cells which were never exposed to 5-HT (top). Injection of the same concentration of GAP-43, 5-10 minutes after a one minute exposure to 5HT resulted in current fluctuations (bottom, 4 of 4 cells).

(B) Higher concentrations of GAP-43 (2 μM final concentration, as in Figure 38D) resulted in an oscillating inward current response (top, the smallest positive response, and bottom, a typical response from 23 positive cells of 25 injected cells). This type of current was never seen in over of 12 hours of recording from more than 20 different buffer-injected and non-injected cells.

(C) EGTA co-injected with GAP-43 (2 μM final concentration) prevents the current response seen in B. (50 mM EGTA in pipette, estimated 0.5 mM in cell after injection, 0 of 12 cells with positive response).

Figure 40. GAP-43 Does Not Block IP_3-Induced Desensitization.

A continuous current trace from one oocyte injected with the high concentration of GAP-43 thirty minutes previously is shown. 5HT produces a large response which returns to baseline after 9 minutes, and this is followed by a current response to the intracellular injection of 4 pmol IP_3. Subsequent bath application of 5HT, or intracellular injection of IP_3 in this GAP-43-injected oocyte produces no change in current. Similar results were observed in 3 of 3 cells examined in this protocol.

Figure 41. GAP-43 inhibits forskolin-stimulated cAMP levels in A431 cells. A431 cells were stably transfected with a *neo* expression cassette alone (control), or with a GAP-43 anda *neo* expression vector (GAP-43-expressing cells). Cyclic AMP levels were measured in the presence of IBMX

and the indicated agents. The data are from 6 control lines and 4 GAP-43 lines.

Figure 42. GAP-43 inhibits isopreternol-stimulated cAMP levels in A431 cells. Control and GAP-43-expressing cell lines as in Figure 41 were assayed for cAMP levels in the presence of the indicated agents.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following description, reference will be made to various methodologies known to those of skill in the art of molecular genetics and neurology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full.

Standard reference works setting forth the general principles of recombinant DNA technology include Watson, *et al., Molecular Biology of the Gene*, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, *et al., Molecular Cell Biology*, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, *Genes II*, John Wiley & Sons, publishers, New York, N.Y. (1985); Old, *et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2d edition, University of California Press, publisher, Berkeley, CA (1981); and Maniatis, *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1982).

By "cloning" is meant the use of *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can

replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Maniatis *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1982).. Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines.

By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of replicating in a host independently of the host's chromosome, after additional sequences of DNA have been incorporated into the autonomous element's genome. Such DNA expression vectors include bacterial plasmids and phages. Preferred for the purposes of the present invention is the lambda gtII expression vector.

By "substantially pure" is meant any antigen of the present invention, or any gene encoding any such antigen, which is essentially free of other antigens or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.

By "functional derivative" is meant the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the cDNA sequences of the present invention, is meant to refer to any nucleotide subset of the molecule. A "variant" of such molecule is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Similarly, a "functional derivative" of a gene of any of the antigens of the present invention is meant to include "fragments," "variants," or "analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity.

A DNA sequence encoding GAP-43 or its functional derivatives, or the membrane-targeting peptide or functional derivatives thereof, may be

recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis, *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1982) and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5′-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3′ to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3′-region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be provided. Where the transcriptional termination

signals are not satisfactorily functional in the expression host cell, then a 3′ region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and a GAP-43 encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the GAP-43 gene sequence, or (3) interfere with the ability of the GAP-43 gene sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

Thus, to express the protein, transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention encompasses the expression of the GAP-43 protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells. Preferred prokaryotic hosts include bacteria such as *E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia*, etc. The most preferred prokaryotic host is *E. coli*. Other enterobacterium such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species may also be utilized. Under such conditions, the GAP-43 will not be glycosylated. The procaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

To express the GAP-43 protein (or a functional derivative thereof) in a prokaryotic cell (such as, for example, *E. coli, B. subtilis, Pseudomonas, Streptomyces*, etc.), it is necessary to operably link the GAP-43 encoding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage l, the *bla* promoter of the b-lactamase gene of pBR322, and the CAT promoter

of the chloramphenicol acetyl transferase gene of pBR325, etc. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage l (P_L and P_R), the *trp, recA, lacZ, lacI*, and *gal* promoters of *E. coli*, the a-amylase (Ulmanen, *et al., J. Bacteriol. 162*:176-182 (1985)) and the s-28-specific promoters of *B. subtilis* (Gilman, *et al., Gene 32*:11-20 (1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward, *et al., Mol. Gen. Genet. 203*:468-478 (1986)). Prokaryotic promoters are reviewed by Glick, (*J. Ind. Microbiol. 1*:277-282 (1987)); Cenatiempo, (*Biochimie 68*:505-516 (1986)); and Gottesman, (*Ann. Rev. Genet. 18*:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold, *et al. (Ann. Rev. Microbiol. 35*:365-404 (1981)).

Most preferred hosts are eukaryotic hosts including yeast, insects, fungi, mammalian cells (especially human cells) either *in vivo*, or in tissue culture. Mammalian cells provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/O-AG14 or the myeloma P3x63Sg8, and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as neuroblastoma cell lines such as IMR 332, that may provide better capacities for correct post-translational processing. COS cells also are convenient eukaryotic hosts for GAP-43 expression, as well as for study of the regulation of GAP-43 expression, and are preferred for this purpose.

For a mammalian host, many possible vector systems are available for the expression of GAP-43. A wide variety of transcriptional and translational

regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of GAP-43 or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express GAP-43 by methods known to those of skill. Thus, in one embodiment, sequences encoding GAP-43 may be operably linked to the

regulatory regions of the viral polyhedron protein (Jasny, *Science 238*:1653 (1987)). Infected with the recombinant baculovirus, cultured insect cells, or the live insects themselves, can produce the GAP-43 protein in amounts as great as 20 to 50% of total protein production. When live insects are to be used, caterpillars are presently preferred hosts for large scale GAP-43 production according to the invention.

As discussed above, expression of the GAP-43 protein in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, *et al., J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, *et al., Nature* (London) *290*:304-310 (1981)); the yeast gal4 gene promoter (Johnston, *et al., Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, *et al., Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)).

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the GAP-43 protein (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as GAP-43 encoding DNA sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the GAP-43 encoding sequence).

The GAP-43 encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such

molecules are incapable of autonomous replication, the expression of the GAP-43 protein may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototropy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, *Mol. Cel. Biol. 3*:280 (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX. Such plasmids are, for example,

disclosed by Maniatis, *et al.* (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, (In: *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include pIJ101 (Kendall, *et al., J. Bacteriol. 169*:4177-4183 (1987)), and streptomyces bacteriophages such as ϕC31 (Chater, K.F., *et al.*, In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, *et al. (Rev. Infect. Dis. 8*:693-704 (1986)), and Izaki, (*Jpn. J. Bacteriol. 33*:729-742 (1978)).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, *et al., Miami Wntr. Symp. 19*:265-274 (1982); Broach, In: *The Molecular Biology of the Yeast Saccharomyce:* Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, *Cell 28*:203-204 (1982); Bollon, *et al., J. Clin. Hematol. Oncol. 10*:39-48 (1980); Maniatis, In: *Cell Biolog:* A Comprehensive Treatise, Vol. 3, Gene Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the vector or DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile (biolistic) bombardment (Johnston *et al., Science 240(4858:* 1538 (1988)), etc.

After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells.

Expression of the cloned gene sequence(s) results in the production of the GAP-43 protein, or in the production of a fragment of this protein. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like).

The expressed protein may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

The invention also relates to cloned genes which encode a fusion protein comprising GAP-43 or fragment thereof and a detectable enzyme such as beta-galactosidase, or any desired homologous or heterologous protein or peptide. Methods for producing such fusion proteins are taught, for example, Bai, *et al., J. Biol. Chem. 261:*12395-12399 (1986), or Huynh, *et al.*, "Construction and Screening cDNA Libraries in lgt10 and lgt11," in *DNA Cloning Techniques: A Practical Approach*, D. Glover (ed.), IRL Press, Oxford, 1985, pp. 49-77.

The GAP-43, functional derivative thereof, or fusion protein comprising GAP-43 or fragment thereof and a detectable enzyme or desired protein or peptide may be isolated according to conventional methods known to those skilled in the art. For example, the cells may be collected by centrifugation, or with suitable buffers, lysed, and the protein isolated by column chromatography, for example, on DEAE-cellulose, phosphocellulose, polyribocytidylic acid-agarose, hydroxyapatite or by electrophoresis or immunoprecipitation. Alternatively, the GAP-43 or functional derivative thereof, or fusion protein comprising GAP-43 and a detectable enzyme or desired protein or peptide, may be isolated by the use of anti-GAP-43 antibodies, or by the use of antibodies directed against the detectable enzyme or desired protein or peptide. Such antibodies may be obtained by well-known methods, some of which as mentioned hereinafter. Thus, for example, the

preparation of polyclonal rabbit anti-GAP-43 sera is disclosed in the examples portion of the present specification.

Another embodiment of the present invention comprises antibodies against the GAP-43 protein. The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of binding an antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24:*316-325 (1983)).

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the GAP-43 protein, or a functional derivative thereof, can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding GAP-43.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al., Nature 256:*495 (1975); Kohler *et al., Eur. J. Immunol. 6:*511 (1976); Kohler *et al., Eur. J. Immunol. 6:*292 (1976); Hammerling *et al., In: Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal with GAP-43 antigen. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands, *et al. (Gastroenterology 80:*225-232 (1981). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the GAP-43 antigen.

DEPOSIT OF HYBRIDOMA CELL LINE

The preferred monoclonal antibodies of this invention are those having the specificity of the monoclonal antibody designated MAb anti-GAP-43 (H5). As an additional embodiment, the invention comprises hybridoma strains which produce the monoclonal antibodies of the invention. The preferred hybridoma cell line according to the invention is designated H-5, which produces monoclonal antibody designated MAb anti-GAP-43 (H5). The H5 cell line has been deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA 20851 on 21 December 1989, and given accession number ATCC HB 10316.

The antibodies of the present invention are well suited for use in standard immunodiagnostic assays known in the art, including such immunometric or "sandwich" assays as the forward sandwich, reverse sandwich, and simultaneous sandwich assays. The antibodies of the present invention may be used in any number of combinations as may be determined by those of skill without undue experimentation to effect immunoassays of acceptable specificity, sensitivity, and accuracy for the GAP-43 antigen or equivalents thereof.

Standard reference works regarding the general principles of immunology include Klein, *Immunology: The Science of Self-Nonself Discrimination*, John Wiley & Sons, Publisher, New York (1982); Kennett, *et al.*, eds., *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, Publisher, New York (1980); Campbell, *Monoclonal Antibody Technology*, in, Burdon, *et al.*, eds., *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13, Elsevier, Publisher, Amsterdam (1984).

By "detecting" it is intended to include determining the presence or absence of a substance or quantifying the amount of a substance. The term

thus refers to the use of the materials, compositions, and methods of the present invention for qualitative and quantitative determinations.

The isolation of other hybridomas secreting monoclonal antibodies of the same specificity as those described herein can be accomplished by the technique of anti-idiotypic screening. Potocmjak, *et al., Science 215:*1637 (1982). Briefly, an anti-idiotypic antibody is an antibody which recognizes unique determinants present on the antibody produced by the clone of interest. The anti-idiotypic antibody is prepared by immunizing an animal of the same strain used as the source of the monoclonal antibody with the monoclonal antibody of interest. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing antibody to these idiotypic determinants (anti-idiotypic antibody). By using the anti-idiotypic antibody of the second animal, which is specific for the monoclonal antibodies produced by a single clone, it is then possible to identify other clones used for immunization. Idiotypic identity between the product of two clones demonstrates that the two clones are identical with respect to their recognition of the same epitopic determinants. The anti-idiotypic antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti anti-idiotypic antibody which will be epitopically identical to the original MAb. Thus, by using antibodies to the epitopic determinants of a monoclonal antibody, it is possible to identify other clones expressing antibodies of identical epitopic specificity. In antibodies, idiotypic determinants are present in the hypervariable region which binds to a given epitope.

Accordingly, the monoclonal antibodies of the present invention may be used to induce anti-idiotypic Abs in suitable animals, such as BALB/c mice. Spleen cells from these animals are used to produce anti-idiotypic hybridoma cell lines. Monoclonal anti-idiotypic Abs coupled to KLH are used as "immunogen" to immunize BALB/c mice. Sera from these mice will contain

anti anti-idiotypic Abs that have the binding properties of the original Ab specific for the shared epitope. The anti-idiotypic MAbs thus have idiotopes structurally similar to the epitope being evaluated.

For replication, the hybrid cells may be cultivated both *in vitro* and *in vivo*. High *in vivo* production makes this the presently preferred method of culture. Briefly, cells from the individual hybrid strains are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid containing high concentrations of the desired monoclonal antibodies. Monoclonal antibodies of isotype IgM or IgG may be purified from cultured supernatants using column chromatography methods well known to those of skill in the art.

The antibodies of the present invention are particularly suited for use in immunoassays wherein they may be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways.

There are many different labels and methods of labeling known in the art. Examples of the types of labels which can be used in the present invention include, but are not limited to, enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds and metal chelates. Those of ordinary skill in the art will know of other suitable labels for binding to antibodies, or will be able to ascertain the same by the use of routine experimentation. Furthermore, the binding of these labels to antibodies can be accomplished using standard techniques commonly known to those of ordinary skill in the art.

One of the ways in which antibodies of the present invention can be detectably labeled is by linking the antibody to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected as, for example, by spectrophotometric or fluorometric means. Examples of enzymes

which can be used to detectably label the antibodies of the present invention include malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotin-avidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase.

The presence of the detectably labeled antibodies of the present invention also can be detected by labeling the antibodies with a radioactive isotope which then can be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe and ^{75}Se.

It is also possible to detect the binding of the detectably labeled antibodies of the present invention by labeling the antibodies with a fluorescent compound. When a fluorescently labeled antibody is exposed to light of the proper wave length, its presence then can be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labeling compounds are fluoroscein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibodies of the invention also can be detectably labeled using fluorescent emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibodies of the present invention also can be detectably labeled by coupling them to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of the chemical reaction.

Examples of particularly useful chemiluminescent labeling compounds are luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibodies of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent antibody is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling include luciferin, luciferase and aequorin.

The antibodies and substantially purified antigen of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the assay to be used.

The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich, immunoassays.

By the term "immunometric assay" or "sandwich immunoassay," it is meant to include simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that the antibodies of the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

Forward sandwich assays are described, for example, in United States Patents 3,867,517; 4,012,294 and 4,376,110. Reverse sandwich assays have been described, for example, in United States Patents 4,098,876 and 4,376,110.

In the preferred mode for preforming the assays it is important that certain "blockers" be present in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, protease, or human antibodies to mouse immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore adds substantially to the specificity of the assays described in the present invention.

It has been found that a number of nonrelevant (i.e. nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g. IgG_1, IgG_{2a}, IgM, etc.) can be used as "blockers." The concentration of the "blockers" (normally 1-100 microgs/microl) is important, in order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in human serum. In addition, the buffer system containing the "blockers" needs to be optimized. Preferred buffers are those based on weak organic acids, such as imidazole, HEPPS, MOPS, TES, ADA, ACES, HEPES, PIPES, TRIS, and the like, at physiological pH ranges. Somewhat less preferred buffers are inorganic buffers such as phosphate, borate or carbonate. Finally, known protease inhibitors should be added (normally at 0.01-10 microgs/ml) to the buffer which contains the "blockers."

There are many solid phase immunoadsorbents which have been employed and which can be used in the present invention. Well known immunoadsorbents include glass, polystyrene, polypropylene, dextran, nylon

and other materials, in the form of tubes, beads, and microtiter plates formed from or coated with such materials, and the like. The immobilized antibodies can be either covalently or physically bound to the solid phase immunoadsorbent, by techniques such as covalent bonding via an amide or ester linkage, or by adsorption. Those skilled in the art will know many other suitable solid phase immunoadsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

For *in vivo, in vitro* or *in situ* diagnosis, labels such as radionuclides may be bound to the antibodies of the present invention either directly or by using an intermediary functional group. An intermediary group which is often used to bind radioisotopes which exist as metallic cations to antibodies is diethylenetriaminepentaacetic acid (DTPA). Typical examples of metallic cations which are bound in this manner are: ^{99m}Tc, ^{123}I, ^{111}IN, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga and ^{68}Ga. The antibodies of the invention can also be labeled with non-radioactive isotopes for purposes of diagnosis. Elements which are particularly useful in this manner are ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr and ^{56}Fe.

The antibodies of the present invention also may be used for immunotherapy in animals, including humans, having a disorder, such as a benign or cancerous neoplasia, which expresses the GAP-43 antigen with epitopes reactive with the antibodies of the present invention.

When used for immunotherapy, the antibodies of the present invention may be unlabeled or labeled with a therapeutic agent. Examples of therapeutic agents which can be coupled to the antibodies of the invention for immunotherapy are drugs, radioisotopes, lectins and toxins.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. Ricin is a toxic lectin which has been used immunotherapeutically. This use is accomplished by binding the alpha-peptide

chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site-specific delivery of the toxic defect. This is described, for example, in Vitetta *et al., Science 238:* 1098 (1987), and Pastan *et al., Adv. Allergy 47:* 641 (1986).

Toxins are poisonous substances produced by plants, animals or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin, for example, is a protein produced by *Corynebacterium diphtheria.* This toxin consists of an alpha and a beta subunit which under proper conditions can be separated. The toxic alpha component can be bound to antibody and used for a site-specific delivery.

Examples of radioisotopes which can be bound to the antibodies of the present invention for use in immunotherapy are: ^{125}Um, ^{131}I, ^{90}Y, ^{67}Cu, ^{217}Bi, ^{211}At, ^{212}Pb, ^{47}Sc and ^{109}Pd.

Of course, the expressed GAP-43 antigen normally is confined within the cell membrane. Accordingly, those of skill will recognize that *in vivo* diagnostic and therapeutic methods employing the antibodies of the invention may require some mechanism by which such antibodies can detect GAP-43 on the intracellular membrane. One such method is to introduce the antibodies or fragments thereof into the cell's membrane or into the cell itself across the cell membrane. This may be accomplished, for example, by attaching the antibody to a ligand for which the target cell contains receptor sites. The antibody can thus be transported into the cell membrane or across the cell membrane along with the ligand.

The choice of a carrier ligand will depend on several factors, as those of skill will appreciate. These include, for example, the kinetics of the ligand and its receptor, and of overall transport, which may include passive or active, with actively transported ligands preferred. The means of attaching the antibody to the ligand also will vary within limits, and may be, for example,

covalent or ionic, bearing in mind that such attachment should not unacceptably alter ligand-receptor affinity.

Examples of receptors suitable for such applications include the receptor for low density lipoprotein (LDL), which has been shown to contain all the information necessary for receptor endocytosis, Davis *et al., J. Cell Biol. 107(6/3):* Abstr. No. 3112 (1988), as well as known brain-specific receptors such as those for dopamine. In this regard, it will be appreciated that the ligand may itself be an antibody or fragment specific for the receptor, to which may be conjugated the antibody of the invention.

Moreover, those of skill may find it particularly desirable to employ antibody fragments of the invention (such as, for example, Fab or $F(ab')_2$ fragments), which are less likely to interfere with the ligand-receptor interaction, and may be more easily transported across the cell membrane. Single-chain antibodies may prove preferable for these and other reasons, as will be appreciated by those of skill.

When an antibody is to be transported into the cell's membrane or into the cell as described above, it will be preferred to diagnostically or therapeutically label the antibody in such a way that the label will be relatively more effective when the antibody is bound to its antigenic site on the GAP-43 protein. This may accomplished, for example, by employing a label which becomes active or detectable as a result of formation of the antigen-antibody complex. Alternatively, the antibody itself may be labeled in such a way that antigen-antibody complex formation induces a conformational change in the antibody to expose or more fully expose the previously unexposed or less fully exposed label. All of the above criteria, and others, will be apparent to those of skill in carrying out these aspects of the invention.

It is also possible to utilize liposomes having the antibodies of the present invention in their membrane to specifically deliver the antibodies to the target area. These liposomes can be produced so that they contain, in addition

to the antibody, such immunotherapy agents as drugs, radioisotopes, lectins and toxins, which would be released at the target site.

Another preferred manner in which the antibodies, and preferably, the GAP-43 encoding nucleotide sequences (and their functional and chemical derivatives) may be introduced into neural cells for diagnostic or therapeutic purposes is by the use of viral, including retroviral, vectors. As an example of suitable viruses may be mentioned the various herpes viruses. Suitable retroviruses include human immunodeficiency virus (HIV). Other suitable viruses and retroviruses are well known to those of skill. The use of viral vectors for introduction of genes into mammalian cells is reviewed, for example, in Varmus, *Science 240(4858)*:1427 (1988); Eglitis *et al.*, *BioTechniques 6, 7:* 608 (1988); Jaenisch, *Science 240(4858)*: 1468 (1988); and Bernstein *et al., Genet. Eng. (N.Y.) 7:*235 (1985).

For the purposes of the present invention, it may be preferred to employ an attenuated viral or retroviral strain. Thus, for example, it is possible to use as vectors for the antibodies or DNA sequences of the invention retroviruses having attenuated cytopathicity, such as HIV-2_{ST} (Kong *et al., Science 240(4858):* 1525 (1988)) or HIV-2_{UCI} (Evans *et al., Science 240(4858):* 1523 (1988)), which enter neural cells by a CD4-dependent mechanism (Funke *et al., J. Exp. Med. 165:* 1230 (1987)). The neurobiology of HIV infections is described, for example, in Johnson *et al., FASEB J. 2(14):* 2970 (1988). Those of skill will be able to target different neural populations having known susceptibilities to viruses by the exercise of routine skill. For example, CD4 is known to have a variant transcript in the human brain, with its highest content in forebrain (Maddon *et al., Cell 47:* 333 (1986).

Ideally, then, the choice of a gene delivery system will be made by those of skill, keeping in mind the objectives of efficient and stable gene transfer, with an appropriate level of gene expression, in a tissue-appropriate

manner, and without any adverse effects. See, for example, Wolff *et al.*, *Rheum. Dis. Clin. North Am. 14(2):* 459 (1988). With respect to delivery to a central nervous system target, many viral vectors, including HIV, offer the advantage of being able to cross the blood-brain barrier (Johnson *et al.*, *FASEB J. 2(14):* 2970 (1988)).

The DNA sequences which encode GAP-43, or a fragment thereof, may be used as DNA probes to isolate the corresponding antigen in humans according to the above-described methods for isolation of rat GAP-43 with labeled probes. The human antigen genes may then be cloned and expressed in a host to give the human antigen. This human antigen may then be used in diagnostic assays for the corresponding autoantibody, and for therapeutic treatment of animals including humans.

The present inventors have undertaken experiments designed to elucidate the regulatory mechanisms which control expression of the GAP-43 gene. Modulation of GAP-43 expression offers a convenient and effective manner in which mammals, including humans, suffering from damaged, diseased or dysfunctioning central or peripheral nervous tissue, may be therapeutically treated. Further, methods of modulating structural remodeling in normal central or peripheral nervous tissue in mammals, including humans, according to the present invention, will be a significant aid to those of skill in further elucidating the mechanisms of neuron structure and function.

The preclinical and clinical therapeutic use of the present invention in the treatment of neurological disease or disorders will be best accomplished by those of skill, employing accepted principles of diagnosis and treatment. Such principles are known in the art, and are set forth, for example, in Petersdorf, *et al.*, eds., *Harrison's Principles of Internal Medicine*, 10th Edition, McGraw-Hill, publisher, New York, N.Y. (1983), especially at Part 6, Section 11 of that work, entitled "Disorders of the Central Nervous System."

The antigens, antibodies and compositions of the present invention, or their functional derivatives, are well suited for the preparation of pharmaceutical compositions. The pharmaceutical compositions of the invention may be administered to any animal which may experience the beneficial effects of the compounds of the invention. Foremost among such animals are humans, although the invention is not intended to be so limited.

The pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

In addition to the pharmacologically active compounds, the new pharmaceutical preparations may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.001 to about 99 percent, preferably from about 0.01 to about 95 percent of active compound(s), together with the excipient.

The dose ranges for the administration of the compositions of the present invention are those large enough to produce the desired effect, whereby, for example, the neoplastic tissue is reduced or eliminated or ameliorated. The doses should not be so large as to cause adverse side

effects, such as unwanted cross reactions anaphalactic reactions and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient. Counterindication, if any, immune tolerance and other variables will also affect the proper dosage. The antibodies can be administered parenterally by injection or by gradual profusion over time. The antibodies of the present invention also can be administered intravenously, intraparenterally, intramuscularly or subcutaneously.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone,

polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl-cellulose phthalate or hydroxypropymethyl-cellulose phthalate, are used. Dye stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of one or more of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection

suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The GAP-43 antigen of the present invention is unique to neuronal cells, and thus provides a convenient and useful marker. Accordingly, antibodies directed against GAP-43 may be used in various techniques well known to those of skill, to identify neuronal cells. Moreover, the antibodies of the present invention will allow detection, determination and therapeutic treatment of neoplasias and other disorders of neuronal origin, and, as such, offer a convenient and useful diagnostic and therapeutic method *in vivo, in vitro* or *in situ*, for preclinical and clinical evaluation and treatment of cancer and other disorders in animals including humans.

The antigen of the invention may be isolated in substantially pure form employing the antibodies of the present invention. Thus, an embodiment of the present invention provides for substantially pure antigen GAP-43, said antigen characterized in that it is recognized by and binds to the antibodies of the present invention. In another embodiment, the present invention provides a method of isolating or purifying the GAP-43 antigen, by forming a complex of said antigen with one or more antibodies directed against GAP-43.

The substantially pure antigen GAP-43 of the present invention may in turn be used to detect or measure antibody to GAP-43 in a sample, such as cerebrospinal fluid, serum or urine. Thus, one embodiment of the present invention comprises a method of detecting the presence or amount of antibody to GAP-43 antigen in a sample, comprising contacting said sample containing said antibody to GAP-43 antigen with detectably labeled GAP-43, and detecting said label. It will be appreciated that immunoreactive fractions and immunoreactive analogues of GAP-43 also may be used. By the term "immunoreactive fraction" is intended any portion of the GAP-43 antigen which demonstrates an equivalent immune response to an antibody directed

against GAP-43. By the term "immunoreactive analogue" is intended a protein which differs from the GAP-43 protein by one or more amino acids, but which demonstrates an equivalent immunoresponse to an antibody of the invention.

In yet another aspect of the present invention, it has been found that the GAP-43 protein contains a novel membrane-targeting peptide domain which directs the GAP-43 protein to the cell membrane, and especially to the region of the growth cone of neuronal cells. The structure of this membrane-targeting domain has been determined, and it has been shown that the peptide is effective in directing normally cytosolic proteins (which are not normally membrane-associated), to the cell membrane. Experiments illustrating this aspect of the present invention are presented in detail in Example VI of the specification.

According to the compositions and methods of this aspect of the invention, it is possible, *inter alia*, to direct any desired protein to the cell membrane, including proteins which are not normally membrane-associated. Further, the compositions and methods of this aspect of the invention are of obvious utility in the therapeutic treatment of neurological damage and disorders *in vitro, in vivo*, and *in situ*, in animals. Those of skill will appreciate that the preceeding description of diagnostic and therapeutic methods is equally applicable to this embodiment of the invention. Further, it will be evident that the membrane-targeting peptide of the present invention will be of use in directing any desired protein or peptide to cell membranes, and will thus be of diagnostic and therapeutic utility in non-neurological indications as well. Examples of such indications include, but are not limited to, any applications wherein the membranes of cells may play an important role, such as immunological indications.

The manner and method of carrying out the present invention may be more fully understood by those of skill by reference to the following

examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

EXAMPLE I

Cloning of the cDNA for Rat GAP-43

A cDNA library was generated from RNA of rat dorsal root ganglia from embryonic day 17 and cloned into the lgt11 expression vector (Huynh *et al.*, in "DNA Cloning A Practical Approach," D.M. Glover, Ed. (IRL Press, Washington, D.C., 1985) pp. 49-78). Three presumptive GAP-43 clones were identified with the antibody to GAP-43 described by Snipes *et al., Soc. Neurosci. Abstr. 12:*500 (1986). The identity of the longest clone, GAP43-2, was confirmed by hybrid-selected translation (Fig. 1). GAP43-2 selected by hybridization a messenger RNA (mRNA) that directed the translation of a polypeptide that migrated in SDS-polyacrylamide gels with the expected mobility of native GAP-43, that is, a molecular size of about 43 kD. This *in vitro* translation product was selectively immunoprecipitated by antibody to GAP-43. The specificity of the immunoprecipitation was demonstrated by competition with unlabeled, purified GAP-43. For additional confirmation, a peptide prepared by cyanogen bromide cleavage of purified GAP-43 was sequenced. The sequence, Arg-X-Lys-Gln-Val-Glu-Lys-Asn-Asp-Glu-Asp-Gln-Lys-Ile, is completely included within the predicted open reading frame of GAP43-2. (The X represents a cycle of sequencing at which the identity of the amino acid could not be determined with certainty.)

The complete nucleotide sequence of GAP43-2 and the predicted amino acid sequence are shown in Fig. 2. The reading frame includes the peptide fragment that was sequenced and is in the same reading frame as the b-galactosidase gene of lgt11. (A cDNA for rat GAP-43 was obtained

independently by J.H.P. Skene and his colleagues. Copies of the sequences were exchanged. The predicted amino acid sequence of the present invention agrees perfectly with that provided by Skene, and the nucleotide sequence differs at only one position in the 3′ untranslated region.) The methionine identified as the start of the open reading frame is the first methionine after the in-frame stop codon (TAA) at nucleotide position 13 and is surrounded by eight of the nine nucleotide consensus sequences suggested by Kozak, *Cell 44*:283 (1986) to be the most favorable context to initiate eukaryotic translation. This suggests that it is the first residue of the GAP-43 coding region. However, the information is insufficient to make this assignment unequivocally, and, therefore, the second methionine (amino acid 5) might play this role. The predicted composition of GAP-43 is highly polar, without evident transmembrane domains or potential N-linked glycosylation sites. This composition is compatible with the observations that GAP-43 is membrane-associated but inaccessible to antibody recognition in the absence of membrane permeabilization (Meiri *et al., PNAS USA 83*:3537 (1986)); thus it may be associated with the inner face of the membrane.

The predicted molecular size of the GAP-43 protein from the open reading frame is 24 kD, which is less than the 43 kD originally observed by Skene and Willard as the apparent molecular size of the molecule in SDS-polyacrylamide gels (Skene *et al., J. Cell. Biol. 89*:86 (1981)). The molecular size has been uncertain because the apparent molecular size of GAP-43 depends on polyacrylamide concentration (Jacobson *et al., J. Neurosci. 6*:1843 (1986)), suggesting that this protein falls in the category of proteins that migrate anomalously on SDS-polyacrylamide gels (Banker *et al., J. Biol. Chem. 247*:5856 (1972); Persson *et al., Science 225*:687 (1984); Smart *et al., Virology 112*:703 (1981)). This property is unlikely to be due to posttranslational modification since the *in vitro* translation product has a mobility similar to that of native GAP-43 (Fig. 1).

To collect information concerning the SDS-polyacrylamide gel migration properties of the protein encoded within the putative open reading frame, GAP-43 RNA was synthesized from the cDNA in an *in vitro* transcription system with the use of the bacteriophage SP6 promoter, by the method of Melton *et al., Nucleic Acids Res. 12:*7035 (1984). An 800-base RNA was generated by transcribing the cDNA cut at the Sau3A site, 65 bases 3′ of the end of the predicted open reading frame (Fig. 2), and a 1100-base RNA by truncating at the HindIII site in the polylinker region at the 3′ end of cDNA. Both the 800-base RNA and the 1100-base RNA directed the synthesis of a polypeptide with an apparent molecular size of 40 kD when translated *in vitro* with reticulocyte lysate and analyzed on a 15% SDS-polyacrylamide gel. The 40-kD translation product in both cases was immunoprecipitated with the antibody to GAP-43. GAP-43 synthesized *in vitro* from newborn rat brain RNA comigrated with these translation products.

Evidence for the belief that GAP-43 is important to the function of growth cones includes enrichment of the protein in growth-cone membranes (Meiri *et al., PNAS USA 83:*3537 (1986); Skene *et al., Science 233:*783 (1986)) and increased transport of the protein in developing and regenerating nerves (Skene *et al., J. Cell. Biol. 89:*86 (1981)). To investigate whether GAP-43 gene expression is regulated coordinately with extension of neurites, its expression was examined in PC12 cells, in which neurite outgrowth was promoted by nerve growth factor (NGF) and to a lesser extent by adenosine 3′,5′-monophosphate (cAMP). These agents act by different mechanisms in inducing neurite outgrowth (Gunning *et al., J. Cell. Biol. 89:*240 (1981)). Concomitant with the neurite growth induced by either agent is an increase in GAP-43 mRNA levels, with the largest increase in cells exposed to both agents (Fig. 3).

To determine the pattern of expression of the GAP-43 gene during normal development, total cellular RNA was isolated from brain, dorsal root

ganglia, heart, and liver of embryonic day 13, embryonic day 17, newborn, and adult rats. GAP-43 was expressed in a neural-specific manner (Fig. 4). At all ages, the major hybridizing band of about 1500 nucleotides is visible only in the neuronal tissues. The faint, large-molecular-size bands may correspond to unspliced precursor molecules, since the present inventors have discovered that the genomic GAP-43 gene contains intronic sequences. The GAP-43 mRNA in neuronal tissue is probably of neural rather than glial origin, since GAP-43 is localized in neurons (Meiri et al., *PNAS USA 83:*3537 (1986)) and no GAP-43 RNA was detected in the glioma cell line C6.

In neural tissues, the amount of GAP-43 mRNA varies with developmental stage. Peak concentrations occur in the perinatal period, with some delay in the central nervous system relative to the peripheral nervous system. The timing of expression accords well with periods of axon growth (Jacobson, *Developmental Neuropathology* (Plenum, New York, 1978)). However, the significant amount of GAP-43 RNA in adult neural tissues is in agreement with observations that GAP-43 protein persists in adult rat cortex, albeit in significantly lower amounts than during the perinatal period (Jacobson *et al., J. Neurosci. 6:*1843 (1986)). The persistence of GAP-43 expression suggests an ongoing role in the adult nervous system. The properties of B-50 and F1, phosphoproteins electrophoretically and antigenically indistinguishable from GAP-43, have been assessed in adult neuronal tissue. These proteins serve as substrates for a protein kinase C-like enzyme, and their phosphorylation is regulated by neuropeptides, neurotransmitters, and during the course of long-term potentiation (Jacobson *et al., J. Neurosci. 6:*1843 (1986); Aloyo *et al., J. Neurochem. 41:*649 (1983); Zwiers *et al., Progr. Brain Res. 56:*405 (1982)). It is not known whether GAP-43 regulation in the adult also occurs by alterations in gene expression. One model for the function of GAP-43 in the mature animal would include an ongoing role in synaptic turnover (Cotman *et al., Science 225:*1287 (1984)) and in other "plastic" changes of the nervous

system, such as learning, that are accompanied by structural growth at the nerve terminal (Bailey and Chen, *Science 220*:91 (1983)).

EXAMPLE II

Cloning of the cDNA for Human GAP-43

EXPERIMENTAL PROCEDURES

Tissue procurement

Human brain tissue was harvested fresh at the time of autopsy and within 10 hours of death. Sections of brain no larger than 2x2x0.5 cm, obtained from specific regions were snap-frozen in isopentane (2-methyl butane) cooled with dry ice and then stored at -90°C. These specimens were used for *in situ* hybridization and immunocytochemistry. In addition, a small portion of tissue from the same regions, obtained fresh or from the frozen sample, was used for Northern blot analysis. Routine histopathology was performed on formalin-fixed, paraffin-embedded tissue immediately adjacent to the frozen blocks.

cDNA cloning

The human GAP-43 cDNA was isolated from cDNA libraries of brainstem of a 1 day old and cerebellum of a 7 year old (both libraries were from American Type Culture Collection). These libraries were screened with ^{32}P-labeled rat GAP-43 cDNA probes (Karns *et al., Science 236*:597 (1987)). Hybridization was for 16 hr at 42°C in 4x Standard Saline Citrate (SSC), 0.8x Denhardt, 10% dextran sulfate, 40% formamide, 20 mg herring sperm DNA per ml, 7 mM Tris (pH 7.6), 1% SDS, and probe at 10^6 cpm/ml. Filters were washed extensively in 2x SSC at room temperature, 1x SSC at 53°C and again 1x SSC at 60°C before autoradiography. Positive clones were subcloned into

both pGem-3Z (Promega Biotec) and M13 mp18 and sequenced by the chain-termination method (Sanger *et al., Proc. Natl. Acad. Sci. USA 74:*5463 (1977)). DNA sequences were analyzed by UWGCG (University of Wisconsin Genetics Computer Group) software package.

Northern blot analysis

Total cellular RNA was isolated from autopsied human tissues by the guanidium thiocyanate procedure (Chirgwin *et al., Biochemistry 18:*5294 (1979)). For Northern blots, 10 mg of total RNA from each tissue were denatured and run on a 1.2% agarose-formaldehyde gel, transferred to Genescreen (New England Nuclear) or Nytran (S & S) in 10x SSC, UV cross-linked, and hybridized overnight at 42°C with randomly primed (Feinberg *et al., Anal. Biochem. 132:*6 (1983)) human GAP-43 cDNA clone Cla. Filters were finally washed to 1x SSC with 0.1% SDS at 60°C. After probing with Cla, the filters were stripped and reprobed with a rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe (Piechaczyk *et al., Cell 42:*589 (1985)) as control.

In situ hybridization

Cryostat sections of human brain tissue were fixed in 4% paraformaldehyde, treated with 0.3% Triton X-100 followed by 1 mg/ml proteinase K, acetylated, and pre-hybridized in 50% formamide/2x SSC. Hybridization using 2 x 10^6 cpm per slide of ^{35}S-labeled antisense or sense riboprobe (Melton *et al., Nucleic Acids Res. 12:*7035 (1984)) was performed in a humidified chamber for 5 hours at 50°C. The tissue sections were then washed in 2x SSC with 10 mM dithiothreitol initially containing 50% formamide, then 50% formamide plus 0.1% Triton-X 100. Single stranded RNA was removed by treatment with 50 mg/ml RNAase A. The sections were further washed in 2x SSC with 1mM DTT for 2 hours, then dehydrated

through graded alcohols containing 0.3 M ammonium acetate. The radioactive signal was detected using NTB2 Kodak emulsion. Emulsion-coated slides were counterstained with hematoxylin.

RESULTS

Human GAP-43 is homologous to a mouse calmodulin binding protein

As described in Example I, the cDNA for rat GAP-43 was cloned and used as a probe to identify related cDNA clones from human brainstem and cerebellum libraries. Overlapping clones were obtained from each of the libraries, which were identical in the overlapping regions. The sequence of the longest clone (Cla from the cerebellum library) is presented in Figure 5A, and a comparison with rat GAP-43 shown in Figure 5B. The identity of GAP-43 with that of a neural specific mouse calmodulin-binding protein, termed P-57, was described by Cimler *et al., J. Biol. Chem. 262:*12158 (1987), so that sequence is also aligned in Figure 5B. P-57 has been described as a neural-specific calmodulin-binding protein with the unusual property that it releases, rather than binds, calmodulin as calcium levels rise (Andreasen *et al., Biochemistry 22:*4615 (1983)). The proteins are highly conserved between human, rat, and mouse. For example, there is 89% identity of amino acids between human and mouse. Additionally there is an unusually high degree of conservation (80%) between 3'-untranslated regions, including 2 energetically favorable stem-loop structures shown in Figure 5C, which may, by analogy to other genes, serve to regulate messenger RNA stability (Reeves *et al., Proc. Natl. Acad. Sci. USA 83:*3228 (1986); Shaw *et al., Cell 46:*659 (1986)).

GAP-43 expression persists in discrete regions of the adult human brain

To minimize RNA degradation, brain tissue was obtained from patients with a postmortem interval of less than 10 hours. Adjacent sections were examined histopathologically. In two infants, 8 days and 1 month old, GAP-43 was uniformly and robustly expressed throughout the brain as assessed by Northern blots. The regions examined included the cerebellum, temporal cortex, temporal association cortex, frontal cortex, orbital frontal region (Area 11), hippocampus, visual cortex (Area 17/18), and spinal cord, some examples of which are shown in Figure 6. In contrast to the brain, levels in the spinal cord were low at these ages, which may be related to earlier maturation of this region (Anand *et al.*, *New Engl. J. Med. 317:*1321 (1987)). GAP-43 was not expressed in any non-neuronal tissues examined either in the newborn or adult (including kidney, lung, liver, and adrenal). In the normal adult brain, GAP-43 expression varied markedly among different regions. For example, in three brains, levels comparable to the neonate were found in Broadman's Area 11 (orbital frontal gyrus) and much lower levels expressed in the visual cortex (Area 17/18). Levels were consistently low in the hippocampus. The latter is of interest because the adult rat hippocampus is enriched in GAP-43. A similar distribution has been reported recently by Neve *et al.*, *Molec. Brain Res. 2:*177 (1987).

Renewed GAP-43 expression after ischemic injury

Brain tissue was examined from two patients with small clinically silent infarcts, both occurring 10 to 14 days antemortem. The histopathological features that characterized these subacute infarcts included the following: [1] a sharp delineation between the infarcted and the intact tissue; [2] loss of neurons; [3] infiltration of necrotic tissue by mononuclear inflammatory cells and lipid-laden macrophages; and [4] activation of fibrous astrocytes along the

edge of the infarct. In one patient the infarct was in the visual cortex (Area 17), and in the other, parietal lobe (Area 3,1,2,5). The tissue utilized for Northern analysis and *in situ* hybridization included both the infarcted tissue and surrounding normal brain from the same Broadman's areas. Figure 7A shows that after a stroke in Area 17, GAP-43 expression is increased to levels comparable to Area 11, the region normally most enriched in GAP-43 in the adult. Figure 7B shows that GAP-43 levels in Area 3,1,2,5 from two normal brains were low (lanes 1 and 2) compared to another patient with a small stroke in that location (lane 3). These observations suggest that GAP-43 increases within days following ischemic infarction.

As described above, GAP-43 is neuron-restricted in its expression, and since neurons were absent in the infarcted regions (Figure 8A1, B), most likely the heightened GAP-43 expression derived from the morphologically uninjured neurons. To examine this hypothesis, *in situ* hybridization was used to study the distribution of GAP-43 expression in the region of infarction. The detailed cellular anatomy of GAP-43 expression is presented in Example III. Throughout most regions of the adult cerebral cortex, including the visual cortex (Area 17), as might be predicted from the Northern analysis, only a few scattered cells expressed GAP-43 (Figure 8C). In the infarcted region of visual cortex, no specific GAP-43 expression was found, although frankly necrotic regions bound both antisense and sense probes non-specifically (Figure 8D, 8G). On the other hand, in the adjacent morphologically normal Area 17 (Figure 8A2) essentially all neurons evidenced GAP-43 expression (Figures 8E, 8F), confirming that regions adjacent to infarcted tissue are the source of the increased GAP-43 expression.

The effect of transient ischemia without infarction upon GAP-43 expression was also investigated. Neurons in certain regions of the brain, such as the cerebellar cortex and Sommer's sector of the hippocampus, manifest selective vulnerability to ischemic and hypoxic insults, particularly

when due to hypoperfusion (Brierley *et al.*, In: *Greenfield's Neuropathology,* Fourth Edition, J.H. Adams, Corsellis, *et al.* Eds., Edward Arnold, London, 1984, pp. 125-156). This type of injury may be transient and full recovery may ensue within weeks. Using ***in situ*** hybridization, the cerebellar cortex of one patient who sustained a cardiac arrest with attendant global anoxia was examined. The patient died several days later, and at autopsy there was evidence of anoxic encephalopathy without infarction. This was manifested by the presence of numerous scattered pyknotic (dark and shrunken) or hydropic (swollen) neurons and vacuolation of the neuropil in the cerebral cortex, hippocampus and cerebellum. In the cerebellum, the ischemic Purkinje cells were hydropic and achromasic. As shown in Figure 9B, there was a striking enhancement of GAP-43 expression in the cerebellum, primarily in the Purkinje cell layer, a region found to be without detectable GAP-43 expression by ***in situ hybridization*** in the normal adult (Figure 9A).

DISCUSSION

Growth cones are nerve terminal structures shared by developing and regenerating nerves (e.g., Ramon *et al.*, *Degeneration and Regeneration of the Nervous System*, Oxford University Press, London (1928); Kater *et al.*, *Biology of the Nerve Growth Cone*, Alan R. Liss, Inc., New York (1985)). They include machinery for motility and transduction of local information and have a protein constituency determined by transport from the cell soma. GAP-43 is one of the rapidly transported proteins which is notable for pronounced enrichment in axonal transport in developing and regenerating nerves. The failure of mammalian CNS neurons to regenerate has been linked to the low and uninducible levels of GAP-43 in adult brain (Skene, *Cell 37:*697 (1984)). Thus it is naturally of special interest to investigate regulation of this protein

in the human because of the problems encountered in treatment of CNS injury and stroke.

GAP-43 and the growth cone

GAP-43 is highly conserved between rat and human and clearly identical to a mouse protein recently identified as a calmodulin-binding protein which has the unusual property of releasing calmodulin when ambient calcium increases (Andreasen *et al., Biochemistry 22:*4615 (1983); Cimler *et al., J. Biol. Chem. 260:*10784 (1985); Alexander *et al., J. Biol. Chem. 292:*6108 (1987)). As suggested by the above authors, one notion for its role in the growth cone might be that it regulates calmodulin activity, and that it does so by releasing it in focal cellular domains. Thus, the affinity of GAP-43 for calmodulin would diminish when calcium rises, for example after an action potential (Belardetti *et al., Proc. Natl. Acad. Sci. 83:*7094 (1986)). The calmodulin-dependent activities of the growth cone could thereby be regulated within the immediate vicinity of calcium entry.

GAP-43 in the adult

GAP-43 expression is highly regulated during development. In general, the highest levels correlate well with the periods of peak axonal elongation. However, its high level of expression in particular regions of the mature brain suggests that GAP-43 has an ongoing role in some adult neurons. One possibility is that GAP-43 expression denotes cells actively engaged in remodeling their structure, especially at nerve terminals. Evidence for this is that in the rat the adult neurons which express GAP-43 include most prominently hippocampal neurons and mitral cells of the olfactory bulb, neurons which do in fact remodel their terminals in the adult. The human hippocampus expresses GAP-43 only at low levels, suggesting that, if GAP-43 is indeed an indicator of such structural remodeling, different regions of the

human brain have retained this function. The other function proposed for GAP-43 is learning, because its phosphorylation state changes in the wake of long-term potentiation (Nelson *et al.*, *Exp. Neurol. 89:*213 (1985)). In fact, structural remodeling may be a facet of long-term learning (Chang *et al.*, *Brain Res. 309:*35 (1984); Goelet *et al.*, *Nature 322:*419 (1983)), and growth of nerve terminal areas has been documented to accompany long-term learning in Aplysia (Bailey and Chan, *Science 220:*91 (1983)). Thus, one intriguing possibility is that neurons of the adult human brain that use structural plasticity for long-term learning are those that express GAP-43. This possibility has been discussed by several investigators, including Nelson *et al.*, *Exp. Neurol. 89:*213 (1985), Jacobson *et al.*, *J. Neurosci. 6:*1843 (1986), and Neve *et al.*, *Molec. Brain Res. 2:*177 (1987), and the present work is consistent with these suggestions. This proposal will require close correlation of various disease states with GAP-43 expression.

GAP-43 and repair in the adult CNS

The restraints upon recovery from CNS injury are poorly understood. Whereas severance of a peripheral nerve of mammals or even central nerves of amphibia may be followed by full repair, no recovery follows such injury in the central nervous system of mammals. The adult neuron does retain the ability to grow, since central nerves will elongate long distances after axotomy so long as they are provided with a peripheral nerve sheath as a guide (Benfey *et al.*, *Nature 296:*150 (1982)). The failure to repair has been attributed to different regulatory controls over a group of axonally transported proteins in the central nervous system as opposed to the peripheral nervous system. Specifically, GAP-43 has been implicated because its levels closely parallel normal outgrowth and regenerative capacity (Skene, *Cell 37:*697 (1984)). The present inventors have shown that mature neurons can express GAP-43 at high levels after certain types of stimulation.

Diseases such as transient ischemia and cerebral infarction are of interest because clinical recovery occurs frequently after such lesions, whereas after axotomy the impairment is permanent. Whether neurological recovery derives from repair of injured cells or sprouting of neighboring uninjured ones cannot be distinguished by clinicopathologic correlation. The distance between the neurons expressing high levels of GAP-43 and the area of cell death suggests that sprouting may be involved. Alternatively, the increased GAP-43 observed in these cases may have derived from ischemic effects upon the soma; effects which are less likely to occur during axotomy. For example, these might include the release of excitatory amino acids and consequent N-methyl-D-aspartate (NMDA) receptor excitation (Olney, in: *Experimental and Clinical Neurotoxicology*, P.S. Spencer and H.H. Schaumberg, eds. (Baltimore, MD: Williams and Wilkins), pp. 272-294 (1980)); Rothman, *J. Neurosci. 4:*1884 (1984)).

EXAMPLE III

Detection of GAP-43 Expression Employing Antibody Directed Against Purified GAP-43 Protein

Previous immunohistochemical studies have suggested GAP-43 to be restricted to neurites, especially in the developing nervous system (Benowitz *et al., J. Neurosci. 8:*339-352 (1988); Gispen *et al., Brain Res. 328:*381-385 (1985); Meiri *et al., Proc. Natl. Acad. Sci. USA 83:*3537-3541 (1986); Skene *et al., Science 233:*783-786 (1986)). In the present example, using *in situ* hybridization and a novel antibody to GAP-43 that reveals perikaryal staining, the inventors identify the cell populations that express GAP-43, and demonstrate that in the adult brain the widespread GAP-43-immunoreactive neurites emanate from a relatively small population of neurons, most of which are regionally restricted.

METHODS

In situ hybridization

Tissues used for *in situ* hybridization and immunocytochemistry were obtained fresh and snap frozen in 2-methyl butane cooled with dry ice. Cryostat sections were fixed with the appropriate fixative immediately prior to use. Brain and spinal cord from embryonic (E) (days 12, 15, 18, and 20), postnatally developing (P) (days 1, 7 and 14), and adult rats were studied simultaneously. The tissue was fixed in 4% paraformaldehyde, treated with 0.3% Triton X-100 followed by 1 mg/ml proteinase K, acetylated, and pre-hybridized in 50% formamide/2x standard saline citrate (SSC). The probe was 1121 bases of GAP-43 antisense RNA, as described above. Hybridization using 2 x 106 cpm per slide of ^{35}S-labeled antisense or sense riboprobe was performed in a humidified chamber for 5 hours at 50°C. The tissue sections were then washed in 2x SSC with 10 mM dithiothreitol initially containing 50% formamide, then 50% formamide plus 0.1% Triton-X 100. Single stranded RNA was removed by treatment with 50 mg/ml RNAase A. The sections were further washed in 2x SSC with 1 mM DTT for 2 hours, then dehydrated through graded alcohols containing 0.3 M ammonium acetate. The radioactive signal was detected using NTB2 Kodak emulsion. Emulsion coated slides were counterstained with hematoxylin and eosin.

Antibody generation, characterization, and immunohistochemical demonstration of GAP-43

Tissue sections adjacent to those used for *in situ* hybridization were used for immunohistochemistry. Polyclonal antiserum was raised in rabbits injected with chimeric GAP-43-ß-galactosidase fusion protein generated in lgt11 (Karns *et al., Science 236:*597-600 (1987); Young *et al., Proc. Natl. Acad. Sci. USA 80:*1194-1198 (1983)). Crude serum was processed by

ammonium sulfate fractionation and DEAE cellulose chromatography (Horowitz *et al., In: Fundamental Techniques in Virology*, pp. 297-315). The antibody was assayed by Western blot analysis (Meiri *et al., Proc. Natl. Acad. Sci. USA 83*:3537-3541 (1986)) using growth cone membrane particles prepared from neonatal rat brain (Pfenninger *et al., Cell. 35*:578-584 (1983)). The Western blots were developed using an alkaline phosphatase/BCIP/NBT kit (Promega). Specificity of antibody binding in the Western blot assay was demonstrated by preabsorption with GAP-43 protein purified from neonatal rat brain (Chan *et al., J. Neurosci. 6*:3618-3627 (1986)). GAP-43 protein was demonstrated in CNS tissue by immunohistochemical staining using the avidin-biotin horseradish peroxidase complex method (Hsu *et al., J. Histochem. Cytochem. 29*:577-580 (1981)) with 3-3′ diaminobenzidine as the chromagen and hematoxylin as a counterstain. Specificity of labeling was confirmed by pre-incubation of the primary antibody with native GAP-43 protein purified from neonatal rat brain.

RESULTS

GAP-43 expression during development assessed by in situ hybridization

At embryonic days 12 and 15, GAP-43 mRNA expression in the CNS was low, but neurons of the dorsal root ganglia exhibited intense labeling, corresponding with their peak period of axonal growth. At E20, GAP-43 levels were uniformly and strikingly high throughout the brain. During the first week of postnatal life, high-level expression persisted, but in contrast to the diffuse labeling observed at E20 and P1, in brains of P7 rats, discrete labeling over individual neurons could be appreciated due to expansion of the neuropil and growth of glial elements. As best as could be determined, all neurons were labeled at this age. At P14, GAP-43 mRNA expression was

diminished such that, the overall signal intensity was lower, and only 50-75% of cortical neurons were labeled.

In the adult CNS, GAP-43 was expressed in relatively few neurons such that throughout most of the cerebral cortex, only scattered cells were labeled, and the intensity of labeling was markedly reduced even compared to P14 brains. The entorhinal cortex, however, had moderately high densities of GAP-43-expressing neurons. While dense focal labeling of neurons was still present in the spinal cord, brainstem, and cerebellum of P14 rats, in adults GAP-43-expressing neurons were either absent, or present in very low densities in these regions. However, in the adult, intense labeling of most neurons persisted in two areas: the hippocampus and olfactory bulb. The pattern of labeling in the hippocampus indicated that neurons throughout the dentate gyrus, CA1, and CA3 expressed GAP-43 mRNA. In the olfactory bulb, it was primarily the mitral cell region that contained high levels of GAP-43 mRNA.

Visualization of GAP-43 expression by immunohistochemistry

The rabbit polyclonal antibody to chimeric GAP-43-ß-galactosidase fusion protein generated in lgt11 specifically labeled GAP-43 on Western blots of neonatal rat brain. Specific immunostaining for GAP-43 antigen was detected in neurons but not in glial cells. Throughout development, immunoreactive GAP-43 was present in both perikarya and neurites. Neurite-restricted immunoreactive GAP-43 has been observed previously (Benowitz *et al., J. Neurosci. 8*:339-352 (1988); Gispen *et al., Brain Res. 328*:381-385 (1985); Skene *et al., Science 233*:783-786 (1986)). Cellular fractionation studies suggest that GAP-43 is also in cell bodies (Alexander *et al., J. Biol. Chem. 262*:6108-6113 (1987)).

Immunostaining with this antibody permitted localization of GAP-43-expressing cells, and comparison with the *in situ* hybridization data. The

regional distribution and density of neurons containing immunoreactive GAP-43 mirrored the developmental pattern observed for its mRNA by *in situ* hybridization. GAP-43 immunolabeling was not detected in E12 embryos, and was present in only small amounts (manifested by faint immunohistochemical staining) in the E15 CNS. At E18, GAP-43 immunoreactivity was more conspicuous, and at E20 it was detected at high levels in both somata and neurites of neural cells. This degree of immunostaining for GAP-43 protein persisted through P7. Subsequently, GAP-43 immunoreactivity diminished in most areas, thereby leaving a more restricted distribution of GAP-43 immunoreactivity. In adults, widespread but faint neuritic labeling was evident throughout the CNS. Neuronal perikarya were labeled heavily in the same regions identified by *in situ* hybridization as expressing high levels of GAP-43.

For example, intense labeling was evident in the hippocampus, in both pyramidal and granule cells throughout CAl, CA4, and the dentate. No labeling occurred when the antibody was preabsorbed with gel-purified GAP-43, isolated as described herein. ß-galactosidase preabsorption did not affect the labeling. There were no or few immunolabeled cells in the cerebellum, brainstem, and spinal cord, low densities of immunolabeled cells in the frontal cortex, somatosensory cortex, visual cortex, and basal ganglia, and moderately high densities in the entorhinal cortex. As noted for the *in situ* hybridization, intense perikaryal staining for immunoreactive GAP-43 persisted most notably in two regions: the hippocampus and olfactory bulb. Both pyramidal and granular cells throughout CA1, CA3 and the dentate gyrus were labeled. In the olfactory bulb, labeling was largely restricted to the mitral cells and to neurites among the granule cells. Thus the pattern of GAP-43 immunoreactivity mirrored that of the *in situ* hybridization.

DISCUSSION

The present example demonstrates that GAP-43 is expressed in all CNS neurons during the perinatal period. As development proceeds, its anatomical distribution becomes progressively restricted, such that, in the adult, GAP-43-containing neurons are inhomogeneously distributed, with the highest level expression largely limited to two discrete regions: the hippocampus and olfactory bulb.

A recent report by Rosenthal *et al., EMBO J. 6:*3641-3646 (1987) also notes inhomogeneous GAP-43 labeling, but somewhat different from that reported here was their finding of higher levels in the cerebellum and frontal cortex, and lack of labeling in the dentate. In previous reports, immunoreactive GAP-43 was detected exclusively in neurites without indication of its cellular origin (Benowitz *et al., J. Neurosci. 8:*339-352 (1988); Gispen *et al., Brain Res. 328:*381-385 (1985); Skene *et al., Science 233:*783-786 (1986)).

The antibody generated by the present inventors to the ß-galactosidase-GAP-43 fusion protein permitted intense labeling of neuronal perikarya. This difference from prior reports may be due to the chimeric nature of the antigen, which perhaps exposes some different epitopes to different degrees. Alternatively, the difference may reflect omission of aldehyde fixation, which was noted by the presnet inventors to diminish perikaryal labeling.

In any case, the procedure of the present invention allowed the present inventors to document that the site of GAP-43 gene expression mirrored that of the GAP-43 immunoreactivity. The distribution of GAP-43 in the CNS differs between rats and humans. In the adult human brain, high levels persist in associative cortical regions more than in the hippocampus (Neve *et al., Proc. Natl. Acad. Sci. USA 85:*3638-3642 (1988)), whereas in adult rats the highest levels of GAP-43 expression are in the hippocampus, olfactory bulb, and entorhinal cortex. The significance of this finding is unclear, but it may

be related to species differences with respect to regional retention of neuronal plasticity. It remains to be determined whether the subsets of CNS neurons which persistently express high levels of GAP-43 in the adult share biological features.

One possibility is that GAP-43 is expressed in cells involved in structural remodeling of synapses. Growth cones persist in certain regions of the adult brain (Sotelo *et al., Lab. Invest. 25:*653-671 (1971)) and direct visualization reveals ongoing synaptic rearrangements of single cells, at least in the peripheral nervous system (Purves *et al., Nature 315:*404-406 (1985)). In fact, there is evidence that such neuronal remodeling is integral to long-term learning (Chang *et al., Brain Res. 309:*35-46 (1984); Goelet *et al., Nature 322:*419-422 (1986); Horn *et al., J. Neurosci. 5:*3161-3168 (1985)) and sexually dimorphic behavior (Kurz *et al., Science 232:*395-398 (1986)).

The complement of proteins in growth cones and synaptosomes are not qualitatively very different (Ellis *et al., J. Neurosci. 5:*1393-1401 (1985); Katz *et al., J. Neurosci. 5:*1402-1411 (1985); Sonderegger *et al., Science 221:*1294-1297 (1983)) and the growth cone bears markers of its synapses-to-be (Hume *et al., Nature 305:*632-634 (1983); Sun *et al., Proc. Natl. Acad. Sci. USA. 84:*2540-2544 (1987)). Mature neurons regulate their architecture in part by changing the constituency of molecules transported to their processes (Grafstein *et al., Physiol. Rev. 60:*1167-1283 (1980); McQuarrie *et al., J. Neurosci. 6:*1593-1605 (1986)). Such changes may be mediated locally (Lasek *et al., In: Cell Motility*, RD Goldman, T Pollard, J Rosenbaum, Eds., Cold Spring Harbor Conferences on Cell Proliferation Series.: Vol 3, Cold Spring Harbor, N.Y., p. 1021-1049 (1976)) and at the level of gene expression, for example, as shown for the tubulins (Miller *et al., J. Cell. Biol. 105:*3065-3073 (1987)) and as shown by the present inventors for GAP-43. The characteristics of adult CNS neurons that manifest plasticity are not known, but given the analogies between growth of neurites during

development and remodeling of synapses in the mature nervous system, at a molecular level a need for growth-related proteins is not unreasonable.

Although there is no independent marker to confirm the linkage of GAP-43 to plasticity, there is evidence that some of the neurons which express high levels of GAP-43 in the adult are capable of synaptic remodeling. Thus, GAP-43 expression is high in both the olfactory nerve and its target, the mitral cells of the olfactory bulb. Since olfactory neurons continue a cycle of death and replacement throughout life (Graziadei *et al., In: M. Jacobson (Ed.), Handbook of Sensory Physiology, Vol IX, Development of Sensory Systems, Berlin, Springer-Verlag* pp. 55-83 (1978)), these synapses must be continuously changing. Entorhinal neurons, which express GAP-43 in the adult, can expand their peripheral fields by sprouting into denervated zones, although it is not clear that they remodel in the absence of injury. Finally, circuitry of the hippocampus is functionally plastic (Benowitz *et al., J. Neurosci. 8:*339-352 (1988); Cotman *et al., Psychol. 33:*371-401 (1982); Lee *et al.,* In: *Electrophysiology of Isolated Mammalian CNS Preparations*, G.A. Kerkut and H.V. Wheal (Eds), Academic, New York, 1981, pp. 189-212; Lee *et al., J. Neurophysiol. 41:*247-258 (1980)) and morphological analysis has confirmed changes in the number and shape of synapses accompanying long-term potentiation (Chang *et al., Brain Res. 309:*35-46 (1984)). Thus, the restricted localization of GAP-43 in the adult CNS would be compatible with the notion that GAP-43-expressing neurons are those actively engaged in nerve terminal remodeling.

EXAMPLE IV

Dual Regulation of GAP-43 Gene Expression by Nerve Growth Factor and Glucocorticoids

In many instances, the phenotype of an individual neuron depends upon its microenvironment. Such "plasticity" is manifest, for example, in the choice of cell fate by precursor cells of the sympathoadrenal system, which assume a neuronal phenotype under the influence of nerve growth factor (NGF) or an endocrine, chromaffin cell phenotype in the presence of corticosteroids. Neurons additionally remodel their connections, a phenomenon termed synaptic plasticity, during normal development and in response to synaptic use (Easter *et al., Science 230:*507-511 (1985)).

One unifying theme for these two types of plasticity is a structural remodeling, dramatic in establishment of the original and ornate neuronal shape and more subtle in the rearrangement of connections. One notion is that the expression of a set of genes is responsible for neuronal plasticity. Regulation of these genes by the microenvironment would then mediate structural changes.

Corticosteroids are necessary for normal development of the mammalian nervous system, influencing cell fate and neuronal structure and integrity (Doupe et al., *J. Neurosci. 5:*2119-2142 (1985); Doupe *et al., J. Neurosci. 5:*2143-2160 (1985); Anderson *et al., Cell 47:*1079-1090 (1986); Bohn *et al., Dev. Neurosci. 1:*250-266 (1978); Scheff *et al., Expt. Neurology 68:*195-201 (1980); Scheff *et al., Expt. Neurology 76:*644-654 (1982); Sapolsky *et al., J. Neurosci. 5:*1222-1227 (1985)). In culture, cells of neural crest lineage, including small intensely fluorescent (SIF) cells and adrenal medullary cells, may exhibit either neuronal or chromaffin phenotypes. Corticosteroids cause them to assume chromaffin characteristics. In the

absence of corticosteroids, the presence of NGF causes them to develop neuronal properties (Doupe *et al., J. Neurosci. 5:*2119-2142 (1985)).

Cells of the clonal line PC12, which is derived from a rat adrenal medullary pheochromocytoma (Greene *et al., Proc. Nat'l Acad. Sci. USA 73:*2424-2428 (1986); Greene *et al.*, in *Advances in Cellular Neurobiology*, Vol. 3, pp. 373-413, Academic Press, New York (1982)), display a similar bipotential fate, becoming more neuronal with NGF and retaining chromaffin characteristics with exposure to corticosteroids.

Surprisingly, the present investigation of GAP-43 expression has revealed that corticosteroids are powerful negative regulators of GAP-43 gene expression in both PC12 cells and cultured sympathetic neurons. Further, it has been discovered that corticosteroids inhibit the stimulatory effect of NGF on GAP-43 expression.

EXPERIMENTAL PROCEDURES

Materials

Enzymes were purchased from Boehringer Mannheim, New England Biolabs, or Bethesda Research Laboratories, and used as specified by the supplier. Tissue culture products were bought from Gibco. Radiochemicals were purchased from New England Nuclear-Du Pont. Agarose and cesium chloride were purchased from Bethesda Research Laboratories. Timed pregnant Sprague-Dawley rats were purchased from Charles River Rat. Steroids were bought from Sigma and NGF from Collaborative Research (2.5s form). All other chemicals were of the highest grade available.

Cell Culture

PC12 cells were grown in Dulbecco's modified Eagles medium (DMEM) with 5% heat-inactivated horse serum and 10% fetal calf serum. Cells were used routinely when at approximately 20% confluence. Cortisol levels, determined by RIA, were 3 nM or less in the serum-containing medium. Cells were grown in a humidified incubator with 5% carbon dioxide at 37°C.

Dissociated neurons from embryonic day 20 rat superior cervical ganglia were cultured in Ham's F12 medium supplemented with NGF (50 ng/ml), 0.6% glucose and 10% fetal calf serum. For steroid experiments, the compounds were usually dissolved in 95% ethanol. Controls performed with ethanol as a vehicle revealed no change in the abundance of GAP-43 or GAPDH RNA.

RNA Blotting

Total RNA was prepared from cultured cells by the guanidine isothiocyanate method (Chirgwin *et al., Biochemistry 18:*5294-5299 (1979)). Twenty micrograms of total RNA from each culture were electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde, transferred by capillarity to Nytran (Schleicher and Schuell), and the nucleic acid immobilized by heat fixation. Prehybridization was done for at least 1 hour in a hybridization solution containing 50% formamide, 5xSSC (1xSSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 1x Denhardt's solution, 1% sodium dodecylsulfate (SDS) and 100 mg/ml denatured salmon sperm DNA at 42°C.

Hybridization was performed for 10-12 hours at 42°C in the same solution, containing 1x105 cpm/ml of ^{32}P-labeled DNA probe prepared by random hexanucleotide priming using the Klenow fragment of DNA polymerase I (Feinberg *et al., Anal. Biochem. 132:*6-12 (1984)). The probes

were made from cloned cDNAs for GAP-43 as described herein or for GAPDH (Piechaczyk *et al., Nucl. Acids Res. 12:*6951-6963 (1984)). Blots were washed with 2xSSC at 65°C twice, each for twenty minutes, and 0.2xSSC for an additional twenty minutes at 65°C. Autoradiography was performed with intensifying screens at -70°C. Blots were stripped of hybridized probe at 80°C for 2 hours in a solution containing 1x Denhardt's solution, 1% SDS, 50 mM tris, pH 7.4, and 0.05% sodium pyrophosphate.

Scanning laser densitometry was performed on an LKB ultra scan. Several exposures of a given blot were scanned and the image intensity plotted versus time. Measurements of image intensity were taken from the linear portion of the curve. Careful prior assessment of GAPDH RNA levels showed them not to change under any of these experimental conditions.

Nuclear Run-On Assay

PC12 cultures were split 48-60 hours prior to each experiment. Cells were either left untreated or treated with either NGF (50 ng/ml) or dexamethasone (1 mM) for 6 hours. Approximately ten million nuclei were prepared from each by the method of Greenberg (Greenberg *et al., J. Biol. Chem. 260:*14101-14110 (1985)) with the following modifications. Lysis was in 10 mM sodium chloride, 10 mM Tris, pH 7.4, 3 mM calcium chloride and 200 units/ml RNAsin (Promega Biotec). Nuclei were resuspended after washing with lysis buffer in 50 mM Tris, pH 8.3, 40% glycerol, 5 mM magnesium chloride, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 2mM dithiothreitol and 200 units/ml RNAsin. Nuclei were counted and stored in liquid nitrogen at a concentration of 50 million/ml.

Labeling of nascent chains in thawed nuclei was performed by adding to the suspended nuclei an equal volume of buffer containing 10 mM Tris, pH 8.0, 5 mM magnesium chloride, 0.3 M potassium chloride, and 10 mM each of adenosine, cytidine and guanosine nucleotide triphosphates. Three hundred

mCi of (^{32}P) uridine triphosphate were then added (3000 Ci/mmol), and the nuclei labeled for 30 minutes at 30°C. Nuclei were then digested with 100 units RQ1 DNAase (Promega Biotec) added in 600 ml of a buffer containing 60 mM Tris, pH 7.5, 15 mM sodium chloride, 10 mM magnesium chloride, and 200 units/ml RNAsin for 45 minutes at 37 °C. The labeled RNA was then digested with proteinase K (Boehringer Mannheim) as described (Greenberg *et al., J. Biol. Chem. 260:*14101-14110 (1985)).

After several rounds of phenol, chloroform-isoamyl alcohol extraction, the nucleic acids were ethanol-precipitated in the presence of sodium acetate. The recovered labeled nucleic acid, which still contained DNA, was subjected to another cycle of RQ1 DNAase (50 units enzyme in 250 ml buffer containing 50 mM Tris, pH 7.5, 10 mM sodium chloride, 7.5 mM magnesium chloride and 200 units RNAsin/ml) for 45 minutes at 37°C, and then proteinase K (by adding 100 ml of buffer containing 5% SDS, 0.5M Tris, pH 7.4, 125 mM EDTA and 0.2 mg/ml proteinase K) for 30 minutes at 42°C. After several cycles of phenol, chloroform-isoamyl alcohol extraction, the labeled RNA was subjected to three cycles of ethanol precipitation with ammonium acetate to remove unincorporated nucleotide triphosphates.

Plasmids containing cloned cDNAs for tyrosine hydroxylase (Lewis *et al., J. Biol. Chem. 285:*14632-14637 (1983)), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pBR322, and an 8 kilobase genomic fragment of the GAP-43 gene, were linearized with appropriate restriction enzymes, phenol extracted, ethanol precipitated and recovered by centrifugation. DNA (250 mg/ml) was denatured by alkali (0.5 N sodium hydroxide) and neutralized by the addition of ten volumes of 1 M ammonium acetate. Nitrocellulose filter circles were loaded with 50 mg of DNA by gravity filtration. Prehybridization of the filters was done in a buffer containing 25 mM sodium PIPES, pH 7.2, 50% formamide, 0.75 M sodium chloride, 2.5 mM EDTA and 100 mg/ml of tRNA at 45°C for 10-12 hours.

Hybridization was performed in the same buffer containing labeled RNA at specific activities ranging from 1-3 x 106 cpm/ml for 4 days at 45°C. Washing and RNAase treatment were performed as described (Greenberg *et al., J. Biol. Chem. 260:*14101-14110 (1985)). Filters done in duplicate were counted after drying in a scintillation counter. Data are expressed as parts per million hybridized after subtracting background from vector containing filters.

RESULTS

Dual regulation of GAP-43 expression

The effects of NGF and glucocorticoids upon GAP-43 mRNA accumulation were measured by RNA blotting. NGF addition resulted in a marked increase over the basal level, whereas dexamethasone caused a prominent diminution in GAP-43 mRNA levels. Quantitation of GAP-43 RNA, as determined by densitometry and corrected for RNA loading, revealed that NGF caused a 3.5 fold increase, while dexamethasone lead to a 5.5 fold decrease. Accumulation of GAP-43 mRNA in the presence of NGF was persistent, unlike that of c-fos (Greenberg *et al., J. Biol. Chem. 260:*14101-14110 (1985)), which peaks within several hours and then rapidly declines despite the continued presence of NGF.

To test the specificity of the steroid effect on accumulated GAP-43 mRNA levels, different steroids of several structural classes were examined over a range of concentrations. Each class of steroid has been shown to selectively affect different types of neurons *in vivo* (McEwen *et al., Physiological Rev. 66:*1121-1188 (1986)).

In one experiment, steroid concentration was 1 mM for 48 hours of treatment. The quantitation was derived by densitometry and normalized for slight variations in RNA input. Estradiol, testosterone, and pregnenolone had no effect on accumulated GAP-43 mRNA levels. Dexamethasone, cortico-

sterone, aldosterone and progesterone reduced the levels of GAP-43 RNA to 6%, 15%, 10% and 15% of control (defining the NGF-stimulated level of GAP-43 RNA as 100%), respectively. These data suggest activation of either the mineralocorticoid or glucocorticoid receptors, although the progesterone effect may be mediated by its own receptor (Arriza *et al., Science 237:*268-275 (1987); Giguere *et al., Cell 46:*645-652 (1986)).

Corticosteroids block the NGF induction of a neuronal phenotype in both SIF and adrenal medullary cells (Doupe *et al., J. Neurosci. 5:*2119-2142 (1985); Doupe *et al., J. Neurosci. 5:*2143-2160 (1985)). To investigate the nature of the interaction of the two agents upon GAP-43 expression, PC12 cells were grown for 36 hours in the presence of NGF (50 ng/ml) and dexamethasone (1 mM). The results indicate that dexamethasone prevents the NGF-mediated increase in GAP-43 mRNA.

NGF and steroid effects are direct

The question whether the effects of NGF and corticosteroids are exerted directly or indirectly was addressed by use of the protein synthesis inhibitor cycloheximide. A concentration of 0.5 mg/ml of cycloheximide inhibits more than 94% of (^{3}H) leucine incorporation into protein without an effect on cell viability at 24 hours. Cycloheximide prevents neither the NGF enhancement nor the dexamethasone suppression of GAP-43 gene expression, indicating that neither effect requires de novo protein synthesis. Controls were compared with NGF-treated cells and with cells treated with NGF and 0.5 mg/ml or 2 mg/ml cycloheximide. Larger sized transcripts were noted after treatment with 0.5 mg/ml cycloheximide, which may represent unspliced precursors. Dexamethasone suppression of GAP-43 expression was more pronounced with cycloheximide and dexamethasone than with dexamethasone alone.

These experiments were repeated with the more potent protein synthesis inhibitor anisomycin at a concentration of 0.1 mM for 6 hours, an exposure period used because cell death occurred by 12 hours. Anisomycin did not affect the NGF-mediated increase in GAP-43 mRNA. Dexamethasone suppression was slower than NGF induction, and not discernible by 6 hours, so the effect could not be assayed for anisomycin sensitivity. Pre-treatment with NGF did not prevent direct steroid repression.

In separate experiments, the time required to achieve the fully suppressed steady state levels of GAP-43 mRNA was found to be longer when the steroid was added after NGF pre-treatment. Cycloheximide was shown to have no effect on basal GAP-43 mRNA levels.

Steroid repression is transcriptional

To assess the level of regulation involved in both the NGF and steroid effect, nuclear run-on experiments were performed. Nuclei were prepared from PC12 cells treated with 50 ng/ml NGF or 1 mM dexamethasone for 6 hours. The labeled RNA from each group was hybridized to nitrocellulose filters containing immobilized DNAs. After hybridization and washing, the specific radioactivity for each filter was calculated and the data expressed as counts hybridized in parts per million. Dexamethasone decreased the rate of transcription of GAP-43 approximately 4.5 fold, whereas NGF had no appreciable effect on the basal rate of transcription. By comparison, tyrosine hydroxylase transcription increased with dexamethasone and that of GAPDH did not change.

In a series of experiments to define the GAP-43 transcription unit, runoff-labeled RNA prepared from newborn brain nuclei was hybridized to a series of contiguous single strand M13 clones spanning the 5′ flanking region through the beginning of the first intron. The results indicated that

transcription is from the coding strand only and not from the flanking segment.

Corticosteroids suppress GAP-43 in sympathetic neurons

Although NGF-treated PC12 cells are considered good models of differentiated neurons, it was desired to determine whether the effects of corticosteroids might be exerted upon primary neurons after they had achieved their fully differentiated state. To do so, dissociated neurons of the rat superior cervical ganglion were cultured, to which 1 mM dexamethasone was added for 48 hours. Total RNA was prepared, fractionated, blotted and probed as before. Dexamethasone reduced the expression of GAP-43 RNA in sympathetic neurons. The morphological appearance of the neurons in the dexamethasone-treated cultures was not different than that of the untreated cells. This suggests that neurite extension or maintenance over the short term may not depend upon the persistence of GAP-43 mRNA, but that long-term effects require evaluation since the GAP-43 gene product may have a long half-life.

DISCUSSION

In this example it is demonstrated that GAP-43 gene expression is subject to both positive and negative control: positive by NGF and negative by glucocorticoids. Both effects are direct, neither requiring new protein synthesis. Interestingly and surprisingly, cycloheximide was shown to further augment the dexamethasone suppression of GAP-43 mRNA. While not intending to be bound by a particular theory, this may be due to inhibition of synthesis on an mRNA-stabilizing protein.

In vivo, the GAP-43 gene is highly regulated. To-date it has been reported only in neurons. The present inventors, however, have obtained data

which support low level expression in other cells that derive from the neural crest. Peak levels in the animal are achieved at the time of neurite growth, relating either to normal development or to regeneration. The molecular regulators of its cell-specific and growth-related expression have not yet been elucidated. Nerve growth factor directly increases expression of several genes, such as c-fos, NGFIA, NGFIB, beta actin and a cloned cDNA related to intermediate filaments (Greenberg *et al., J. Biol. Chem. 260:*14101-14110 (1985); Milbrandt, *Science 238:*797-799 (1987); Milbrandt, *Neuron 1:*183-188 (1988); Leonard *et al., J. Cell. Biol. 106:*181-193 (1988)).

The present data indicate that there are several notable differences between GAP-43 regulation and that of these other genes. For some, such as c-fos and NGFIA and NGFIB, NGF induction is rapid, exerted within minutes, and declining after several hours. This is in contrast to the NGF effect on GAP-43 expression, which is slower in onset and persistent.

Additionally, a wide range of stimuli can cause an increase in c-fos, including calcium entry, other growth factors and serum withdrawal and repletion, and these effects are seen in a variety of cell types (Greenberg and Ziff, *Nature 311:*433-438 (1984)). Genes such as c-fos have been likened to the immediate-early genes of DNA viruses (Goelet *et al., Nature 322:*419-422 (1986)), some of which themselves encode transcriptional regulators that reprogram the cellular machinery to a dedicated function.

The delayed response of GAP-43 to NGF suggests that it may fall into a different class of NGF-regulated genes than do c-fos, NGFIA, NGFIB, etc., and may play a role in longer-term adaptation rather than in immediate responses. Unlike these other genes, although NGF does cause a large increase in GAP-43 mRNA accumulation, its effect upon transcription rate is negligible. It is therefore likely that its action is mediated through a post-transcriptional mechanism.

The effects of corticosteroids upon the nervous system are widespread (for review see McEwen *et al., Physiological Rev. 66:*1121-1188 (1986)). Since steroids act through their receptors as transcriptional regulators, it is important to determine which neuronal genes are regulated by corticosteroids. In considering cells of neural crest lineage, it is of particular interest to determine whether the antagonistic effects of corticosteroids and NGF on cell phenotype are mirrored at the level of gene expression, i.e., whether the same gene may be bimodally regulated by the two agents.

The present inventors have shown that GAP-43 transcription is suppressed by corticosteroids, and that the concomitant presence of NGF does not prevent this suppression. This is similar to the glucocorticoid inhibitory effect on NGF-mediated neuronal differentiation of cultured chromaffin cells (Doupe *et al., J. Neurosci. 5:*2119-2142 (1985)). Thus, GAP-43 is dually regulated by NGF and corticosteroids in a manner at least compatible with the known divergent effects of these modulators of cell fate. It is of interest that another neural-specific gene, designated SCG 10, is bimodally regulated in PC12 cells (Stein *et al., Develop. Biol. 127:*316-325 (1988)). Like GAP-43, SCG 10 gene expression is stimulated by NGF and repressed by glucocorticoids, although the levels of control differ somewhat between the two genes.

Corticosteroids do not noticeably affect PC12 cell shape. Since GAP-43 is suppressed, it is clear that normal levels of mRNA are not needed for neurite extension in PC12 cells. However, it is not clear that these results may be interpreted to mean that GAP-43 is unnecessary for neurite growth. First, low levels of GAP-43 RNA are still present after steroid-suppression. Second, the protein may have a long cellular half-life. Additionally, PC12 cells are transformed and likely subject to different structural constraints from those exerted upon their *in vivo* counterparts.

EXAMPLE V

The Neuronal Growth-Associated Protein GAP-43 Imparts Growth Cone-Like Morphology to Non-Neuronal Cells

In the present example, the inventors set out to test the hypothesis that GAP-43 might contribute directly to the establishment of the unique neuronal phenotype, perhaps at the level of the cytostructure. Thus, in yet another aspect of the invention, expression vectors encoding rat GAP-43 are introduced into several types of non-neuronal cells.

Expression vectors were constructed using rat GAP-43 cDNA (Karns, *et al., Science 236:*597 (1987)) inserted into plasmids containing the SV40 origin of replication under the control of the adenovirus major late promoter, the SV40 early promoter, or the cytomegalovirus promoter. The results were similar using all of these vectors.

COS 7 cells (Gluzman, *Cell 23:*175 (1981)) were transfected as described (Zuber, *et al., Science 234:*1258 (1986)) and examined for GAP-43 immunoreactivity using rabbit anti-GAP antibody as described above. Control transfections were done identically using a similar vector expressing the T-cell-specific membrane protein CD8 (Seed, *et al., Proc. Natl. Acad. Sci. USA 84:*3365 (1987)). Cells were examined 1 hour after plating.

Control COS cells were essentially round after immunofluorescent labeling of the CD8-transfected cells with antibody to CD8. In GAP-43 transfected cells, GAP-43 immunoreactivity was prominent in about 5-20% of the cells, depending upon transfection efficiency. Some GAP-43 appeared to be membrane-associated, an observation confirmed by Western blot analysis comparing cytosolic and membrane fractions.

Cells that expressed high levels of GAP-43 had a distinctive structure with many cells extending processes from their cell perimeter. To ensure that this was not due to better visualization of the cell surface by antibody to

GAP-43, the surface of all cells was labeled by rhodaminated wheat germ agglutinin. CD8-transfected cells, mock transfected cells, or cells in the same GAP-43 transfected dish that did not express GAP-43, did not have these extensive processes, although shorter or single processes did occur. Long, thin processes appeared to be associated only with high level GAP-43 expression. A similar association of process outgrowth with high level GAP-43 expression was found when WOP cells, 3T3 cells expressing polyoma T antigen (Valle *et al., Mol. Cell. Biol. 1:*417 (1981)), were transfected with CDM8-GAP, a GAP-43 expression vector that included the polyoma origin of replication. In these transient transfection assays, the efficiency of transfection and level of expression both vary, making difficult the quantitation of the effect.

To overcome the problem of quantitation, a series of clonal, stably transformed CHO cell lines was generated that constitutively expressed GAP-43. Control cell lines 30 minutes after plating were generally round. In GAP-43-expressing lines, GAP-43 expression clearly correlated with process extension. Many cells expressing GAP-43 extended filopodial processes that were narrow and between 20 and 75 mm in length. In both control and cell lines expressing GAP-43, the perimeter often included broad, thin, ruffled lamellipodia.

Clonal cell lines constitutively expressing GAP-43 were established by co-transfection of CDM8-GAP and Neomycin resistance expression plasmids into CHO cells by the Ca-PO4 co-precipitation method and G418 selection (Maniatis, *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). In control transfected cells, the plasmid pCDM8 (Seed, *Nature 329:*840 (1987)) was used instead of CDM8-GAP. After the cells became confluent, they were passaged with trypsin and plated on poly-D-lysine-coated glass coverslips.

Process formation was assessed from four independent lines transfected with a control plasmid and four independent lines expressing the highest amounts of GAP-43, as determined by Western blot. Living cells were examined by the use of Nomarski optics. All CHO cell lines with GAP-43 immunoreactivity had a greater tendency to extend processes than did control cell lines. In addition, cells expressing GAP-43 often had multiple processes (ranging between 6% and 11% of cells) as compared to control cell lines (from 0.5% to 1%), and the process length was longer than in the control cells.

The neuronal protein GAP-43 therefore causes a change in the shape of these non-neuronal cells. Filopodia and lamellipodia extend directly from the cell soma, such that the cell protrusions resemble growth cones.

In non-neuronal cells, GAP-43 is removed from its normal biological context, and is expressed in a deregulated fashion, so the changes observed here may not mimic the effect of GAP-43 in its neuronal context. However, there is evidence that GAP-43 is, in fact, related to growth cone function, in that it is enriched in growth cones (Katz, *et al., J. Neurosci. 5:*1402 (1985); DeGraan, *et al., Neurosci. 61:*235 (1985); Meiri, *et al., Proc. Natl. Acad. Sci. USA 83:*3537 (1986); Skene, *et al., Science 233:*738 (1986)), is at its highest levels in neurons extending axons *in vivo* or *in vitro* (Skene, *et al., J. Cell Biol. 89:*86 (1981); Benowitz, *et al., Neurosci. 1:*300 (1981); Meiri, *et al., J. Neurosci. 8:*2571; Benowitz, *et al., T.I.N.S. 10:*527 (1987)), and increases in PC12 cells with NGF exposure concomitant with neurite growth, as described above.

Although the inventors do not intend to be bound by a particular theory, one interpretation of these data is that GAP-43, a neuron-specific molecule, is able to contribute a completely novel and neuron-like structure to these cells. Another explanation is that GAP-43 interacts with more general

mechanisms that control cell shape (Bray, *et al., Science 239:*883 (1988); Smith, *Science 242:*708 (1988)).

Many cells can extend filopodia or lamellipodia, a tendency that depends upon several factors, including the phase of cell cycle, plating conditions, and levels of second messengers (Allred, *et al., Surfaces of Normal and Malignant Cells* R.O. Hynes, Ed. (John Wiley & Sons, New York, 1979), p. 21). For this reason, cells were assayed under exactly the same strict plating conditions. The cellular mechanisms that permit cells to extend processes are complex, and include components which are likely to be present in all cells. Therefore, the similarity of fibroblast processes to those of growth cones is not surprising. In fact, growth cone structure has been suggested to utilize cellular mechanisms, such as flow of cortical actin and selective adhesion, that may be used as a general means to impart cellular motion (Bray, *et al., Science 239:*883 (1988); Smith, *Science 242:*708 (1988)). How GAP-43 might interact with such machinery remains to be determined.

EXAMPLE VI

Identification of a Novel Membrane-Targeting Peptide

In yet another aspect of the present invention, it has been found that the GAP-43 protein contains a novel membrane-targeting peptide domain which directs the GAP-43 protein to the cell membrane, and especially to the region of the growth cone of neuronal cells. The structure of this membrane-targeting domain has been determined, and it has been shown that the peptide is effective in directing normally cytosolic proteins (which are not normally membrane-associated), to the cell membrane.

According to the compositions and methods of this aspect of the invention, it is possible, *inter alia,* to direct any desired protein to the cell membrane, including proteins which are not normally membrane-associated.

Further, the compositions and methods of this aspect of the invention are of obvious utility in the therapeutic treatment of neurological damage and disorders *in vitro, in vivo,* and *in situ,* in animals.

It is well known that most membrane-associated proteins contain a highly hydrophobic domain which directly intercalates with the cell membrane. Surprisingly, however, it has been discovered that GAP-43, while it is associated with cell membranes, and especially with the growth cones of developing or regenerating neuronal cells, lacks any such highly hydrophobic region.

It has now been discovered that the GAP-43 protein is encoded in three exons, as shown in Figure 2. The short (10 amino acid residues) amino-terminus exon has surprisingly been discovered to encode a membrane-targeting peptide domain. Experiments in which large portions of the second GAP-43 exon were removed did not affect membrane binding of the remaining protein. Similarly, it was found that replacing the carboxy-terminus of GAP-43 had no effect on membrane binding. However, a synthetic GAP-43 gene lacking the initial four amino acids (MET LEU CYS CYS), and beginning at the MET of position five failed to bind to the membranes of neuronal or non-neuronal host cells (see Figure 11), indicating that the first exon is responsible for this membrane-targeting function.

By "membrane-targeting peptide," then, is meant any amino acid sequence as follows:

MET LEU CYS CYS MET ARG ARG THR LYS GLN

or a functional derivative thereof, which, when attached at or near the amino-terminus end of a desired protein or peptide, will effect the direction of said protein or peptide to the cell membrane.

The membrane-targeting peptide of the invention may be attached to a desired protein or peptide by well known methods, including but not limited to direct synthesis by manual or, preferably, automated methods. An alternate preferred method by which the membrane-targeting peptide of the invention may be attached to the desired protein or peptide involves modifying the gene encoding the desired protein or peptide, so that the expressed gene product will include the membrane-targeting peptide at its amino-terminus end. This may be accomplished by well-known methods, including but not limited to blunt-ended or sticky-ended ligation methods as described herein.

Thus, in another aspect, the present invention provides for cDNA coding for a membrane-targeting domain comprising the nucleotide sequence

atg ctg tgc tgt atg aga aga acc aaa cag

or a functional derivative thereof.

Because of the degeneracy of the genetic code, of course, it will be possible to vary the nucleotides while still achieving the desired results. Similarly, those of skill will appreciate that, in certain instances, it may be desirable to alter the nucleotides when expression is contemplated in a particular host, because of preferred codon usage. These and other such modifications are contemplated as within the scope of the present invention.

In order to further elucidate the GAP-43 membrane-targeting domain, non-neuronal cells (including COS cells, NIH 3T3 cells, and CHO cells) and neuronal cells (PC12 cells) were transfected with plasmids containing the GAP-43 gene in which mutations were introduced into the nucleotide sequence of the cysteines at positions three (C3) or four (C4), to result instead in expression of alanine at those positions (Figure 11).

It was found that mutation of either C3 or C4 results in a significant reduction in membrane binding. The most marked effect was seen when C4

was altered. Mutation of both C3 and C4 completely abrogated the phenomenon. The mechanism of this effect is unclear, but is apparently unrelated to simple alterations in oxidation state at those positions, since redox experiments failed to alter membrane binding.

Further, a synthetic GAP-43 gene was constructed which lacked the initial four amino acids (MET LEU CYS CYS), and began at the MET of position five. The expressed protein failed to bind to the membranes of neuronal or non-neuronal host cells. (See Figure 11.) This shows that the first four amino acids are necessary for membrane binding. As discussed below, experiments with normally cytosolic proteins have demonstrated that the ten amino acid peptide is clearly sufficient. Preliminary data indicate that the first four amino acids are also sufficient for membrane binding.

Thus, in another embodiment, the invention comprises an amino acid sequence comprising

MET LEU CYS CYS

or a functional derivative thereof, which sequence may be attached at the amino-terminus end of a desired protein or peptide in order to allow membrane binding of said protein or peptide.

Moreover, peptides including the first five, six, seven, eight or nine amino acids of exon 1 also will allow membrane binding when attached to a desired protein or peptide. Those of skill will appreciate that the sufficiency of these intermediate length peptides for directing membrane binding in particular applications may be determined by the exercise of merely routine skill, with the benefit of the teaching of the present invention. Accordingly, the same and their equivalents are to be considered as within the contemplated scope of the present invention.

In an additional experiment, the first GAP-43 exon described above was ligated at the amino-terminus end of the gene encoding chloramphenicol acetyl transferase (CAT), a protein which is normally cytosolic, and not membrane-associated. Plasmids containing this sequence were used to transfect neuronal and non-neuronal cells (Figure 11). Immunofluorescence assay revealed that the expressed CAT protein was membrane-associated in transfected cells. This demonstrates that the amino acids of the first GAP-43 exon are sufficient to accomplish membrane targeting of a desired protein or peptide.

Moreover, experiments have shown that the first 40 amino acids of GAP-43 will direct CAT to the same location as GAP-43 in transfected PC12 cells. These cells resemble neuronal cells in putting out long processes tipped by growth cones. GAP-43 is normally especially enriched in neuronal cell growth cones, and data suggest that the membrane-targeting peptides of the present invention are responsible for this observed growth cone enrichment.

To further elucidate the selective growth cone accumulation phenomenon described herein, the present inventors employed mutational analysis and laser scanning confocal microscopy of fusion proteins that included regions of GAP-43 and chloramphenicol acetyltransferase (CAT). It has consequently been verified that a short stretch of the GAP-43 amino terminus suffices to direct accumulation in growth cone membranes, especially in the filopodia.

Constructions that encoded varying amounts of the GAP-43 amino terminus fused to a reporter peptide were expressed in COS and PC12 cells. Chloramphenicol acetyl transferase (CAT) was chosen as the reporter peptide because it is cytosolic when expressed in eukaryotic cells and is very stable. Plasmids were constructed that encode fusion proteins of the first 10 amino acids of GAP-43, MLCCMRRTKQ, fused to the amino terminus to the

complete CAT protein (GAP10CAT), or the first 40 amino acids of GAP-43 fused to CAT (GAP40CAT).

Immunoblotting was carried out as follows: Chimeric proteins with the amino terminus of GAP-43 fused to CAT associate with COS cell membranes. CAT, GAP10CAT and GAP40CAT were transiently expressed in COS cells. Immunoblots of membrane (M) and cytosolic (C) fractions from each transfection were prepared using anti-CAT antibody. In the CAT-transfected cells, immunoreactivity is found only in the cytosolic fraction and co-migrates with purified CAT protein. In the GAP40CAT and GAP10CAT cells, nearly all of the immunoreactivity is membrane-associated and migrates more slowly than CAT, as expected for fusion proteins with Mr 4000 or 1000 greater than CAT. Molecular weight standards of 116, 84, 58, 48.5, 36.5 and 26.6 kilodaltons were used.

Membrane association of GAP and GAP40CAT was evaluated in PC12 cells. Stably transfected PC12 cells expressing CAT, GAP40CAT, or GAP were selected as described herein. Immunoblots of membrane (M) and cytosolic (C) fractions were stained with anti-CAT or anti-GAP antibodies. CAT-transfected cells (CAT) contained immunoreactivity in the cytosolic, but not in the membrane fraction, and this immunoreactive CAT co-migrated with purified CAT. In contrast, GAP40CAT transfected cells (G40CAT) contained membrane-associated CAT immunoreactivity which migrated more slowly. Fractions from rat brain (BR) demonstrated that most, but not all, endogenous GAP-43 immunoreactivity is membrane-associated. In transfected PC12 cells over-expressing GAP-43, nearly all of the GAP-immunoreactivity is membrane-associated and co-migrates with purified GAP-43.

In the GAP-43 expression plasmid, pGAP, the GAP-43 coding sequence replaced the stuffer at the Xba I sites of the CDM8 plasmid described by Seed, *Nature 329*:840-846 (1987). The inserted GAP-43 sequence included the entire coding sequence of rat GAP-43, from the Nla III

site at the start of translation to the Sau 3AI site 68 bp downstream from the termination codon, as described herein. For the CAT expression plasmid, pCAT, the Hind III to Bam HI fragment containing the CAT coding sequence and polyadenylation site from pSV2CAT (Gorman, *et al.*, *Mol. Cell. Biol.* 2:1044-1051 (1982)) replaced the Hind III to Bam HI fragment of CDM8 containing the stuffer and polyadenylation site.

pGAP40CAT and pGAP10CAT include the first forty or ten amino acids of GAP-43, respectively, fused in-frame with CAT in pCAT by the use of polylinkers. For transient transfection of COS cells, DEAE dextran and chloroquine was used as described (Zuber, *et al. Science 234:*1258-1261 (1986)). For stable transfection of PC12 cells a neomycin resistance plasmid co-transfected with the plasmid of interest on a 1 to 10 ratio was used as described herein. During selection of PC12 cells, 400 ug/ml of active Geneticin (GIBCO) were used. Transient transfection of PC12 cells was performed by electroporation with the Bio-Rad Inc. electroporation system using 300 volts and 960 microfarad. After 8 hour the medium was changed. Twenty-four hours after electroporation the cells were plated on poly-D-lysine-coated coverslips in the presence of 50 ng/ml NGF and analyzed 24 hours later.

For immunochemical assays, rabbit anti-GAP-43 antibodies were made by immunizing rabbits against four peptides including aa 1 to 24, aa 35 to 53, aa 53 to 69, and aa 212 to 228 of rat GAP-43. Anti-GAP-43 antibody was affinity-purified on GAP peptide agarose. Anti-GAP antibody was bound to a resin that contained 10 mg/ml of each peptide coupled to agarose by the cyanogen bromide method and the antibody was eluted at pH 3.5. Rabbit anti-CAT antibodies were obtained from 5 Prime-3 Prime, Inc. Secondary antibodies were obtained from Organon Teknika, Jackson Immunologicals, and Vector labs.

For cell fractionation, COS or PC12 cells were scraped from 100 mm confluent petri dishes and pelleted at 2000 x g for 10 minutes. The pelleted cells were homogenized by Polytron in 10 mM Tris- HCl, lmM EDTA, pH 7.6 (300 ul/dish) and centrifuged at 250,000 x g for 30 minutes at 4°C. The supernatant was collected as the cytosol fraction. The pellet was washed by homogenization and centrifugation in the same buffer, and then resuspended to the same volume as the cytosol fraction. Rat brain was obtained from 1 day old rats and homogenized by Polytron in 10 mM Tris-HCl, 1mM EDTA, pH 7.6 (10 ml/gram wet weight tissue). The cytosol and washed membrane fractions were prepared by centrifugation as described for the cell extracts. GAP-43 protein was purified from rat brain by a modification of the method of Andreasen *et al., (Biochemistry 22*:4615-4618 (1982)) and used as a positive control for immunostaining. The same volume of cytosol or membrane fraction (usually 100 ul) was electrophoresed on polyacrylamide gels (Laemmli, *Nature 227*:680-685 (1970)). Proteins were electrophoretically transferred to nitrocellulose and excess sites were blocked with 4% BSA. Membranes were then incubated for 24 hour at 4°C with 40 ug/ml affinity purified anti-GAP, or a 1:1000 dilution of anti-CAT antibodies. Bound antibody was detected using anti-rabbit Vectastain horseradish peroxidase method according to the manufacturer's instructions. Tetramethyl benzidine (Kirkegaard and Perry, Gaithersburg, MD) was employed as peroxidase substrate.

Immunoblotting revealed that CAT expressed in COS cells or PC12 cells is present only in the cytosolic fraction. By contrast, the chimeric proteins GAP10CAT and GAP40CAT are membrane-associated. The fusion protein is extracted by detergent, but not by sodium chloride, calcium chloride, or EGTA. Thus, the nature of this membrane binding is similar to that of native GAP-43 in rat brain (Perrone-Bizzozero, *et al., J. Neurosci. Res. 20*:346-350 (1988); Oestreicher, *et al., J. Neurochem. 41*:331-340

(1983); Chan, *et al.*, *J. Neurosci.* *6*:3618-3627 (1986); Skene, *et al.*, *J. Cell Biol.* *108*:613-624 (1989)).

The cellular distribution of GAP-43 and the GAP-CAT chimeric proteins in NGF-treated transfectants of PC12 cells was investigated by confocal microscopy in order to determine whether the amino terminus accounts for the growth cone enrichment of GAP-43 in neuronal cells. By this assay, CAT remains cytosolic, whereas GAP-43 is distributed in a punctate pattern with notable enrichment in growth cones, a pattern similar to that of native GAP-43 in neurons. The amino terminus of GAP-43 fused to CAT caused the resulting fusion protein to acquire a distribution that closely resembled that of GAP-43 itself. Perinuclear labeling for both GAP-43 and the chimeric protein was detected at a low level, and may be due to localization to the Golgi, as has been observed for native GAP-43 (Van Hooff *et al., J. Cell Biol.* *108*:1115-1125 (1989)). Glutaraldehyde fixation provided better histologic preservation of the finer processes of the growth cones, and revealed that the chimeric protein accumulates especially within filopodia.

Subcellular localization of CAT, GAP-43 and fusion proteins in transfected PC12 cells was carried out as follows: Confocal immunofluorescence of (A) CAT, (B) GAP-43, (C) GAP40CAT, and (D) GAP10CAT in PC12 cells revealed that CAT labeling is diffuse and cytosolic whereas GAP-43 is localized to the membrane in a punctate fashion with some enrichment in the growth cones. When either the amino terminal 40 amino acids (GAP40CAT) or 10 amino acids (GAP10CAT) were fused to CAT, the immunofluorescent distribution resembled that for GAP-43, including enrichment in growth cones. All cells were treated with NGF for 24 hours prior to fixation. Anti-CAT antibody was used for CAT, GAP40CAT and GAP10CAT, whereas anti-GAP-43 antibody was used for GAP-43. Control PC12 cells of this variant expressed undetectable levels of GAP-43 and CAT immunoreactivity. PC12 cells were transferred to poly-D-lysine coated

coverslips 24 hours before immunofluorescence in the presence of 50 ug/ml nerve growth factor (NGF). Fixed with 3.7% formaldehyde for 7 minutes, and permeabilized with 0.1% Triton-X-100 for 3 minutes. The samples were blocked with 4% BSA in PBS for 1 hour, incubated for 1 hour in primary antibody, rinsed with PBS, incubated in 0.3% H_2O_2 in PBS for 15 minutes (to reduce background), rinsed again and incubated 1 hour in secondary antibody. After washing with PBS several times, coverslips were rinsed with water and mounted with Gelvatol containing 0.4% n-propyl gallate to decrease bleaching. Immunofluorescence was not detectable above background when cells did not contain specific antigens or when the primary or secondary antibodies were omitted.

Localization of GAP40CAT within the growth cone of a PC12 cell was demonstrated using a higher power comparison of PC12 cells expressing GAP40CAT viewed with Nomarski optics and scanning confocal immunofluorescence, labeled with anti-CAT antibodies. Cells had been treated with NGF for seven days. One growth cone appeared brightly labeled, but a smaller one did not. Unequal labeling of different growth cones, even of the same cells, occurs for native GAP-43 in neurons (Goslin *et al.*, *Nature 336*: 672-674 (1988)) as well. Comparison of the Nomarski and immunofluorescent images showed that filopodia were especially labeled. Similar results were seen for GAP10CAT.

For high resolution confocal microscopy, the cells were fixed with freshly made 4% paraformaldehyde and 0.5% glutaraldehyde, which was essential to preserve the fine structure of the filopodia, followed by 0.1% Triton-x-100 for 3 minutes and 10 minutes with 2 mg/ml sodium borohydrate in PBS. Confocal analysis employed a Biorad MRC-500 scanning confocal imaging system and a Zeiss Axioplan microscope.

These experiments confirm the present inventors' surprising discovery that the first 10 amino acids of GAP-43 suffice to direct growth cone

accumulation. The present inventors are not aware of other proteins that have a sequence closely related to the GAP-43 amino terminus, although at least one other non-integral membrane protein that accumulates in growth cone membranes, SCG 10 (Stein, *et al.*, *Neuron 1*:463-476 (1988)) has two cysteines in close proximity (at positions 22 and 24).

In polarized epithelial cells, different proteins accumulate in the apical and basolateral plasma membranes (Matlin, *J. Cell Biol. 103*:2565-2568 (1986); Rodriguez-Boulan, *et al.*, *Proc. Natl. Acad. Sci. USA 75*:5071-5075 (1978); Simmons, *et al.*, *Ann Rev. Cell Biol. 1*:243-288 (1985)) a process believed to depend upon sorting signals within the protein, similar to the signals which direct traffic of membrane and secreted proteins to their particular destinations (Wickner, *et al.*, *Science 230*:400-407 (1985); Verner, *et al.*, *Science 241*:1307-1313 (1988); Pfeffer, *et al.*, *Ann. Rev. Biochem. 56*:829-852 (1987)). In the case of epithelial cells, such signals would also recognize different regions of the plasma membrane as apical or basolateral.

In neurons, the growth cone membrane is also distinctive in its protein make-up. One interesting possibility is that the growth cone membrane has binding sites that recognize and bind the palmitylated amino terminus of GAP-43. While the present inventors do not intend to be bound by any particular theory, it seems less likely that the palmitylated residues interact with the lipid bilayer directly, because that would likely cause a more uniform membrane distribution for GAP-43. Along these lines, the fatty acid moiety of another acylated protein, N-myristylated VP4 of poliovirus, has been shown by X-ray diffraction to interact with specific amino acid residues of other viral proteins and not with the lipid bilayer (Schultz, *et al.*, *Ann. Rev. Cell Biol. 4*:611-647 (1988); Chow, *et al.*, *Nature 327*:482-486 (1987)). Since GAP-43 and GAP-CAT fusion proteins bind to the membrane of non-neuronal cells, similar or identical binding sites must be present in other cell types, but

because GAP-43 is neuron-specific, these sites would presumably be targets for different proteins in non-neuronal cells.

It is notable that the sorting domain of GAP-43 causes enrichment especially in filopodia. This is the normal location of GAP-43 in these cells, as evidenced by electron microscopy (Van Hooff, *et al.*, *J. Cell Biol. 108:*1115-1125 (1989)). Given the observation that transfected GAP-43 enhances the propensity of non-neuronal cells to extend filopodia as described herein, it will be of interest to correlate GAP-43 location with motile activity of particular filopodia.

Thus, the present invention provides, in another aspect, for a method of introducing a desired protein or peptide into the membrane region of a neuronal or non-neuronal cell, and for a method of directing a desired protein or peptide to the growth cone areas of neuronal cells. In one exemplary embodiment is provided a method for directing a desired protein or peptide to the membrane of a cell, comprising

(a) ligating to the amino-terminus of said protein or peptide a membrane-targeting peptide comprising an amino acid sequence selected from the group consisting of

I. MET LEU CYS CYS MET ARG ARG THR LYS GLN;

II. MET LEU CYS CYS MET ARG ARG THR LYS;

III. MET LEU CYS CYS MET ARG ARG THR;

IV. MET LEU CYS CYS MET ARG ARG;

V. MET LEU CYS CYS MET ARG;

VI. MET LEU CYS CYS MET;

VII. MET LEU CYS CYS; and

VIII. functional derivatives thereof; and

(b) introducing the resulting protein or peptide comprising said membrane-targeting domain into a cell;

wherein the resulting protein or peptide of step (b) is directed to said membrane of said cell by said membrane-targeting domain.

In another non-limiting exemplary embodiment, the present invention provides nucleotide sequences encoding the membrane-targeting peptide comprising the above amino acid sequences or their functional or chemical derivatives, as well as the addition of these sequences by well known methods to nucleotide sequences encoding proteins or peptides other than GAP-43 (as well as GAP-43 itself), and the expression of the resulting sequences in prokaryotic or eukaryotic hosts by methods well known to those of skill.

As described herein, of course, the desired protein or peptide may be diagnostically or therapeutically labeled, and the utility of the composition and methods of this aspect of the invention will be apparent to those of skill, and may be readily utilized for *in vitro, in vivo,* or *in situ* diagnostic or therapeutic purposes in animals including humans with the exercise of merely routine skill.

EXAMPLE VII

Cloning of the Entire Rat Genomic DNA of GAP-43 and Identification of a Regulatory Site

The work of the present inventors as described herein strongly suggests that GAP-43 regulation occurs at the level of gene expression. Until the present time, however, nothing has been known about cis or trans-acting elements that might regulate its expression. Naturally, it would be of great interest to define elements of the GAP-43 gene that confer its responsiveness to growth factors, cause cellular restriction of expression, and regulate the gene during development of the nervous system. In order to identify regulatory elements, the entire rat genomic DNA of GAP-43 has been cloned.

Accordingly, genomic GAP-43 has been isolated, and its intron-exon boundaries and transcriptional start sites have been mapped. It has surprisingly been discovered that the promoter is quite unusual in its structure, containing a repetitive sequence capable of forming unusual conformations, and lacking some canonical promoter components. Transcription can initiate from more than one site, and some of the start sites are utilized differently in the central and peripheral nervous systems.

Further, the inventors have investigated whether the GAP-43 promoter contains regions recognized by brain-specific nuclear proteins. Regions of the GAP promoter have been examined by gel electrophoresis mobility shifts, and a domain which binds protein(s) present in brain but not in liver nuclear extracts has been identified. The binding activity diminishes with brain maturation. The binding site is limited to a stretch of about 20 nucleotides, which also is specifically protected in DNase protection assays by brain nuclear extracts and not by liver extracts. The region has a sequence similar to binding sites recognized by a class of DNA binding proteins known as POU.

These results suggest that brain-specific nuclear proteins bind to a specific region upstream of GAP-43.

EXPERIMENTAL PROCEDURES

Genomic cloning and mapping

All methods used for cloning were as described by Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology*, John Wiley & Sons, publisher (1987). All enzymes were purchased from New England Biolabs. Genomic clones containing the three GAP-43 exons were isolated from a library constructed by inserting size fractioned SauIIIA partial digests of rat genomic DNA into the BamHI site of bacteriophage EMBL-3.

The library was initially screened on Colony Plaque Screen filters (DuPont/NEN) following standard protocols with random primed GAP-43 cDNA, as described hereinabove. To find exon I, the library was replated and duplicate lifts were probed sequentially with three oligonucleotides complementary to the 5' most region of the cDNA (#4, -68 to -39; #2, -38 to -9 and #5, +1 to +20 in Figure 14). Clones positive for at least two oligonucleotides were selected for further analysis. Inserts from positive phage were subcloned into the SalI site of the pBluescript vector (Stratagene) for mapping with a variety of restriction enzymes.

H-DNA gels

Two 25 cm long, 1.4% agarose gels were poured using 45 mM Tris base (adjusted to pH 7.4 or pH 4.0 with acetic acid) as a buffer. Loading buffer was electrophoresis buffer containing 5% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol. Gels were loaded with the digests of exon 1 containing plasmid bs1.5R1X4 listed in the legend of Figure 15, run for 16 hr at 20V, stained with ethidium bromide in Tris acetate pH 9, de-stained and photographed.

Sequencing

The GAP-43 promoter was sequenced by the dideoxy method of Sanger *et al., Proc. Natl. Acad. Sci. USA 74:*5463-5467(1977) using Sequenase as described by the supplier (USB). Subclones of the bacteriophage clones containing the first exon were constructed by standard methods in pBluescript (Stratagene) for double stranded sequencing and in M13 vector (Messing, *Meth. Enzymol. 101:*20-78 (1983)) for single-stranded sequencing.

RNAse mapping

The RNAse protection analysis was done as described in Krieg *et al.*, *Meth. Enzymol. 155:*397-415 (1987). For the protections, a genomic piece of GAP-43 from the XbaI site at -475 from the translation start site to the SspI site at +83 (in the first intron) was cloned into the XbaI and EcoRV sites of pSP72 (Promega).

RNAse protection analysis showed three major GAP-43 transcripts at -47/48, -51/52 and -78 bases from the translational start site. Protections were performed on tRNA, RNA prepared from newborn rat lung, dorsal root ganglia, and cerebral cortex. The probe extended 475 bases upstream from the translational start site (XbaI site). An over exposure showed additional longer transcripts which were much more abundant in the cerebral cortex as opposed to the DRG. Markers were MSPII digested pBR322.

Other RNAse protection analyses were carried out showing the heterogeneity of GAP-43 transcripts in different areas of the nervous system and in PC12 cells. RNA from control PC12 cells was compared with that obtained from NGF treated PC12 cells, tRNA, DRG, cerebellum, cortex, and hippocampus. RNA samples derived from the CNS had a higher proportion of the longer transcripts than samples from DRG or PC12 cells.

In another RNAse protection analysis, the genomic piece of GAP-43 from the NdeI site at -233 to the same SspI site at +83 was cloned by digesting the plasmid described above with NdeI and HindIII and filling with Klenow fragment of DNA polymerase. The HindIII site was reformed. In all cases, transcripts were elongated with T7 polymerase after linearizing the vectors with HindIII. Thus, all transcripts extending beyond this site accumulated as a single band at -234. RNA samples from newborn rat heart, liver, lung, cerebellum, spinal cord, cortex, hippocampus, and dorsal root ganglia were used. The longer upstream start sites as a group constituted the

start sites of a significant fraction of RNA in the central nervous system tissues but not in the dorsal root ganglia.

RESULTS

Cloning of GAP-43 genomic sequences

A rat genomic library was screened with probes derived from the GAP-43 cDNA, as described herein above. Initial screening with radiolabeled full length cDNA provided two classes of phage, which subsequent analysis showed to correspond to the second and third exons of the gene. Because the first exon proved to be small, and hence underrepresented in the cDNA probe, additional rounds of screening using three oligonucleotide probes derived from the 5′ most region of the cDNA were necessary in order to obtain clones containing the 5′ end of the gene.

A map of the GAP-43 gene is shown in Figure 13a, with representations of the phage used to map it. The gene spans at least 50 kb and contains 3 small exons. The first is about 80 bp (see below for a description of the variability of the 5′ end), the second is 565 bp, and the third is 672 bp, and they are separated by 2 introns of greater than 24 kb and 20 kb, respectively.

The first exon contains the 5′ untranslated sequences of the mRNA and encodes the first 10 amino acids of the protein. This short amino terminal region of the protein contains the "sorting sequence" that directs binding of GAP-43 (and heterologous fusion proteins) to growth cone membranes, as described hereinabove. The second exon encodes the bulk of the protein and includes a region identified by Alexander *et al.*, *J. Biol. Chem.* *263:*7544-7549 (1988) as the calmodulin binding site. The third exon encodes the carboxy-terminal 28 amino acids and contains 587 bases of untranslated sequence and the poly-A addition site.

The intron-exon boundaries shown in Figure 13b were identified by sequencing and are in agreement with consensus splice sites (Mount, *Nucl. Acids Res. 10:*459-472 (1982)). The polyadenylation site shown here was verified by RNAse protection and agrees with the one predicted by Rosenthal *et al., EMBO 6:*3641-3646 (1987) as the major site. A tandem pair of the consensus motif (YGTGTTYY) often found immediately 3′ of poly A addition sites (McLauchlan, *Nucl. Acids Res. 13:*1347-1368 (1985)) is underlined in the figure.

The GAP-43 promoter contains H-DNA

The sequence of the 5′ region of the gene is displayed in Figure 14. It contains no TATA or CAAT boxes, but does contain a sequence, TATTCATG (overlined), which is identical to the consensus Pit-1 binding site. This octamer binds a class of proteins thought to regulate transcription of several genes, including prolactin and growth hormone (Bodner *et al., Cell 55:*505-518 (1988); Ingraham *et al., Cell 55:*519-529 (1988)).

A striking feature of the promoter sequence is that more than 80% of the coding strand is composed of purines (underscored by asterisks in the figure), with two uninterrupted purine homopolymer stretches spanning from -118 to -188, and from -238 to -370, respectively. Some areas of these homopolymer stretches that are not simply alternating G and A contain tandem repeats, which possess some mirror symmetry (for example -168 to -118). Hairpin forming palindromes centered at -112, -232 and -509 flank the homopolymer regions and may influence secondary structure.

Purine-pyrimidine homopolymer stretches, especially those with mirror symmetry (Mirkin, *Nature 330:*495-497 (1987)), have the potential to assume a triple stranded conformation termed H-DNA (for reviews *see* Wells *et al., J. Biol. Chem. 263:*1095-1098 (1988); Htun *et al., Science 243:*1571-1576 (1989)).

The first indication that the GAP-43 promoter contained regions of strong secondary structure *in vitro* came while sequencing it. Sequencing is routinely accomplished using the double stranded dideoxy method, but when this technique was applied to the GAP-43 promoter, readable sequence would come to an abrupt halt upon reaching the homopolymer region at -250. Only after subcloning small fragments into M13 for single stranded sequencing were the inventors able to arrive at the sequence.

Htun *et al.*, *Science 241:1791-1795* (1988) devised a simple gel system to demonstrate that H-DNA will introduce a severe kink in DNA. Their assay is based upon the enhanced stability of H-DNA at low pH. When fragments of DNA which contain an H-forming region are electrophoresed at low pH, an H-DNA induced kink will retard mobility as compared to the mobility at a pH not favoring H-DNA formation (Htun *et al.*, *Science 241*:1791-1795 (1988)). The present inventors exploited this mobility shift to demonstrate that the purine homopolymer region from -240 to -370 in the GAP-43 promoter is capable of forming stable H-DNA structures in linear DNA *in vitro*.

Figure 15 is a representation of the restriction digest fragments of the GAP-43 promoter which were analyzed by gel electrophoresis, as described hereinbelow. The potential H-DNA forming homopurine-homopyrimidine regions are shown as thickened lines. In carrying out gel electrophoresis, aliquots of the digests represented in Figure 15 were loaded on 1.4% agarose gels that had been equilibrated with Tris-acetate at either pH 7.4 or 4.0 and run in parallel. Bands that shifted at pH 4 exhibited smearing that may result from the B to H transition (Htun *et al.*, *Science 241:*1791-1795 (1988)). Only bands containing homopolymer region I (i.e., the fragments containing the upstream (-240 to -370) homopurine stretch) exhibited an altered mobility at pH 4.0 in this assay. There was no visible shift in the markers or in fragments of the plasmid from outside the promoter region, or even in the fragments containing regions II and III when they were separated from region

I. Thus, only the upstream region exhibited a shift on its own. Note that the fragments containing regions II and III did not shift at pH 4. Progressive removal of DNA outside the homopolymer region increased the relative shift in mobility. A much greater shift in mobility was observed when region II was included in a fragment with region I than when region I alone was present in a similarly sized fragment. In this assay, regions II and III were not able to effect a shift on their own, but they may act cooperatively with the upstream region.

Heterogeneity of GAP-43 transcription initiation

Another notable feature of the GAP-43 upstream sequence is the absence of the TATA motif. Genes that lack a TATA sequence to direct initiation of transcription often have multiple mRNA start sites. This proved to be true for GAP-43. RNAse protection analysis was used to determine the transcriptional start sites for GAP-43 in several tissues. RNA from lung, dorsal root ganglia (DRG) and cerebral cortex (CTX) was analyzed with a probe extending to -475 bases from the translation start site. Using this probe, three major bands were protected, corresponding to transcriptional start sites at -47/-48, -51/-52, and -78. These same sites were identified by primer extension. Additional minor bands become visible after longer exposure.

Several transcripts at around -230 are present to a much greater extent in mRNA from the cerebral cortex as compared to the dorsal root ganglia. This is interesting in light of observations that suggest the regulation of GAP-43 gene expression in the central and peripheral nervous system is different (Skene *et al., J. Cell Biol. 89*:86-95 (1981); Skene *et al., J. Cell Biol. 89*:96-103 (1981)). Hence, RNA from other areas of the CNS, as well as from PC12 cells (which are believed to derive from sympatho-adrenal precursor cells), was analyzed. RNA from the hippocampus, cortex and cerebellum has a higher proportion of the transcripts initiating from the area

around -230 than RNA from DRG or PC12 cells, although the amount of each of these longer messages is relatively small. When a probe was used that pools all messages that start beyond -234, the difference between start sites in the CNS and PNS becomes more apparent. This analysis showed that the longer GAP-43 transcripts together actually account for a significant fraction of the total GAP-43 transcripts, and that these transcripts are much more prevalent in RNA from the CNS than that from the DRG or PC12 cells. In sum, the 5'end of GAP-43 mRNA is heterogeneous, and upstream start sites are used more commonly in the central nervous system as compared to the peripheral nervous system.

DISCUSSION

The present embodiment of the invention is directed to the isolation and characterization of genomic sequences containing the GAP-43 gene. Three small exons corresponding to the ~1.5 kb mRNA are separated by introns of at least 24 and 20 kb, respectively. The promoter region is rather unusual. There are several long homopurine-homopyrimidine stretches in the upstream region which are potentially capable of forming triple stranded "H-DNA" (Wells *et al., FASEB J.2*:2939-2949 (1988)). It is here demonstrated that one of these regions does, in fact, form H-DNA *in vitro*. The promoter lacks a canonical TATA box, and has multiple transcription initiation sites. The utilization of some of these sites differs in various parts of the nervous system.

The rat GAP-43 gene is a single copy gene that consists of three exons and two introns spanning at least 50 kb. The present inventors have obtained some evidence that the exons correspond to functional domains in the protein. The first exon, which encodes only the first 10 amino terminal residues, contains the stretch responsible for membrane targeting of GAP-43. Cysteines at positions 3 and 4 in the protein are acylated and may be involved in membrane binding, as described hereinabove. The amino terminus is

necessary for membrane binding of GAP-43, and contains sufficient information to target heterologous fusion proteins to the same membrane domains as GAP-43, including those of the growth cone, as described hereinabove.

The second exon includes the calmodulin binding region from amino acid 43 to 51 (Alexander *et al., J. Biol. Chem. 263:*7544-7549 (1988)) as well as a serine at position 41 that is a substrate for protein kinase C (Coggins *et al., Soc. Neurosci.* Abstract (1988)). Exons I and II contain regions that are well conserved between fish and several mammalian GAP-43 proteins.

The promoter of GAP-43 is unusual in sequence and structure. The lack of a TATA box and consequent use of multiple start sites cause the GAP-43 promoter to resemble promoters of constitutively expressed housekeeping genes. However, the GAP-43 promoter lacks the consensus Sp-1 binding sites (GGGCGGG) that have been correlated with the promoters of housekeeping genes (Dynan, *Trends Genet. 2:*196-197 (1986)). Furthermore, the tightly regulated expression of GAP-43 in development, its specificity to neurons, and its inducibility in particular neurons in the adult sugest that it does not belong to this class of genes.

GAP-43 is regulated differently in the central and peripheral nervous systems. For example, axotomy of mammalian central neurons does not cause increased GAP-43 expression and transport, whereas axotomy of a peripheral nerve does (Skene *et al., J. Cell Biol. 89:*96-103 (1981). As described hereinabove, GAP-43 does not appear to be irreversibly repressed in the CNS, and may play a role in plasticity other than in axonal growth (Benowitz *et al., T.I.N.S. 10:*527-532 (1987)), but it is clear that there is a difference in regulation centrally and peripherally. Hence, the usage of different start sites suggests the possibility that the mRNA from different neurons may differ at the 5′ end, in turn regulating ribosome binding or stability.

It is interesting to note that Thy-1, a gene expressed in, although not limited to, neurons, has been demonstrated to be expressed in a developmentally regulated, tissue-specific fashion at the transcriptional level, and also lacks a TATA box and Sp-1 binding sites (Spanopoulou *et al., Molec. Cell. Biol. 8:*3847-3856 (1988)). Also, like GAP-43, the choice of transcriptional start sites in the Thy-1 promoter can vary between expressing tissues, with upstream start sites being more prominent in the brain (Spanopoulou *et al., Molec. Cell. Biol. 8:*3847-3856 (1988)). This suggests an additional level of control in brain versus other tissues for both GAP-43 and Thy-1.

A potential upstream regulatory element present in the GAP-43 promoter is the consensus Pit-1 binding site (TATTCATG). This and related sequences are recognized and bound by transcription factors known collectively as POU proteins. This group originally included Pit-1, Oct-1 and Oct-2 in mammals, and unc86 in nematodes (reviewed in Herr *et al., Genes Develop. 2:*1513-1516 (1988)), and has recently been expanded by the finding of cDNAs encoding proteins that share the two peptide regions that characterize this family (He *et al., Nature 340:*35-42 (1989)). As described in the following example, the present inventors have identified and cloned brain-specific proteins that bind this region of GAP-43 and may regulate its transcription.

Another remarkable feature of the GAP-43 promoter is the presence of long homopurine-homopyrimidine stretches. These are interesting because they may bind proteins specific to GAGA stretches (Biggin *et al., Cell 53:*699-711 (1988); Gilmour *et al., Science 245:*1487-1490 (1989)), and because they have the potential to take on a triple stranded conformation called H-DNA. Such homopolymer regions have been found to be overrepresented in the 5′ ends of eukaryotic and eukaryotic viral genes, leading to the speculation that they may somehow be involved in transcriptional control

(Wells *et al.*, *FASEB J.* 2:2939-2949 (1988); Htun *et al.*, *Science* 243:1571-1576 (1989)). For instance, it has been postulated that adoption of the H configuration, perhaps stabilized by protein interactions, would cause a kink in the DNA. This kink could phase nucleosomes by exclusion from the kinked region, thereby making the DNA around the kink more accessible to transcriptional factors (Htun *et al.*, *Science 241:*1791-1795 (1988); Han *et al.*, *Cell 55:*1137-1145 (1988)). Additionally, such a kink could serve to bring upstream sequences into closer apposition to those downstream, allowing an interaction between the sequences or proteins bound to them (Htun *et al.*, *Science 241:*1791-1795 (1988)). Alternatively, H-DNA could serve as a repressor of transcription by directly blocking access to DNA in its immediate vicinity (Maher *et al.*, *Science 245:*725-730 (1989)).

Thus, additional embodiments of the present invention comprise, *inter alia*, a nucleotide sequence as shown in Figure 13 encoding genomic GAP-43, or a functional or chemical derivative thereof, as well as a nucleotide sequence as shown in Figure 14 encoding the GAP-43 promoter, or a functional or chemical derivative thereof. It will further be appreciated that the GAP-43 promoter of the invention will be of great utility, not only in modifying the activity of GAP-43 itself, but as a means of achieving desired alterations in expression of other structural genes, using methods well known to those of skill.

Stated more broadly, this aspect of the invention is directed to a promoter substantially as shown in Figure 14, characterized in that it contains multiple start sites and a consensus Pit-1 binding site, but lacks a TATA box and consensus Sp-1 binding sites, and further characterized in that it comprises long homopurine-homopurimidine stretches capable of taking on triple stranded (H-DNA) conformation. Structural genes, or fragments thereof, comprising, at their amino-terminus end, in phase, the nucleotide sequence of the GAP-43

promoter as described herein, and functional or chemical derivatives thereof, also form intended embodiments of the invention.

In yet additional embodiments, there are provided DNA expression vectors comprising the structural gene as described above, host cells transfected with these vectors, and the proteins produced thereby.

EXAMPLE VIII

A Major Component of the Neuronal Growth Cone Membrane is the GTP Binding Protein, G_o

The neuronal growth cone contains specialized transduction machinery which converts signals from the microenvironment into directed growth of axons or dendrites. Subcellular fractions from neonatal rat brain that are enriched in growth cone membranes have simple and distinctive protein composition. The two major non-cytoskeletal proteins in growth cone membrane preparations have molecular weights of 40,000 and 35,000. By electrophoretic, immunologic and partial protein sequence criteria, these proteins have been identified as the alpha and beta subunits of the GTP binding protein, G_o. Immunohistologic staining of neuronally differentiated rat pheochromocytoma cells demonstrates high concentrations of the alpha subunit of G_o at the distal tips of cellular processes. These data suggest that regulation of growth cone motility may utilize a G_o signal transduction mechanism.

The complex state of neuronal connectivity achieved during brain development, and refined through synaptic plasticity, requires selection of specific targets by neuronal axons. The mechanisms by which axons transduce information form their extracellular milieu into directed growth are poorly understood. The distal tip of a neuronal axon has a unique ultrastructure termed the growth cone, which is thought to be critical for this process (Bray,

*et al., Ann. Rev. Cell Biol. 4:*43 (1988)). Fortunately, the membrane of the axonal growth cone, and therefore its transduction system, can be fractionated from other neuronal constituents (Pfenninger, *et al., Cell 35:*573 (1983); Gordon-Weeks, *et al., Neuroscience 13:*119 (1984); Ellis, *et al., J. Cell Biol. 101:*1977 (1985)). It is composed of only a few major proteins, and several of these proteins have been identified: tubulin, actin, and the neural-specific, growth-related protein, GAP-43 (Pfenninger, *et al., Cell 35:*573 (1983); Ellis, *et al., J. Cell Biol. 101:*1977 (1985); Simkowitz, *et al., J. Neurosci. 9:*1004 (1989); Cheng, *et al., J. Biol. Chem. 263:*3935 (1988); Meiri, *et al., Proc. Natl. Acad. Sci. USA 85:*3537 (1986); Skene, *et al., Science 233:*783 (1986)). Characterization of the other major growth cone membrane constituents could explain axonal response to extracellular cues.

A growth cone membrane fraction was prepared from neonatal rat brain (Pfenninger, *et al., Cell 35:*573 (1983); Ellis, *et al., J. Cell Biol. 101:*1977 (1985); Simkowitz, *et al., J. Neurosci. 9:*1004 (1989); Cheng, *et al., J. Biol. Chem. 263:*3935 (1988)). This preparation has a simple protein composition by SDS-PAGE (Ellis, *et al., J. Cell Biol. 101:*1977 (1985); Simkowitz, *et al., J. Neurosci. 9:*1004 (1989); Cheng, *et al., J. Biol. Chem. 263:*3935 (1988)). The most intensely stained ban, migrating at 50-55,000 daltons, has been identified as tubulin (Simkowitz, *et al., J. Neurosci. 9:*1004 (1989); Cheng, *et al., J. Biol. Chem. 263:*3935 (1988)). There are also prominent proteins with M_rs of about 35,000 and 40,000, which have been termed p34 and p38, and are specifically enriched in the growth cone membrane (Simkowitz, *et al., J. Neurosci. 9:*1004 (1989)). These are the two unidentified proteins which were characterized further. Apart from cyto-skeletal proteins, they are the most prominent proteins in the growth cone membrane preparation.

It was noted that p34 and p38 have similar molecular weights to the alpha and beta subunits of the GTP-binding protein, G_o (Stryer, *et al., Ann.*

*Rev. Cell Biol. 2:*391 (1986); Gilman, *Ann. Rev. Biochem. 56:*615 (1987)). Co-electrophoresis of the growth cone membranes with purified bovine grain G_o demonstrated that p34 co-migrates with the beta subunit, and p38 with the alpha subunit of G_o. Immunoblotting demonstrated that p34 reacts with an anti-beta subunit antiserum, and that p38 reacts with an anti-alpha subunit G_o antiserum. Furthermore, the predominant protein species of p38 must be $alpha_o$, because equal protein concentrations of p38 and $alpha_o$, as determined by Coomassie blue staining, exhibited identical immunoreactivity. The same was true for p34 and the beta subunit of G_o. $Alpha_i$ subunit was about 10-fold less reactive than $alpha_o$ with this antiserum (Gilman, *Ann. Rev. Biochem. 56:*615 (1987)), so that it cannot account for a major percentage of the $alpha_o$ immunoreactivity.

To verify these immunologic and electrophoretic data, partial protein sequences were obtained from electrophoretically purified p34 and p38 (Figure 16). Both p34 and p38 were amino termimally blocked. Sequence was obtained from tryptic fragments separated by HPLC and from *Staph. aureus* V8 protease partial digestion fragments separated by SDS-PAGE. The sequence for each of three peptides from p38 was identical to that of $alpha_o$, confirming that $alpha_o$ is the major component of the p38 protein. Other known alpha subunits have similar but distinct sequences. The three p34 peptides had a sequence identical to that of the beta subunit of G proteins. Two peptides were from regions where $beta_1$ and $beta_2$ subunits are identical and the third contained a mixture of the sequences for $beta_1$ and $beta_2$. Thus, the alpha and beta subunits of G_o are major constituents of the growth cone membrane subcellular fraction.

Although these preparations are substantially enriched in growth cone membrane, they are not pure (Pfenninger, *et al., Cell 35:*573 (1983); Gordon-Weeks, *et al., Neuroscience 13:*119 (1984)). Previous immunohistology of unfractionated tissue has demonstrated that G_o is concentrated in the neuropil

of adult rat brain (Worley, *et al., Proc. Natl. Acad. Sci. USA 83:*4561 (1986)), that a related G protein, G_{olf}, is localized to the terminal region of primary olfactory neurons in the adult (Jones, *et al., Science 244:*790 (1989)), and that G_o stains throughout cultured primary neurons but is concentrated at regions of cell-cell contact (Jones, *et al., Science 244:*790 (1989)). These studies are consistent with, but do not prove, G_o localization in growth cones. Therefore, immunohistologic methods were employed on NGF-treated PC12 cells to examine G_o distribution in the intact cells. NGF causes these cells to extend long processes tipped with growth cones. The neuronal protein GAP-43 is enriched in these growth cones as it is in those of primary neurons (Van Hooff, *et al., J. Cell Biol. 108:*1115 (1989)). $Alpha_o$ immunofluorescence was highly concentrated in these growing tips of PC12 cells (although it was not found exclusively there).

METHODS

Preparation of growth cone membranes

Growth cone membranes were prepared with minor modifications from previous methods (Pfenninger, *et al., Cell 35:*573 (1983); Ellis, *et al., J. Cell Biol. 101:*1977 (1985); Simkowitz, *et al., J. Neurosci. 9:*1004 (1989); Cheng, *et al., J. Biol. Chem. 263:*3935 (1988)). Sprague-Dawley rats less than 24 hours old were decapitated and the brains were homogenized at 4°C with 6 passes in a glass/teflon homogenizer in 5 volumes of 0.32 M sucrose, 1 mM Tris-HCl, 1 mM MgCl, pH 7.6. The following protease inhibitors were employed throughout the procedure: 100 ug/ml soybean trypsin inhibitor, 1 ug/ml pepstatin A, 30 uM leupeptin, and 1 mM PMSF. The crude brain homogenate was layered over a step gradient of sucrose at 0.75 M, 1.0 M and 2.2 M. The gradient was centrifuged at 250,000 x g for 40 min, and the 0.32/0.75 M interface was collected as the growth cone particle fraction. This fraction was lysed in 5 mM Tris-HCl, pH 7.6, and the membranes were

collected by centrifugation at 250,000 x g for 40 min. The membranes were washed by resuspension in 20 ug/ml saponin and 0.3 M Na_2So_3 and again collected by centrifugation. Bovine brain G_o was prepared as described (Bray, *et al., Ann. Rev. Cell Biol. 4:*43 (1988)).

Production of anti-sera

The production and characterization of anti-bovine brain alpha$_o$ and anti-beta antiserum in rabbits has been described (Huff, *et al., J. Biol. Chem. 260:*10864 (1985)). Immunoblot samples were electrophoresed through 10% polyacrylamide gels with SDS and then electrophoretically transferred to nitrocellulose. Non-specific protein binding sites were blocked with 10 mg/ml bovine serum albumin, and the blots were incubated with 1:400 anti-alpha antiserum or 1:100 anti-beta antiserum (Huff, *et al., J. Biol. Chem. 260:*10864 (1985)) overnight at 4°. Bound antibody was detected by the avidin biotin complex method (Vectastatin, Burlingame, CA) using tetrabenzidine as a peroxidase substrate.

Amino acid sequencing of growth cone membrane proteins

Growth cone membranes were fractionated as described above. When proteins were transferred to polyvinylfluoridine (PDVF) membranes, the spots for p34 and p38 yielded no sequences, presumably because the proteins are amino terminally blocked. Therefore, protein sequences were obtained from proteolytic fragments for p34 and p38. For tryptic digestion, the proteins were transferred to nitrocellulose, stained with ponceau S and the appropriate bands were excised. At the Harvard Microchemistry Facility, tryptic digestions were performed on the nitrocellulose membranes, and the released peptides were separated by reverse phase HPLC and sequenced on a gas phase automated sequenator (Moos, *et al, J. Biol. Chem. 263:*6005 (1988)). Further amino acid sequence was obtained for p34 following partial digestion with

Staph. Aureus V8 protease (Boehringer, Mannheim). Polyacrylamide cubes containing p34 were digested *in situ* with V8, fractionated by SDS-PAGE, electroblotted to PVDF membrane (Millipore, Bedford, MA) and visualized with Coomassie blue (Kennedy, *et al., Proc. Natl. Acad. Sci. USA 85:*7008 (1988)). Peptide fragments were excised and sequenced on an Applied Biosystems (Foster City, CA) 470A gas phase sequencer at the Howard Hughes Medical Institute Protein Chemistry Core Facility of Columbia University to yield sequence p34-B.

Alpha$_o$ immunostaining of PC12 cells

PC12 cells were grown on poly-D-lysine treated coverslips for 48 hours in the presence of 100 ng/ml nerve growth factor. The cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), and then permeabilized with 0.1% Triton X-100. After incubation with 5 mg/ml bovine serum albumin in PBS, the cells were incubated with 1:1000 anti-bovine brain alpha$_o$ antiserum for 1 hour at 23°C, rinsed with PBS, and incubated with 0.3% H_2O_2 for 15 minutes to reduce background. Bound rabbit immunoglobulin was detected by use of the Texas red conjugated donkey anti-rabbit IgG (Jackson Immunologicals).

RESULTS

SDS-PAGE reveals two bands which co-migrate with G$_o$ alpha and beta subunits

Proteins of the axonal growth cone membrane were identified by SDS-PAGE. Two separate preparations of axonal growth cone membranes, purified bovine brain G$_o$, and crude brain homogenate were electrophoresed through a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Enrichment of two bands, termed p34 and p38, in the

growth cone membrane preparation relative to crude brain was observed. These proteins comigrated with the alpha and beta subunits of purified G_o. Under these conditions, actin and GAP-43 comigrated with an apparent Mr of 43,000.

Anti-G_o immunoblots of growth cone membrane show G_o reactivity

Immunoblotting revealed $alpha_o$ immunoreactivity migrating at the position of alpha Coomassie blue staining in both the purified G_o preparation and the growth cone membrane preparation. The gels were loaded such that Coomassie blue labeling of $alpha_o$ was identical to that of P38, and similarly matched for other pairs. The Coomassie stained gels were run in parallel. Note that the pairs also were immunostained to the same degree, as the total protein was increased, demonstrating that p38 is as immunoreactive as authentic $alpha_o$ with this antiserum. This suggests that most or all of p38 is $alpha_o$. There was a small amount of immunoreactivity migrating just above $alpha_o$ in the G_o sample which was likely to be due to slight contamination and cross-reaction with $alpha_i$. This was not seen in the growth and growth cone membrane (A) fractions, which were previously shown to stain identically with Coomassie blue for beta and p34, respectively, and also showed similar immunoreactivity with anti-beta antiserum.

The partial protein sequence for p34 and p38 is identical to that of G_o.

The partial protein sequence for p34 and p38 is shown in Figure 16. The sequence of three peptides from p38 matches the sequence of three peptides from $alpha_o$ from rat brain (Goh, *Science 244:*980 (1989)). The sequence of three peptides from p34 is compared to that of $beta_1$ and $beta_2$ subunits from bovine brain (Fong, *et al., Proc. Natl. Acad. Sci. USA 84:*3792 (1987)). Note that two peptides are identical to regions in which $beta_1$ and

$beta_2$ are identical. The other peptide contains a mixture of the sequences for $beta_1$ and $beta_2$.

Alpha$_o$ immunoreactivity is concentrated in the tips of PC12 processes

Alpha$_o$ staining of PC12 cells differentiated with nerve growth factor revealed high concentrations of the antigen at the distal tips of the cellular processes. There was also a lower level of diffuse staining in the region of the cell body surrounding the nucleus. The specificity of the antibody has been demonstrated (Huff, *et al., J. Biol. Chem. 260:*10864 (1985); Worley, *et al., Proc. Natl. Acad. Sci. USA 83:*4561 (1986)), but as a further control, identical samples were prepared with the addition of excess purified bovine brain G_o (30 ug/ml) to the incubation with antiserum, or with the substitution of normal rabbit serum for antiserum. These controls exhibited essentially no staining of the cellular processes.

DISCUSSION

The results presented in the present example demonstrate that a G protein, specifically G_o, is a major constituent of the growth cone membrane. In fact, there is more G_o than any other non-cytoskeletal protein in the growth cone membrane. G proteins, in general, couple transmembrane receptors to intracellular signalling systems, although the role of G_o, which is expressed primarily in brain, has not been clear (Stryer, *et al., Ann. Rev. Cell Biol. 2:*391 (1986); Gilman, *Ann. Rev. Biochem. 56:*615 (1987); Neer, *Nature 333:*129 (1988); Ross, *Neuron 3:*141 (1989)). G_o can interact with a number of cell surface receptors and may affect a variety of intracellular signalling systems including phospholipase C, phospholipase A_2, potassium channels and calcium channels (Skene, *et al., Science 233:*783 (1986); Stryer, *et al., Ann. Rev. Cell Biol. 2:*391 (1986); Neer, *Nature 333:*129 (1988); Ross, *Neuron 3:*141 (1989); Brown, *et al., Am. J. Physiol. 254:*H401 (1988)). The

strikingly high levels of G_o in the growth cone membrane, which are comparable to those of the retinal G protein, transducin, in rod and cone outer segments, strongly suggest a G_o-based transduction system in growth cones.

G proteins have been proved crucial to developmental morphogenesis in the slime mold, *Dictyostelium*, where chemotaxis towards cAMP is transduced via a G protein (Snaar-Jagalska, *et al., F.E.B.S. Lett. 232:*148 (1988); Snaar-Jagalska, *et al., F.E.B.S. Lett. 236:*139 (1988); Kesbeke, *et al., J. Cell Biol. 107:*521 (1988)). Similarly, signals from pathways or targets in the developing nervous system may bind to a G_o-linked receptor, or receptors, in the growth cone membrane, and thereby alter the level of intracellular second messengers, and hence growth cone motility. In general, these signals, receptors, and second messengers are unknown at present. One class of candidate receptors are the cell adhesion molecules, N-CAM and L1, which are localized to the neuronal growth cone (Letourneau, *et al., Development 105:*505 (1989)). Antibodies to thse molecules alter calcium levels and phosphotidylinositol metabolism in PC12 cells, and the effect of these antibodies is blocked by the G protein antagonist pertussis toxin (Van Hooff, *et al., J. Cell Biol. 108:*1115 (1989)).

The persistence of G_o expression in the adult nervous system (Worley, *et al., Proc. Natl. Acad. Sci. USA 83:*4561 (1986)) implies roles other than the regulation of axonogenesis. Another growth cone enriched molecule, GAP-43, also exists in discrete regions of the adult brain (Benowitz, *et al., Trends Neurosci. 10:*527 (1987); Skene, *Ann. Rev. Neurosci. 12:*127 (1989)). The localization of GAP-43, the nature of its gene regulation, and especially the correlation of its phosphorylation state with long-term potentiation in the hippocampal slice (Routtenberg, *N.Y. Acad. Sci. 444:*980 (1989)) has suggested a role for GAP-43 in synaptic plasticity in the adult (Benowitz, *et al., Trends Neurosci. 10:*527 (1987); Skene, H.J.P., *Ann. Rev. Neurosci. 12:*127 (1989)). It is noteworthy that pertussis toxin blocks long-term

potentiation, perhaps implicating G_o in this process as well (Goh, *Science 244:*980 (1989)). Hence, G_o may transduce regulatory signals for axonal extension during neuronal development and for synaptic plasticity in the adult nervous system.

EXAMPLE IX

Pertussis Toxin-Sensitive G Proteins Mediate Neuronal Growth Cone Collapse

Inhibition Of G Proteins By Pertussis Toxin or Intracellular GDPβS Stimulates Neuronal Growth

The nerve growth cone controls neuronal form by sensing signals from neighboring cells, matrix and targets, and by transducing this information into structural changes. The consequent directed growth directs establishment of proper connections during development. Adult CNS neurons have relatively unimpressive regenerative capacity, which is a vexing clinical problem.

Although positive and negative growth regulators have been identified, the inhibitors may be critical in explaining poor regeneration within the adult CNS. The most prominent non-cytoskeletal protein associated with the growth cone membrane is G_o, a member of the heterotrimetric G protein family. The great abundance of G_o in the growth cone, and its regulation by the growth associated protein, GAP-43, suggest that G_o might be a key intermediary in the transduction of morphogenetic information. This model predicts that G protein activation or inhibition should affect neuronal growth. The following experiments demonstrate that G proteins act as mediators of neuronal growth in both CNS and PNS cells.

METHODS

Cell Culture: Embryonic chick sympathetic neurons and retinal ganglion cells were isolated as follows. Sympathetic chains, isolated from the thoraco-lumbar regions of day eight to twelve chick embryos or retina dissected from day 7 chick embryos, were incubated for 15 minutes in 0.25% trypsin and 0.5% collagenase in HBSS at 37°C. The tissue was then triturated in growth medium (F12 with 10% fetal bovine serum, glutamine, penicillin/streptomycin, and, for sympathetic neurons, 5 ng of NGF/ml), 15 times with a fire polished pasture pipet. Cells were diluted to a density of 1 x 10^4 cells/ml, preplated on an untreated tissue culture substrate for 3 hours to allow fibroblasts to attach, and then plated onto glass chamber slides coated with either poly-L-lysine (5 mg/cm^2 for 30 minutes at 20 °C) or laminin (4 mg/cm^2 for 60 minutes at 20 °C).

Conditions for inhibiting or stimulating G proteins: Pertussis toxin treatment was performed by incubating neurons in floating culture with either 200 ng/ml of freshly prepared toxin (Cal Biochem), or PBS alone as control for 3 hours and then plating cells in parallel. Aluminum flouride (AlF) or mastoparan were added to cultures 45 minutes after plating. Conditions for electroporation of primary neurons were developed to facilitate entry of relatively small molecules (MW < 1000) into live cells. Using sulfo-rhodamine (a highly ionic rhodamine analog which does not penetrate intact cells) as a tracer for temporary permeability, conditions were optimized to maintain > 80% cell viability and achieve > 95% cell permeability. The integrity of the plasmalemma was only temporarily violated as sulfo-rhodamine could be detected within neurons when it was present during the electroporation, but not when it was added as little as 1 minute post-electroporation. Electroporations were performed in growth medium at 500 V and 1 microFarad in the presence of 1 mM GTPγS, 1 mM GDP beta-S or

PBS alone. Fifteen discharges were each spaced by 5 seconds and then cells were plated. To assess the efficiency of electroporation, cells were incubated with or without electroporation in the presence of sulfo-rhodamine, plated, allowed to attach, washed with growth medium and fixed after 18 hours in culture.

Fixation and Staining: Cells were fixed in 4% paraformaldehyde with 12% sucrose (w/v) for 12 minutes at room temperature, and then permeabilized with 5% glacial acetic acid in ETOH at -20°C for 7 minutes. For immunohistochemistry, fixed and permeabilized cells were pre-incubated with 10% normal goat serum in PBS for 10 minutes, incubated with primary antibody for 60 minutes followed by three washes with phosphate buffered saline, then incubated with secondary antibody for 60 minutes. Primary antibodies used were a rabbit polyclonal raised against GAP-43, or an anti-neurofilament mouse monoclonal. Secondary antibodies were FTIC-goat anti-rabbit, Texas Red conjugated goat anti-rabbit or RTIC-goat anti-mouse (Molecular Probes Inc.).

RESULTS

In chick cells, pertussis toxin inhibits receptor mediated activation of G_o and G_i via irreversible ADP-ribosylation. Treatment with pertussis toxin causes a significant increase in both the total number of neurites per cell and the total length of neurites per cell. AlF acts as an activator of G-proteins and has been shown in other cell types to stimulate G-protein mediated events. Mastoparan, a wasp venom toxin, stimulates G_o and G_i through a reversible interaction with their receptor binding domain. Both aluminum fluoride and mastoparan dramatically reduced process formation. Although prolonged exposure to either agent was clearly toxic, the inhibitory effect on neurite

outgrowth was reversible. Cell death in the mastoparan and AlF treated cultures was approximately 60%.

The non-hydrolyzable guanine nucleotide analogs GTPγS and GDPβS bind to and maintain G proteins in the active and inactive states, respectively. Electroporation of GTPγS, GDPβS or control buffer (PBS) was performed as described in cultures of embryonic chick sympathetic neurons. Neurons were plated immediately after electroporation, fixed one hour post-plating and examined by phase-contrast or Normarski optics.

G-protein inhibition caused an increase in the total number of neurites per cell, the total length of neurites per cell, and the average neurite length in cells. In contrast, neurite extension was significantly diminished in cells receiving GTPγS.

Retinal ganglion cells exhibit similar increases in neurite extension when treated with pertussis toxin or with electroporation of GDPβs. GTPγs electroporation reduces neurite outgrowth, but to a lesser degree than in sympathetic neurons.

DISCUSSION

Neuronal plasticity depends upon a complex interaction involving such factors as G proteins and inhibitory or stimulatory ligands. Thus, the net effect of G protein modulation, upon neuronal plasticity, depends on the particular environment and the balance between inhibitory and stimulatory ligands. These results demonstrate that, under certain conditions, G protein inhibition enhances neurite growth, and G protein activation reduces neurite extension. These results are most likely due to the effect of PT in overcoming a particular inhibitory ligand in this system. However, the most important discovery is the role that G proteins play in regulation of neuronal plasticity

in both sympathetic neurons, which are from the peripheral nervous system, and in retinal ganglion neurons, which are from the central nervous system.

There are many cell-specific receptors that guide growth, some of which are known to be coupled via G proteins. For example, serotonin application to Heliosome neurons causes growth cone collapse and cessation of axon elongation (Haydon *et al., Science 226:*561-564 (1984)), which is compatible with the observations reported here. Interestingly, pertussis toxin has been shown to modify LTP, a process now believed to involve physical changes in nerve terminal size.

Given the abundance of G_o in growth cones, and the effect upon neurons from both the PNS and CNS, it appears likely that G proteins may be relatively universal mediators of growth cone responsiveness. The pronounced stimulation effects of pertussis toxin, suggest one effect of G proteins may be to mediate inhibitory, or "stop" signals. Such signals are important in neuronal development and plasticity. Oligodendrocyte proteins have been isolated of 35 K and 250 K MW which inhibit growth of central and peripheral nervous system neurons. Although each of these systems may bear specific receptors for negative signals, perhaps they share the G protein transduction machinery. This is of clinical significance, since it is important to devise means to encourage regeneration of CNS neurons from many sites, and inhibition of G proteins may provide such tools.

Pertussis Toxin and GAP-43/G_o Modulation of Growth Cone Function

GAP-43 stimulates GTPγS binding, GTPase activity and GDP release in the same fashion as do transmembrane G-linked receptors. However, GAP-43 differs from receptors in that it acts on the isolated alpha$_o$ subunit just as effectively as on the heterotrimeric G_o. Pertussis toxin blocks receptor/G_o interaction, but not GAP-43/G_o interaction. The relative ability of fifteen synthetic peptides to stimulate G_o demonstrates that the first 25 amino acids of GAP-43 are important for its effect on G_o. It has been shown that the major form of GAP-43 isolated from brain is phosphorylated by protein kinase C at position 41. Dephosphorylation of GAP-43 does not alter its potency for stimulating G_o. Calmodulin can bind to GAP-43, but 20 uM calmodulin does not alter the GAP-43 effect on G_o. G_o may integrate extracellular and intraneuronal growth clues in the growth cone. GAP-43 could be an upstream regulator of G_o activity or a downstream effector of activated G_o.

Pertussis Toxin-Sensitive G Proteins Mediate Growth Cone Collapse

Many signals are utilized to demarcate and guide the assembly of neuronal circuitry and structure. A growing body of recent evidence suggests that inhibitory signals, those which stop or deflect growing neurites, are critical, in addition to those which are stimulatory or permissive for growth. Such inhibitory activities are components of brain membranes, myelin, and somites (Cox *et al., Neuron 4*:31-37 (1990); Raper *et al., Neuron 4*:21-29 (1990); Schwab, *Trends Neurosci. 13*:452-456 (1990); (Davies *et al., Bioessays 13*:11-15 (1991); Davies *et al., Neuron 4*:11-20 (1990)). Their role has been speculated to be critical to the prevention of regrowth of injured axons in the CNS, to pathway guidance through somites, and to the recognition of targets (Aveldano *et al., J. Neurochem. 57*:250-257 (1991)).

Different assays have been developed to detect this inhibitory activity. Kaphammer *et al.* found that collapse of growth cones can be induced by brain

membranes, an effect exerted both upon CNS and PNS neurons (Kapfhammer *et al., J. Neurosci. 7*:201-212 (1987). Others, using growth in cell culture chamber systems, have partially isolated myelin-associated proteins which inhibit growth of neurons (Schwab, 1990). The transduction mechanisms which are responsible for registering inhibitory signals and triggering growth cone collapse are largely unexplored. It is conceivable that inhibitory mechanisms might be shared between neurons of different types, although the specific signals and their receptors might differ.

G_o is the most prominent non-cytoskeletal protein of the growth cone membrane, which suggests that G_o may be integral in the response to environmental signals that direct pathway and target selection. The inventors have examined the role of G proteins in growth cone collapse and found that collapse stimulated by both brain membranes and myelin has a large component dependent upon pertussis toxin-sensitive G proteins.

METHODS

The membranes were prepared from E10 chick brains as described previously. (Raper *et al., Neuron 4*:21-29 (1990)). The crude collapsing activity was obtained from detergent-solubilized membrane extracts as described by Raper and Kapfhammer. (Raper *et al., Neuron 4*:21-29 (1990)). Briefly, the membranes were suspended in 2% CHAPS/PBS and the extract was centrifuged (100,000 g for 1 hour). The supernatant was dialyzed against PBS and then F12 medium to remove the detergent. The dialysate was used as the crude growth cone collapsing activity. Each DRG was removed from E7 chick embryos and explanted onto laminin-coated glass chamber slides in F12 medium including NGF, fetal calf serum, streptomycin, and penicillin.

Collapse assays were done 18-26 hours later. Growth cone cllapse was assayed as described previously. (Raper *et al., Neuron 4*:21-29 (1990); Sarndahl *et al., J. Cell Biol. 109*:2791-2799 (1989)). Briefly, 50 μl of the

diluted crude collapsing activity or MP (Sigma) was added into 0.5 ml of culture medium. The cultures were incubated at 37°C and 5% CO_2 for 30 min. and then fixed with 4% paraformaldehyde in PBS containing 10% sucrose. Fixed preparations were scored quantitatively, using standard methods previously described. (Cox *et al., Neuron 4*:31-37 (1990); Raper *et al., Neuron 4*:21-29 (1990)). In some experiments, DRG explants were preincubated with PTX (100 or 200 ng/ml; Calbiochem) for 2 to 4 hours before the collapse assay.

CNS myelin was prepared from adult rat cerebral cortex (Norton *et al., J. Neurochem. 21*:749-757 (1973)), and myelin proteins were solubilized by 60 mM octyl glucoside in PBS (pH 6.7) containing 0.1 M sodium sulfate at 4°C for 1 hour (Aveldano *et al., J. Neurochem. 57*:250-257 (1991)). The supernatant was collected and dialyzed using the same method as that for chick brain membranes. The solubilized myelin fraction was diluted and added to chick DRG explants.

RESULTS

Under basal conditions, about 26% of growth cones are collapsed, with the remainder being fan shaped. Direct stimulation of G proteins, particularly G_o and G_i, with the wasp venom peptide mastoparan, dramatically enhances the proportion which are collapsed, as shown in Figure 22(b). This effect of mastoparan is completely reversed by pretreatment with pertussis toxin. This suggests that, under certain conditions, stimulation of G proteins predisposes to growth cone collapse.

The percentage of collapsed growth cones under basal conditions is unchanged by exposure to pertussis toxin, as shown in Figure 17. Brain membranes, isolated by standard techniques (Raper *et al., Neuron 4*:21-29 (1990)), increase the percentage of collapsed growth cones, and do so in a dose-dependent way, with maximal effects exerted by about 1.2 mg/ml of

brain extract, as shown in Figure 18. Preincubation of the dorsal root ganglion explants with pertussis toxin markedly attenuates this collapse. Preincubation with 100 ng/ml shifts the dose response curve by six-fold and with 200 ng/ml by ten-fold. This suggests that the collapsing activity of the brain extracts exerts a significant proportion of its effect via pertussis toxin-sensitive G proteins. Additional mechanisms that are non-pertussis toxin-sensitive may be brought into play at the highest concentrations of brain extract.

Brain extracts have been shown also to cause collapse of retinal neurons from chick, a finding of clinical relevance in terms of the regrowth of an inured optic nerve (David *et al., Science 14*:931-933 (1981)). As shown in Figure 20, about 26% of growth cones from the explanted retina are collapsed under basal conditions. Although less sensitive to brain extracts than are the DRG, retinal growth cones also collapse with exposure to brain extract, as shown in Figure 20. As also shown, this collapse is markedly attenuated by pretreatment with pertussis toxin, suggesting that central nervous system as well as peripheral nervous system growth cone collapse is mediated in large part by pertussis toxin-sensitive G proteins.

One of the best characterized inhibitory activities in the nervous system is that due to the 35 kD and 250 kD proteins of myelin (Schwab, *Trends Neurosci. 13*:452-456 (1990)). Recently, the inhibitory activities of these proteins have been shown to correlate with a growth cone collapsing ability. The inventors have found that CNS myelin protein, solubilized by octyl glucoside, causes growth cone collapse of the dorsal root ganglia. As shown in Figure 19, the addition of the myelin extract to the dorsal root ganglion cultures causes an increase in collapsed growth cones from 26% to 68%. Pretreatment of the dorsal root ganglia with pertussis toxin reduces the percentage of growth cones which collapse with exposure to myelin from 68% to 38%.

DISCUSSION

These results suggest that growth cone collapse is in part, mediated by a G protein-sensitive system. Since pertussis toxin does not effect the number of collapsed growth cones under basal conditions, it appears that growth cones might cycle between collapse and non-collapsed states normally by some mechanisms that are independent of G proteins. The stimulation of G proteins by mastoparan, which directly and most potently affects G_o and G_i, causes enhancement of growth cone collapse. Two different types of inhibitory substances, crude membranes or partially purified myelin, are potent collapsing agonists and their effects are blocked to a large degree by pretreatment with pertussis toxin. Both dorsal root ganglia and retinal cells respond to these inhibitors, and in both cases the collapsing effect is abrogated by pertussis toxin, suggesting that the intracellular cascade that regulates collapse may be universal.

Neither the inhibitory ligands themselves nor their receptors on the nerve growth cone have been identified. This work suggests that such receptors may be members of the family that are linked to G proteins. These include predominantly, the seven membrane spanning domain receptors, and at least one example of a single membrane spanning receptor (Okamoto *et al.*, *Cell 62*:709-717 (1990)). The collapse of growth cones induced by serotonin and dopamine would be consistent with these results, since these soluble agonists act via G protein-coupled receptors. Components of the cell membrane or matrix may also exert their inhibitory effects by stimulation of G protein-coupled receptors. L1 and N-CAM, for example, can change PC12 cell fate, and the entry of calcium by pertussis toxin-sensitive means (Doherty *et al.*, *Cell 67*:21-33 (1991)). The nature of the linkage between such receptors and G proteins remains to be determined, and it is conceivable that such receptors might be present at quite low quantities on the membrane, since

their signals could well be amplified by the G proteins. It is feasible to isolate such receptors by virtue of their linkage to G proteins (Birnbaumer *et al., Crit. Rev. Biochem. Molec. Biol. 25*:225-244 (1990)) or by conservation of structure within the seven membrane spanning family (Lefkowitz *et al., J. Biol. Chem. 263*:4993-4996 (1988). This would permit the direct identification of the receptors responsible for registering inhibitory signals.

The biological relevance of growth cone collapse has been presumed to reside in its relationship to inhibition of growth. Inhibition might affect either the absolute length of axons or their direction of growth, or the nerve terminal remodelling that refines connectivity. This terminal plasticity continues throughout life, and may underly long-term memory. Furthermore, perturbation of neuronal plasticity may be associated with certain types of neuronal degeneration. Hence, the systems that regulate morphogenesis may be similar to those responsible for long-term changes in the function of neuronal connections.

Victims of strokes, at best, recover only partial neuronal function (when the edema resolves), a recovery that does not include significant regrowth of axons in the central nervous system. Therefore, agents which modulate this pertussis toxin-sensitive G-protein system would prevent the actions of the inhibitory components of myelin and brain membranes that retard such growth. In this manner, these agents, by modifying the inhibition of neuronal growth caused by these components, facilitate neuronal growth, and hence, recovery from CNS injury or degenerative disorders.

G proteins facilitate the growth cone collapse stimulated by myelin and brain membranes. Therefore, one embodiment of the invention relates to the use of agents which regulate the action of G proteins, to modulate the inhibitory action on neuronal growth executed by components of myelin and brain membranes. In a particular aspect of this embodiment, GAP-43 would

be used to regulate G_o activity, thereby modulating the inhibitory action of myelin and brain membranes.

EXAMPLE X

GAP-43 is a Novel Internal Regulator of Protein Binding

In another aspect, the present invention is directed to the surprising discovery that GAP-43 acts within the cell to modify the binding capacity of other cell proteins, including that of G_o. As far as the present inventors are aware, this constitutes the first report of an important new class of internal regulatory proteins ("IRP"), of which GAP-43 is representative, comparable in effect and utility to external cell receptors. Those of skill will easily recognize that the IRP compositions and methods of this aspect of the invention allow the internal modulation of protein activity, and, thereby, of cell activity and function, in neuronal and non-neuronal cells.

Further, it has surprisingly been found that synthetic peptides comprising the amino terminus amino acids of GAP-43 duplicate exactly the modulation in GTP binding by G_o that is caused by the intact GAP-43 protein. For example, a peptide comprising the first 24 amino acids of GAP-43 stimulates GTPγS binding to the same level as GAP-43, acting as a full GAP-43 agonist.

Biologically active synthetic IRP peptides according to this embodiment comprise the following sequences:

I. MLCCMRRTKQVEKNDEDQKIEQDGV;

II. MLCCMRRTKQVEKNDEDQKIEQDG;

III. MLCCMRRTKQVEKNDEDQKIEQD;

IV. MLCCMRRTKQVEKNDEDQKIEQ;

V. MLCCMRRTKQVEKNDEDQKIE;

VI. MLCCMRRTKQVEKNDEDQKI;
VII. MLCCMRRTKQVEKNDEDQK;
VIII. MLCCMRRTKQVEKNDEDQ;
IX. MLCCMRRTKQVEKNDED;
X. MLCCMRRTKQVEKNDE;
XI. MLCCMRRTKQVEKND;
XII. MLCCMRRTKQVEKN;
XIII. MLCCMRRTKQVEK;
XIV. MLCCMRRTQVE;
XV. MLCCMRRTKQV;
XVI. MLCCMRRTKQ;
XVII. MLCCMRRTK;
XVIII. MLCCMRRT;
XIX. MLCCMRR;
XX. MLCCMR;
XXI. MLCCM;
XXII. MLCC; and
XXIII. functional derivatives thereof.

A related embodiment of the invention is directed to nucleotide sequences encoding the synthetic IRP peptides described above, which sequences will easily be determined by those of skill who have appreciated the teachings of the present invention.

A further aspect of the invention is directed to the discovery that a consensus amino acid sequence is found in GAP-43 and beta adrenergic receptors, said sequence comprising

hydrophobic-leu-cys-cys-x-basic-basic

or functional derivatives thereof. It will be further appreciated from the present teachings that the cysteines of the IRPs and IRP peptides of the invention may be prone to palmitoylation.

Those of skill also will appreciate that, by varying the structure of the IRP proteins and peptides of the invention, the target protein activity may be enhanced or, if desired, inhibited in an unprecedented manner. Thus, in another embodiment, there is provided a method of stimulating the binding activity of a desired protein, comprising introducing into an environment comprising said desired protein and its binding substrate an effective amount of an IRP peptide. The desired protein is preferably a G protein, and, most preferably, G_o. The preferred binding substrate is GTP, and GTPγS is most preferred. The environment is preferably that inside a living cell, which may be a central or peripheral neural cell.

Those of skill will appreciate that the methods of the invention may be carried out *in vitro, in situ,* or *in vivo,* with the latter being most preferred, keeping in mind the generally accepted principles of administration well known in the art, as discussed herein.

Those of skill who have the benefit of the teachings of the invention will appreciate that internal regulation of protein activity offers significant opportunities for the efficacious treatment of disorders in mammals, including humans, and that such treatment is especially valuable in preventing, ameliorating, or reversing the effects of neural disease or dysfunction, inasmuch as the compositions and methods of the invention are directed, *inter alia,* to mechanisms involved in neuronal growth and synaptic plasticity. It may be desirable, for a given medicinal indication, to reduce, as well as enhance, neural growth or plasticity. This may be accomplished, for example, by administering antibodies directed against the IRP peptides of the invention, or against the sites at which such IRP peptides have their physiological effect. Also, by such means, it is possible to regulate the activity of a desired protein

with an exquisite degree of control. Thus, in another aspect, the invention is directed to antibodies, preferably monoclonal antibodies, directed against the IRP peptides of the invention, and to functional or chemical derivatives thereof, said antibodies or their said derivatives being optionally detectably or therapeutically labeled.

In another aspect, the invention is directed to pharmaceutical compositions comprising the IRP peptide of the invention, together with a pharmaceutically acceptable carrier, and optionally comprising one or more therapeutically effective agents, as well as to pharmaceutical compositions comprising an antibody directed against the IRP peptides of the invention, together with a pharmaceutically acceptable carrier, and optionally comprising one or more therapeutically effective agents. Use of the pharmaceutical compositions of the invention will be accomplished by those of skill wihtout undue experimentation, keeping in mind those principles of administration as set forth herein and as are well known in the art.

In another aspect, the invention is directed to a method of modulating structural remodeling in a neural cell, comprising administering to said cell an effective amount of the compositions of the invention.

In yet another embodiment of the invention, the GAP-43 sequences of the invention have been used to isolate a G-like protein from neural cells. Using a GAP-43 column, a protein of MW 39,000 has been found to bind specifically a GAP-43 with high affinity. Cell extracts were introduced into columns containing GAP-43 in a buffer comprising 50 mM Tris, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The protein elutes in a single band with equimolar EDTA buffer. The protein does not react with polyclonal antibody to G protein. It is thus a distinct and novel protein associated with growing neurons, and forms an additional embodiment of the invention.

It also will be appreciated that IRP peptides according to the invention may be produced by any known means, for example, using recombinant

genetic methods as described hereinabove, and that nucleotide sequences encoding the IRP peptides of the invention may be deduced and optomized for a desired host expression system with the exercise of merely routine skill.

The significance of the novel compositions and methods of the invention in modifying cellular transduction systems such as G_o is enhanced when considered in conjunction with the demonstration herein that G_o is a major noncytoskeletal protein present in neuronal growth cones. The discovery that GAP-43, the function of which has not previously been known, modulates G_o activity, is evidence that GAP-43 is a long sought "missing link" between first and second messengers in cellular transduction systems. Although not wishing to be bound by any particular theory, it may be that persistent activation of G_o by GAP-43 could make the cells ignore their environment and hence grow constitutively. The present work also suggests that there is a family of other molecules that contain sequences similar to the amino terminus of GAP-43, and hence regulate G_o from inside the cell. It is of great interest that the amino terminus of GAP-43 bears a significant resemblance to the cytosolic domains of several G protein-linked receptors (such as the beta receptor). This suggests that GAP-43 may interact with G_o in a similar place in the molecule as do the cytosolic domains of the G protein-linked receptors.

It will further be appreciated that the novel modulatory effects of internal regulatory proteins and internal regulatory peptides (also called "IRP peptides" herein) may be, for example, stimulatory or inhibitory. In other words, the IRPs and IRP peptides, or other substances having such internal regulatory action, may have any of the possible pharmacologic effects, such as are known to those of skill in the art and are described in standard reference works including, for example, *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980), and Goodman *et al.*, *The Pharmacological Basis of Therapeutics*, 7th ed., Macmillan Publishing

Co., New York (1985), or the current editions thereof. As such, they may act as agonists, partial agonists, reverse agonists, antagonists, etc. Those of skill will recognize that the characterization of such effects may be accomplished using well known standard assay methods, such as those described herein with respect to GTP binding to G_o, for any binding protein of interest.

There is thus provided by the present invention a method of screening for a substance capable of modulating the binding activity of a desired protein, comprising introducing into an environment comprising said desired protein and its binding substrate a substance which it is desired to screen, and measuring the increase or decrease in substrate binding relative to substrate binding in the absence of said substance. The desired protein may be a G protein, and, preferably, is G_o.

METHODS AND RESULTS

GAP-43 Stimulates GTPγS Binding to G_o.

With the major growth cone membrane proteins identified, the inventors sought to determine whether these components were capable of forming intermolecular complexes. In particular, G_o and GAP-43 were examined since they are the major non-cytoskeletal proteins in the growth cone membrane. Attempts to physically isolate a GAP-43/G_o complex by gel exclusion chromatography, immunoprecipitation and affinity chromatography were unsuccessful.

To identify transient GAP-43/G_o interactions in solution, (35S)GTPγS binding to purified G_o was measured (Huff, *et al., J. Biol. Chem. 260*: 10864-10871 (1985); Northrup, *et al., J. Biol. Chem. 257*: 11416-11423 (1982)) in the presence of varying concentrations of purified GAP-43. GAP-43 itself does not bind (35S)GTPγS, but GAP-43 stimulates specific (35S)GTPγS binding to G_o to a level 310+40% (n=7) of control (Fig. 21).

This effect is saturable and is half maximal in the presence of 150-800 nM GAP-43. If G_o and GAP-43 were free in solution and not membrane-bound in vivo, then their concentrations would be about 2 uM in whole brain. Therefore, the affinity of GAP-43 for G_o measured in the assays described herein is consistent with in vivo conditions. All assays were conducted in the presence of 200 ug/ml BSA, so a nonspecific protein effect by GAP-43 cannot explain the stimulation of GTPγS binding. G_o is known to be partially inactivated during preincubation at 30°C without GTPγS (O'Dowd, *et al., J. Biol. Chem. 263*: 15985-15992 (1988)). GAP-43 does not affect the degree of G_o thermal instability. Although assessed under different conditions, ligand-receptor complexes which interact with G proteins stimulate GTPγS binding to approximately the same extent as does GAP-43 (Florio, *et al., J. Biol. Chem. 264*: 3909-3915 (1989)).

A GAP-43 Decapeptide Interacts With G_o.

To determine whether the cysteines of the amino terminal decapeptide, which are critical for membrane association, are also required for G_o regulation, a decapeptide with threonines substituted for the two cysteines was synthesized. This peptide had no effect on (35S)GTPγS binding to Go, demonstrating the specificity of the 1-10 peptide effect and the necessity of the cysteines (Fig. 21).

The other group of proteins known to stimulate GTPγS binding to G proteins are hormone and neurotransmitter receptors. Overall hormone and neurotransmitter receptor structure (e.g., a large extracellular region and seven transmembrane domains), is much different from that of GAP-43. The inventors thus searched for homologies between these proteins, focusing on those domains thought to interact with G proteins. For the receptors, site-directed mutagenesis and peptide competition studies have implicated the carboxyl end of the third cytoplasmic loop and the proximal end of the

cytoplasmic tail in G-protein coupling (Konig, *et al., Proc. Natl. Acad. Sci. U.S.A. 86*:6878-6882 (1989); O'Dowd, *et al., J. Biol. Chem. 263*:15985-15992 (1988); Strader, *et al., J. Biol. Chem. 262*:16439-16443 (1987)). B2-adrenergic receptor linkage with G_s is interrupted most specifically by a point mutation at a palmitylated cysteine in the cytoplasmic tail of the protein (*O'Dowd, et al., J. Biol. Chem. 264*:7564-7569 (1989)). The cysteines in the amino terminus of GAP-43 which are required for G_o regulation are also subject to palmitoylation in vivo (Skene, *et al., J. Cell Biol. 108*:613-624 (1989)). A comparison of the GAP-43 amino terminus with the amino terminal portion of the cytoplasmic tail from a series of receptors reveals a consensus sequence of hydrophobic-leu-cys-cys-x-basic-basic (Fig. 22). Within this sequence, the cysteines, where studied, undergo palmitoylation (Skene, *et al., J. Cell Biol. 108*:613-624 (1989); O'Dowd, *et al., J. Biol. Chem. 264*:7564-7569 (1989); Ovchinnikov, *et al., FEBS Letts. 230*:1-5 (1988)).

Other endogenous regulators of GTPγS binding to G proteins are unknown. However, the peptide toxin, mastoparan, reversibly activates G proteins. GAP-43 is not homologous to this peptide.

Although their overall structure, with a large extracellular region and seven transmembrane domains, is much different from that of GAP-43, the inventions nevertheless searched for homologies between these proteins and the amino terminus of GAP-43. The interaction of the b_2-adrenergic receptor with G_s is interrupted most specifically by a point mutation at a palmitylated cysteine in the cytoplasmic tail of the protein. The cysteines in the amino terminus of GAP-43 are also palmitylated. There is a consensus sequence shared by GAP-43 and these receptors which consists of hydrophobic-leu-cys-cys-x-basic-basic, where the cysteines are prone to palmitoylation.

Calmodulin Reduces GAP-43-Stimulated (35S)GTPγS Binding

GAP-43 has previously been shown to bind calmodulin, an interaction which is enhanced in the absence of calcium (Alexander, *et al., J. Biol. Chem.* *262*:6108-6113 (1987)). The physiologic relevance of this association is unclear. The addition of low concentrations of Ca++-calmodulin or EGTA-calmodulin to G_o causes no change in the ability of GAP-43 to stimulate (35S)GTPγS binding. However, higher concentrations of calmodulin lead to dose-dependent reduction in GAP-43-stimulated (35S)GTPγS binding (Figure 23).

DISCUSSION

The present data provide a growth cone mechanism for the coordination of extracellular signals with the expression of intracellular growth associated proteins during neuronal morphogenesis. The strikingly high levels of the alpha and beta subunits of G_o in the growth cone membrane suggest a major role for G_o in neurite regulation. The G_o concentration in the growth cone membrane exceeds that of another G protein, transducin, in the highly specialized outer segment of retinal photoreceptor cells.

In systems where G protein function is clearly defined, it is a link between the binding of extracellular signals to transmembrane receptors and the regulation of enzymes or ion channels which modulate intracellular second messengers. There are many heterotrimeric alpha-beta-gamma G proteins, differing primarily in their alpha subunits. In general, the alpha polypeptide exists in a GDP-bound state until an agonist-receptor complex causes the exchange of GTP for GDP. The GTP-bound activated alpha subunit then exerts its action on second messenger systems. Endogenous alpha subunit GTPase activity terminates signal transduction.

G_o is predominantly expressed in brain, where it is the major form of G protein. In adults, it is found in the neuropil (-), and the present data localize G_o to the tips of neurites in growing cells where it is the major non-cytoskeletal protein. G_o may respond to a variety of receptors and in turn regulate a number of intracellular systems, including calcium channels, potassium channels, phospholipase G and phospholipase A_2.

There is evidence that some matrix and soluble effects on the growth cone involve G-protein transduction. Antibodies to the growth cone localized cell adhesion proteins, N-CAM and L1, alter calcium levels and phosphotidylinositol metabolism in PC12 cells. The effect of these antibodies is blocked by the G_o/G_i inhibitor pertussis toxin (Schuch, *et al., Neuron 3:*13 (1989)). In certain heliosome neurons, serotonin, which acts via a G-coupled receptor, is a potent inhibitor of neurite extension.

The restricted localization of G_o suggests that the protein's regulation or action is mediated by one or more neuronal-specific molecules. GAP-43 is expressed only in neurons, and the protein is enriched in the growth cone. Therefore, the present inventors wondered whether GAP-43 might interact with G_o. GAP-43 enhances GTPγS binding to G_o. Furthermore, a small region of GAP-43, defined by a synthetic decapeptide, exerts this action. Stimulation of GTPγS binding by GAP-43 is similar to that by agonist-receptor complexes, and the decapeptide sequence has homology with these receptors. Although not intending to be bound by any particular theory, the most likely interpretation is that, *in vivo*, GAP-43 mimics transmembrane receptors and activates G_o, creating a GTP-bound alpha subunit which then triggers an intracellular second messenger system. Alternatively, GAP-43 binding to G_o might function primarily to disrupt G_o-receptor or G_o-effector interactions. It is also conceivable that GAP-43 is an effector of G_o activation by receptor in some as yet unknown manner.

The modulation of a G_o cone transduction system by a growth associated protein, GAP-43, provides a mechanism to integrate extracellular signals with an intracellular program for neuronal growth. Further regulation of the system could occur via other modifications, such as phosphorylation of the receptor by receptor kinases such as BARK, or phosphorylation of GAP-43 by protein kinase C. In this model, GAP-43 might synergistically enhance the response of G_o to extracellular ligands, or decrease responsiveness to ligands by overriding the dependency of G_o on receptor. In the later case, removal of GAP-43, as occurs during synapse formation, would restore sensitivity to extracellular ligands. The net effect of GAP-43 action on receptor effectiveness would depend on the relative concentrations of the components.

GAP-43 is unique among G-protein regulators in that it is an intracellular protein with no presently known capacity to respond directly to extracellular ligands. However, the intracellular regulation of membrane bound GTPase proteins does have precedence. Normal RAS proteins are stimulated by a widely distributed 120 kD intracellular protein, GAP, Despite the similarity in their names, GAP and GAP-43 are unrelated proteins.

The cysteines in the region of GAP-43 and receptors which stimulate GTPγS binding to G_o are subject to palmitoylation. In the present experiments, the amino terminal peptides and probably the GAP-43 (prepared by pH 11 extraction of membranes) exist in a non-palmitylated state. The relative ability of palmitylated versus non-palmitylated GAP-43, and G-linked receptors, to stimulate G proteins is unknown. It is possible that rapid palmitoylation-depalmitoylation plays a regulatory role for these proteins.

The persistence of G_o expression in the adult nervous system (Worley, *et al., Proc. Natl. Acad. Sci. USA 83:*4561 (1986)) implies roles other than the regulation of neurite outgrowth during development and regeneration. GAP-43 also exists in discrete regions of the adult brain, and the immunohistochemical maps for the two proteins are strikingly similar, if the

cerebellum is excluded (Benowitz, *et al., Trends Neurosci. 10:*527 (1987); Skene, *Ann. Rev. Neurosci. 12:*127 (1989)). The localization of GAP-43, the nature of its gene regulation, and especially the correlation of its phosphorylation state with long term potentiation in the hippocampal slice (Routtenberg, *Ann. N.Y. Acad. Sci. 444:*203 (1985)) has suggested a role for GAP-43 in synaptic plasticity in the adult (Benowitz, *et al., Trends Neurosci. 10:*527 (1987); Skene, *Ann. Rev. Neurosci. 12:*127 (1989)). It is noteworthy that the G_o/G_i antagonist, pertussis toxin, blocks long-term potentiation, perhaps implicating G_o in this process as well (Goh, *et al., Science 244:*980 (1989)). Hence, G_o may transduce intracellular and extracellular signals for neurite extension during development and for synaptic plasticity in the adult nervous system.

From the foregoing, those of skill will appreciate that, although specific embodiments of the invention have been described herein for illustrative purposes, various modifications may be made without deviating from the spirit and scope of the invention. Acordingly, the invention is not limited except as by the appended claims.

EXAMPLE XI

GAP-43 Modulates Cell Shape Via A Short G Protein Activator Domain

GAP-43 enhances filopodial formation in non-neuronal cells

Previous experiments described in Example V, showed that GAP-43 can enhance filopodial formation in non-neuronal cells. This phenomenon depends on incubation time after plating (Figure 28). COS cells expressing transfected genes were detected by immunofluorescent staining, and then the positive cells were examined under Nomarski optics. At all times, a greater

percentage of GAP-43-expressing COS cells have filopodia than do control cells. The difference is detectable as soon as the first cells attach, and then decreases rapidly over the ensuing 30 minutes. CHO cells stably transfected with GAP-43 expression vectors also exhibit more filopodia than do control cells in this period shortly after plating (Figure 24).

To determine whether the production of filopodia is dependent on contact with a substratum, cells were fixed in suspension, and filopodia quantitated. More filopodia-bearing cells are seen among the GAP-43 expressing cells under these conditions (shown as zero time point in Figure 24). The presence of filopodia is not significantly altered by varying the length of time between trypsinization and analysis from 5 minutes to 120 minutes. Thus, GAP-43 is not merely altering the recovery from trypsinization.

EXPERIMENTAL PROCEDURES

GAP-43 expression vectors

The synthesis of many of the plasmids has been described previously. In brief, the GAP-43 plasmids were all derived from CDM-8 and contain an SV40 origin of replication, and the rat GAP-43 cDNA sequence under the control of a CMV promoter. The point mutations of arg^6 to gly, of arg^7 to gly and of lys^9 to gly were created from the GAP-43 vector by oligonucleotide-directed mutagenesis, as described previously for the $cys^{3,4}$ to thr substitutions. The CAT and GAP-43/CAT expression vectors were constructed as described previously. The GAP-43(1-10)/CAT constructs encode a stretch of 17 amino acid residues, VDLQASLARFSGAKEAK, linking GAP-43 to CAT. The GAP-43(1-40)/CAT and GAP-43(1-6)CAT proteins are linked by ARVDLQASLARFSGAKEAK. All mutations and fusions were confirmed by DNA sequencing.

Non-neuronal cell culture and transfection

A431 epithelial cells were maintained in DMEM, 7.5% fetal bovine serum. DNA transfections were by the calcium phosphate procedure, and included a neomycin resistance gene expression vector, pDOJ (Bloch *et al., Mol. Cell Biol. 9*:5434-5439(1989)), and a 5-fold excess of a GAP-43 expression vector (Zuber *et al., Science 244*:1193-1195 (1989a)). Stable transfectants were selected in 400 μg/ml G418 and then screened for protein expression by immunoblotting. After selection, clones were maintained without G418. Those clones with the highest levels of expression were used in subsequent experiments.

The control and GAP-43-expressing CHO cell lines have been described previously. COS-7 cells were transfected with equal amounts of different DNAs by the DEAE-dextran method or by electroporation. Forty hours after transfection, cells were analyzed in the filopodial and spreading assays.

Immunoblots

Twenty μg of total protein from various A431 lines was separated by SDS-PAGE, and then transferred to nitrocellulose and stained for GAP-43 as described previously (Strittmater *et al., J. Biol. Chem. 266*:22465-22471 (1991)). Membrane and soluble fractions from COS cells were analyzed by immunoblot as described previously (Zuber *et al., Nature 341*:345-348 (1989b)).

Analysis of filopodial formation

Filopodial formation was assessed by modification of previous methods. Cultures of CHO, or COS-7 cells were trypsinized at 30-50% confluency with 0.25% trypsin for 5 minutes at 37°C. The cells were then diluted into at lest 20 volumes of serum-containing medium and plated onto glass slides precoated with poly-L-lysine at 50 mg/ml in PBS for 2 hour. After various incubation times at 37°C (8 minutes in routine assays) the medium was aspirated and the cells fixed. CHO cells were fixed with 2% Glutaraldehyde in PBS, and stained with 0.1% Coomassie Blue in 25% isopropanol, 10% acetic acid. COS-7 cells were fixed with 3.7% formaldehyde in PBS and then incubated for 1 hour in 5% normal goat serum, 0.1% Tyriton X-100, PBS with 1:1000 rabbit anti-GAP-43 serum, or with 1 μg/ml anti-CAT antibody (5'-3', Inc.). Bound antibody was detected by fluorescence after incubating with fluorescein-labelled goat anti-rabbit IgG.

CHO cells with filopodia were quantitated by observation at 400X magnification with Nomarski optics. COS cells expressing the protein of interest were identified by fluorescence microscopy and then positive cells were scored positive or negative for any spike-like protrusion from the cell body greater than 2 μ in length. In each experiment, 50-200 cells were counted. Each cell line or transient expression assay was examined in at least three separate experiments.

To measure filopodia formation in the absence of substratum contact, trypsinized COS cells were centrifuged at 1000 x g and the pellet was resuspended in fixative and the cells were stained as described above.

GAP-43 inhibits non-neuronal cell spreading

Filopodia are transient, so that few GAP-43 expressing COS or CHO cells exhibit them after 30 minutes. However, over the next 1 to 4 hours, the shape of GAP-43 cells can be distinguished from control cells by another attribute, the rate of cell spreading. When control COS cells are plated on poly-L-lysine coated glass, cell spreading occurs over the first 60 minutes (Figure 25). The rate of spreading by GAP-43-expressing cells is slower, although the degree of spreading eventually reaches the same level. One day after plating, cell shape is indistinguishable between control and GAP-43 cells.

To examine the generality of these changes in cell spreading, stably transfected clones derived from CHO and A431 cells were also studied. Control A431 cells spread more extensively than CHO or COS cells and therefore provide the most convenient assay system for cell spreading. GAP-43 expressing A431 clones spread less rapidly than do control lines, and a clear diminution in cell area is apparent 2 hours after plating on laminin-coated glass (Figure 25). CHO clones expressing GAP-43 also exhibit a decrease in spreading, but since spreading is less extensive in control CHO cells, this is more difficult to quantitate. Immunoblot analysis of GAP-43-expressing A431 cells demonstrates a GAP-43 concentration that is 10-50% of that found in whole neonatal rat brain, so that these effects on non-neuronal cell shape occur at physiologic levels of GAP-43 (Figure 25).

Several factors which are known to alter cell spreading were considered as explanations for this GAP-43 effect. In all cases, control and experimental cultures were maintained at the same cell density before and after trypsinization, so that differences in cell density cannot account for the results. The stage of the cell cycle could conceivably alter spreading, but GAP-43 cells have the same doubling time (23 $\pm$ 1 hour, SEM, n = 6 lines) as control lines (22 $\pm$ 2 hours, SEM, n = 6 lines), so this is an unlikely mechanism. Substrate coating (poly-L-lysine-coated glass, versus laminin-coated glass,

versus tissue culture plastic) alters the rate of spreading but not the degree of difference between GAP-43 and control. No difference was detected in the adhesion of metabolically labelled control and GAP-43 cells (Figure 25). This is consistent with the observation that induction of filopodia occurs in GAP-43-expressing cells prior to contact with the substratum.

Analysis of cell spreading

Cultures of CHO, A431 or COS-7 cells were trypsinized, diluted and plated as for the filopodial assay. Glass slides were precoated with laminin at 10 μg/ml in PBS for 1 hour, or with poly-L-lysine at 50 mg/ml in PBS for 1 hour. Routine experiments employed laminin. After 70 minutes (COS cells) or 120 minutes (CHO and A431 cells) at 37°C in 5% CO_2, the medium was aspirated and the cells were fixed. The cells were fixed and stained as described for the filopodial assay.

The size of the stained cells was determined from micrographs of randomly chosen fields by an observer unaware of the identity of the samples. For 75 consecutive cells, the maximal and minimal diameter passing through the nucleus was recorded. The trends in the data were identical regardless of whether maximal or minimal data were analyzed, but all data in this example is based on the minimal diameter. Cells were classified as "spread" if the minimal diameter exceeded 20 μ.

Adhesion of A431 cell lines

Twenty-four hours prior to the attachment assay, A431 cultures at 20-30% confluency were incubated with medium containing 1 μCi/ml of (^{35}S)methionine. To initiate the assay, cells were washed twice with PBS, incubated for 5 minutes with 0.25% trypsin and then washed again with growth medium and plated on laminin-coated plastic at a density that would yield approximately 20% confluency if all cells attached. After 45 minutes at

37°C, the plates were washed three times for 30 seconds with PBS, adherent cells were released in 2% Triton X-100, and radioactivity was determined by liquid scintillation spectrophotometry. The 45 minute incubation produced attachment of about a third of the cells. By 90 minutes, 70-80% of the total radioactivity incorporated into cells was adherent.

Growth rate of A431 cell lines

Different cell lines were plated at 5-10% of confluency, and then trypsinized at 24 hour intervals over the next five days and counted with a hemocytometer. The exponential rate of increase between 10 and 50% confluency was used to determine the doubling time.

The amino terminus of GAP-43 is necessary and sufficient to induce filopodia and to inhibit cell spreading

The ability of GAP-43 to alter non-neuronal cell shape provides a bioassay for determining its functional domains. This is useful since there is no simple way to test the action of such mutants on neuronal growth cones directly, due to the high levels of endogenous GAP-43 in all studied neuronal culture systems.

As several potentially functional domains have been described in GAP-43, the inventors sought to determine which domain causes these changes in cell shape. It has previously been shown that calmodulin binds to the region 39-55 (Alexander *et al., J. Biol. Chem. 263*:7544-7549 (1988)), and protein kinase C phosphorylates serine[41] (Coggins *et al, J.Neurochem. 53*:1895-1901 (1989)). In addition, as shown in the previous examples, the first ten amino acids of GAP-43 account for membrane targeting and contain two palmitoylated cysteines, and also stimulate G_o.

An initial screen examined the effect of GAP-43 deletions on activity in the two bioassays. A large internal deletion of GAP-43 from residue 41

through 189 removes both the protein kinase C phosphorylation site and the calmodulin binding domain. When transiently expressed in COS cells, this protein is as effective as intact GAP-43 in enhancing filopodia and in decreasing cell spreading (Figure 26). Thus, in these assay systems, neither phosphorylation nor calmodulin binding is necessary for GAP-43 action on cell shape. Of course, one or both activities could modulate the effectiveness of some other domain of GAP-43 in causing these changes.

It is known from phylogenetic studies that the C-terminal portion of GAP-43 shows little sequence conservation (LaBate *et al, Neuron 3*:29-310 (1989)). Therefore, the activity of the internal deletion GAP-43 mutant is more likely to reside in the N-terminal 1-40 fragment than in the 190-226 C-terminal fragment. To test this, a mutant GAP-43 with a deletion at the N-terminus from residue 2 through 5 was expressed; the deletion should remove both the membrane binding and the G protein activator function. This modified GAP-43 was devoid of activity in both cell shape assays (Figure 26).

To test whether the amino terminal domain from 1-40 is sufficient for GAP-43 action, a chimeric protein containing an N-terminal fragment of GAP-43 fused to the N-terminus of chloramphenicol acetyl transferase (CAT), was expressed in COS cells. This fusion protein caused the same increase in filopodia and decrease in spreading as does intact GAP-43. CAT is inactive in these assays. A series of GAP-43.CAT fusion proteins were examined to better define the active domain. The GAP-43(1-10)/CAT protein was fully active, but he GAP-43(1-6)/CAT protein caused no change in filopodia or spreading when compared to control cells expressing CAT.

The internal deletion protein and the GAP-43(1-40)/CAT fusion protein are capable of inducing filopodia in stably-transfected clones of CHO cells as effectively as in transiently-transfected COS cells (Figure 26).

Basic residues arg^6 and lys^9 are required for cell shape modulation

The above studies emphasize the contribution of the 6-10 region of GAP-43 to its ability to modulate cell shape. Three of the amino acid residues in this region (met^1-leu^2-cys^3-cys^4-met^5-arg^6-arg^7-thr^8-lys^9-gln^{10}) are basic. Because a similar array of basic residues (BBxB) is present in the canonical G protein activator domain of receptors (Okamoto *et al., Cell 62*:709-717 (1990a)), the inventors tested the importance of these residues for GAP-43 action.

Substitution of glycine for arg^6 or lys^9 completely blocks the action of GAP-43(1-10)/CAT and GAP-43 on cell shape (Figure 27). In contrast, substitution for arg^7, which results in a molecule with the same amino acid composition but a different sequence from the arg^6 substitution, does not abrogate GAP-43 or fusion protein effects. All of the combinations of double and triple basic site substitution are also inactive for the GAP-43(1-10)/CAT protein (only the triple basic substitution is shown in Figure 27). Thus, there is a highly specific sequence requirement in this region for two basic sites.

Cysteine residues cys^3 and cys^4 are also required for GAP-43 effect on cell morphology

In addition to the cluster of basic residues, the amino terminus of GAP-43 contains two adjacent cysteine residues at position 3 and 4. These cysteines undergo reversible cycles of palmitoylation (Skene *et al., J. Cell. Biol. 108*:613-624 (1989)) and this is almost certainly required for the membrane localization of the protein. It has previously been shown that substitution of threonine for one or both cysteines shifts GAP-43 from membrane to cytosolic fractions. In addition, these cysteines are important for G protein activation by GAP-43. Palmitoylation of these residues reversibly blocks interaction of GAP-43 and G_o, and a GAP-43(1-10) peptide with threonines substituted for

cysteines does not stimulate G_o. Thus, substitution of threonines for cys^3 and cys^4 in GAP-43 disrupts both G protein activation and membrane binding.

Given that the cysteine residues are necessary for G protein activation and membrane binding, the inventors set out to determine whether these residues are necessary for the effect of GAP-43 on cell morphology. If these residues are important for GAP-43 action on cell morphology, mutation of the cysteines should prevent GAP-43 action. Accordingly, deletion of the cysteines and two adjacent residues inactivated GAP-43 (Figure 26). To specifically delineate the importance of the cysteines, GAP-43 molecules with point mutations of the cysteines were expressed in COS cells. These proteins had no effect on filopodia formation or cell spreading (Figure 28).

Cell shape modulation is a separate action from the membrane binding activity of GAP-43

The N-terminal ten amino acids of GAP-43 are known to direct membrane binding G protein activation, and cell shape changes. G protein activation is correlated with activity in the cell shape assays, and experiments were conducted to determine whether membrane binding has the same sequence requirements.

As demonstrated previously the GAP-43(1-40)/CAT and the GAP-43(1-10)/CAT fusion proteins are localized to membrane fractions, as opposed to the soluble CAT molecule (Figure 29). The inactive GAP-43(1-6)/CAT fusion protein is detectable only in membrane fractions. Immunoblot analysis of COS cells expressing GAP-43 with the single basic amino acid substitutions reveals that all three mutants are found primarily in membrane fractions (Figure 30). The membrane localization of three inactive proteins, GAP-43(1-6)/CAT, arg^6-substituted GAP-43 and lys^9-substituted GAP-43, demonstrates that the morphoregulatory activity is not conferred simply by those amino acid residues which cause membrane binding.

The G protein activator region of GAP-43 is required to alter cell shape

The inventors tested different synthetic peptides derived from the amino terminal GAP-43 sequence for their ability to stimulate G_o *in vitro*. These experiments allow comparison of the structure required for filopodial extension described above with that for required G protein activation. If the action of GAP-43 on cell shape is due to changes in G protein activity, the structural determinants should be very similar in the two assays. The inventors found that the 1-25 and 1-10 peptides stimulate G_o, just as the amino terminal-CAT fusion proteins cause filopodial extension. Shorter peptides, such as 1-6, do not have effects in either assay (Figure 34).

The inventors also investigated the contributions of the cysteine residues at position 3 and 4 to GAP-43 stimulation of G_o (Figure 35). The effect of threonine substitution varies with the length of the peptide in which the substitution is made. One or both cysteines can be replaced in the 1-25 peptide without loss of activity, but not in the 1-10 peptide where the dithreonine peptide is inactive (Figure 35). Thus, the cysteines may have a weak contribution to the active domain which can be compensated for by the secondary structure present in the larger peptides.

The function of these cysteines was also studied by modifying their side-chains in both GAP-43 protein and peptide. It had been previously shown that large hydrophobic modifications of the cysteines with palmitate renders the 1-25 peptide and the protein inactive. These studies showed that small, neutral reagents such as iodoacetamide and N-ethylmaleimide do not alter GAP-43 protein activity, even though they remove the free sulfhydryls at position 3 and 4 as shown in (Figure 35). This supports the conclusions which resulted from the threonine-substituted 1-25 peptide. Thus, the basic residues in the 6-9 region appear to be the core region of the G protein activating domain in GAP-43.

Modification of residues near this region can block or even reverse the effect of the the activating domain. Preliminary results indicate that modification of the 1-25 peptide with p-chloromercuriphenylsulonate (PCMPS), causes inhibition of G_o. (Figure 35). The 1-10 peptide may also have inhibitory activity and be competitive with GAP-43 in its actions on G_o, when the cysteine sulfhydryls are been fully oxidized, thus introducing a negative charge at positions 3 and 4. These studies suggest that agents can be developed which will modulate the effect GAP-43 in cells.

The mutational analysis localizes the active portion of GAP-43 to the first ten amino acid residues. Remarkably, this stretch alone can confer GAP-43 activity on the otherwise inert protein, CAT. None of the other putative functional domains of GAP-43 are necessary to do so. Within this region the basic residues arg^6 and lys^9 are required, but arg^7 is not.

Recent data on G protein-coupled receptors of the seven-transmembrane domain family and on the IGF-II receptor have defined a G protein activator sequence consisting of a basic-basic-x-basic (BBxB) or BBxxB motif (Okamoto *et al., et al., Cell 62*:709-717 (1990); Okamoto *et al., Cell 67*:723-730 (1991), Figure 35). This importance of this BBxB motif is consistent with receptor mutagenesis studies (Ross, *Neuron 3*:141-152 (1989)) and is related to information on the stimulation of G proteins by the cationic amphiphilic peptide, mastoparan (Higashijima *et al., J. Biol. Chem. 265*:14176-14186 (1990)). GAP-43 has such a BBxB sequence at position 6-9, the N-terminal region which activates G_o and modulates cell shape.

Within the IGF-II receptor motif, the first and third basic residues are critical for G_i stimulation (Okamoto *et al., Cell 62*:709-717 (1990)), whereas the second is not (Okamoto *et al., Biochem. Biophys. Res. Comm. 179*:10-16 (1991b)). Similarly, if either the first or third basic residues in the BBxB region of GAP-43 is mutated, the protein loses activity in the cell shape assays, whereas the second basic residue is not essential.

The G protein activating function of the IGF-II and β2-adrenergic receptors also depends on basic amino acids N-terminal to the BBxB or BBxxB sequence (Okamoto *et al., Cell* 62:709-717 (1990); Okamoto *et al., Cell* 67:723-730 (1991a)). No such basic residues are present N-terminal to this region in GAP-43, but he α-amino group of the protein is just five residues away and may contribute a positive charge. The high degree of correspondence between the sequence required for G protein activation in receptors and that required for GAP-43 to modify non-neuronal cell shape strengthens the hypothesis that GAP-43 acts by modifying G protein transduction.

GAP-43 activation of G_o can be distinguished from receptor stimulation of G proteins by several criteria, including pertussis toxin sensitivity, βγ subunit dependence and phospholipid requirements (Strittmatter *et al., J. Biol. Chem.* 266:22465-22471 (1991)). There are also some differences in the sequences of the G protein activator region of receptors and GAP-43. For GAP-43, the cysteine residues at position 3 and 4 appear to be involved in G_o activation in addition to the basic residues. In the cell shape assays, GAP-43 is inactive without these cysteines. It may be that this dependence reflects a necessity for membrane association, which is also mediated by these cysteines, or that it reflects direct interruption of G protein interactions, or both.

DISCUSSION

The major finding of the present study is that the G protein activator region of GAP-43 accounts for its modulation of non-neuronal cell shape. This provides further evidence that GAP-43 regulates G protein transduction within cells, and supports the hypothesis that GAP-43 alters neuronal growth cone motility by such a mechanism.

GAP-43 alters the morphology of non-neuronal cells

The study of GAP-43 gene expression and protein localization have led to the hypothesis that it might influence growth cone motility. Thus, assessment of non-neuronal cell morphology was conducted in order to develop a functional bioassay for examining the molecular mechanism of GAP-43 action. This bioassay more fully characterizes the previous observation that GAP-43 enhances the propensity of non-neuronal cells to form filopodia. Also described is a GAP-43-induced decrease in the rate of cell spreading.

Although the growth cone is notable for its profusion of filopodia and lamellipodia (Lockerbie, *Neurosciencec 20*:719-729 (1987); Bray *et al., Annu. Rev. Cell Biol. 4*:43-61 (1988)), these structures are seen in other motile cells. Both growth cone motility and non-neuronal cell shape depend to a major degree on the same cytoskeletal mechanisms (Smith, *Science 242*:708-715 (1988); Bray *et al., Science 239*:883-888 (1988); Bray *et al., Annu. Rev. Cell Biol. 4*:43-61 (1988)). Thus, the filopodial formation caused by GAP-43 is most likely due to an increased tendency to exhibit one facet of the non-neuronal cell shape repertoire, rather than the endowment of these cells with a "pseudo-neuronal" phenotype. It seems reasonable to suggest that these effects are related to the action of GAP-43 at the neuronal growth cone, given that both filopodial extension and lamellipodial spreading are prominent features of the growth cone, and that the levels of GAP-43 expressed in these cells are within the range found in brain.

Enhancement of filopodia formation by GAP-43 is seen before contact with a substratum, and diminished spreading is exhibited over the subsequent hours after plating. It seems likely that the two morphogenic activities reflect the same underlying mechanism, since they are affected similarly by mutations in GAP-43. Their temporal disparity suggests that filopodia may need to resolve before cells can spread, or that filopodia and cell spreading are alternate cellular forms and that GAP-43 shifts an equilibrium towards

filopodial extension. These changes are observed during periods when cellular structure is being reorganized, and do not appear to result from alterations in substratum adhesion. They could reflect an indirect alteration of cytoskeletal dynamics by GAP-43.

Other interactions of GAP-43

Although they overlap, the membrane targeting domain of GAP-43 is not identical to its morphoregulatory domain. Three proteins, the GAP-43(1-6)/CAT fusion, the arg^6 substituted GAP-43 and the lys^9 substituted GAP-43, are localized to the membrane but are inactive in the filopodial and spreading assays. Thus, the membrane targeting region is not sufficient, by itself, to effect cell shape changes. This region may be necessary, but this is unclear at present, because all of the cysteine mutations which prevent membrane binding also alter G protein activator function. It is interesting to note that if membrane binding by the GAP-43(1-6)/CAT protein is dependent on palmitoylation, then a very short sequence is capable of directing palmitoylation.

Serine which is subject to phosphorylation by protein kinase C, and the calmodulin binding region of GAP-43 are not required for its action on non-neuronal cell shape. These sites may regulate activities not tested in this assay, or they might modulate the activity of the amino terminal domain of GAP-43, increasing or decreasing the concentration of GAP-43 required for cell shape modulation. Such a quantitative change might be too subtle to be readily detected in these assays.

G protein-mediated effects on cell shape and growth cone motility

The data presented here suggest that a G protein activating region of GAP-43 accounts for its action on non-neuronal cell shape. GAP-43 stimulates both G_i and G_o; thus even though there is little or no G_o in these non-neuronal cells, there is at least one potential G protein target for GAP-43 action.

Several studies suggest that G protein activity modulates non-neuronal cell shape. The spreading of macrophages has been linked to the activation of G protein-coupled receptors and their second messengers (Petty *et al., J. Cell Physiol. 138*:247-256 (1989)). F-met-leu-phe peptides modify chemotaxis and actin polymerization via G proteins (Bengtsson *et al., Proc. Natl. Acad. Sci. USA 87*:2921-2925 (1990)). G protein activity determines the degree of focus formation among clusters of fibroblasts (Klebe *et al., Proc. Natl. Acad. Sci. USA 88*:9588-9592 (1991)). Mutations in G_α subunits are responsible for mutation in Dictyostelium (Deverotes, *Science 245*:1054-1058 (1989)), both of which disrupt developmental morphogenetic events. Second messengers known to be dependent on G protein activation have well-characterized actions on actin cytoskeletal dynamics (Hartwig *et al., Curr. Opin. Cell Biol. 3*:87-97 (1991)).

There are also several reasons to suggest that modulation of G_o and/or G_i would alter growth cone motility. The soluble extracellular molecules, serotonin, dopamine and thrombin, activate G protein-coupled receptors in certain cells and cause growth collapse and cessation of neurite elongation (Haydon *et al., Science 226*:561-564 (1984); Lankford *et al., Proc. Natl. Acad. Sci. USA 95*:2839-2843 (1988); Rodrigues *et al., Proc. Natl. Acad. Sci. USA 87*:9693-9697 (1990); Suidan *et al., Neuron 8*:363-375 (1992)). Direct regulation of G protein activity with GDPβS, GTPγS, pertussis toxin, aluminum fluoride or mastoparan alters the rate of neurite outgrowth from chick embryonic sympathetic neurons. The effects of NCAM and N-cadherin

on neurite growth are blocked by pertussis toxin. Thus, it is clear that modulation of G_o and/or G_i by GAP-43 could change growth cone activity.

Generally, G proteins respond to complexes between extracellular ligands and transmembrane receptors (Gilman, *Annu. Rev. Biochem. 56*:615-649 (1987); Ross, *Neuron 3*:141-152 (1989)). G protein regulation by intracellular proteins such as GAP-43 is a novel mechanism. Whether other examples exist is unknown, but is not an unreasonable speculation given that heterotrimeric G proteins appear to regulate intracellular processes which occur independently of extrinsic signals (Columbo *et al., Science 255*:1695-1697 (1992)).

GAP-43 Amino Terminal Peptides Stimulate Neurite Growth

Since the amino terminal domain alters non-neuronal cell shape and activates G proteins, the inventors added amino terminal peptides to cultured cell lines which can be differentiated to a neuronal phenotype. The 1-10 peptide increased neurite outgrowth from differentiated N1E-115, NG108 and PC12 cells (Figure 36). A similar peptide known to be inactive in G_o assays, 1-10 with both cys changed to thr, has no effect. The longer peptides, 1-20 and 1-25, do not stimulate outgrowth like the 1-10 peptide. Presumably this occurs because the peptides must act intracellularly, like GAP-43 protein, and the 1-10 peptide has greater access to the cell interior due to its smaller size. To test this hypothesis directly and confirm the intracellular action for the 1-10 peptide, the inventors added the peptides to neuroblastoma cells briefly permeabilized with phosphatidylcholine as described previously. As expected, after this treatment, the longer peptides do not have a stimulatory action which is equal to that of the 1-10 peptide (Figure 36).

The previous Example X demonstrated that GAP-43, an IRP peptide, modulates the activity of G proteins, and through this action, modulates neuronal structural remodeling. The above experiments confirm the surprising

discovery by the inventors that the amino terminal domain of GAP-43 alone, increases neurite outgrowth, and that this action occurs as a result of the regulation of G-proteins.

Therefore, in one embodiment, the invention relates to a particular IRP peptide, comprising an N-terminal amino acid sequence consisting of

MET LEU CYS CYS MET ARG ARG THR LYS GLN.

This N-terminal peptide of GAP-43 has all of the attributes, and is used for all of the purposes previously described for other IRP peptides. This peptide is particularly advantageous in that, because of its small size, it is useful in those situations in which intracellular access requires a smaller peptide.

For example, in one embodiment, the N-terminal peptide of GAP-43 may be used within a cell to modulate the binding capacity of cellular proteins, including G-proteins, and thereby regulate cellular activity and function in nueuronal and non-neuronal cells. These modulatory effects may be either inhibitory or stimulatory, thus acting as agonists, partial agonists, reverse agonists, or antagonists, with respect to a particular physiological effect.

In another embodiment, the N-terminal peptide of GAP-43 may be used to modulate the structural remodeling or plasticity of central or peripheral neuronal cell. It will be appreciated that this modulation may result in either stimulation or inhibition or neuronal growth, depending upon the particular G protein cascade which is modulated.

Furthermore, using the guidance provided in the disclosure, one may obtain modified N-terminal GAP-43 peptides which also modulate G-protein activity, and thereby neuronal growth. Therefore, another embodiment of the invention relates to the N-terminal peptide of GAP-43 in which the cysteines at positions 3 and 4 are chemically modified or replaced with other amino acids. In particular, these modified peptides include the N-terminal peptide

in which cys^3 and cys^4 are palmitoylated or treated with other sulfhydryl modifiers. Such sulfhydryl modifiers include iodoacetamide, N-ethylmaleimide, and p-chloromercuriphenylsulonate. Other modified peptides include N-terminal peptides of GAP-43 in which cys^3 and cys^4 are replaced with amino acids, such as THR, ASP or GLU.

In another embodiment, these modified GAP-43 N-terminal peptides may be used in the same manner as other IRP peptides, and in all the methods previously described. For example, the modified compounds may be used to modulate the binding capacity of proteins, such as G proteins, within a cell so as to cause stimulation or inhibition of a desired effect. These compounds may also be used intracellularly to modulate neuronal structural remodeling. Modulation of neuronal structual remodeling may result in either stimulation or inhibition of neuronal growth, depending upon the G protein system which is modulated.

It is also appreciated that, by using the guidance provided in the foregoing examples, one of skill could develop additional N-terminal peptides of GAP-43 containing other amino acid substitutions, which modulate G protein activity.

In another aspect, the invention is directed to pharmaceutical compositions comprising the N-terminal peptide of GAP-43 and the modified derivatives thereof, together with a pharmaceutically acceptable carrier, and optionally comprising one or more therapeutically effective agents. Use of these pharmaceutical compositions of the invention will be accomplished by those of skill without undue experimentation, keeping in mind the principles of administration as set forth herein and as are well known in the art.

Those of skill will appreciate that the methods of the invention may be carried *in vitro*, *in situ* or *in vivo*, with the latter being most preferred, keeping in mind the general accepted principles of administration which are well known in the art.

As appreciated by those of skill, modulation of neuronal growth provides significant opportunities for the efficacious treatment of neural disorders in mammals, including humans. Thus, the above embodiments are especially valuable in preventing, ameliorating, or reversing the effects of neural disease or dysfunction. For example, stimulation of neuronal growth may be especially beneficial in the treatment or prevention of neural developmental abnormalities, neuronal repair from acute injury, or degenerative diseases of the brain. Furthermore, one of skill in the art would appreciate that it may be desirable in certain clinical situations, to inhibit, rather than stimulate, neuronal growth, or to alternatively inhibit and stimulate neuronal growth in the same subject.

EXAMPLE XII

GAP-43 Both Directly Modulates G Protein-Related Second Messenger Systems Within Cells And Amplifies the Sensitivity of G-Protein-Coupled Receptor Transduction

Certain cellular functions are regulated by signal molecules, such as neurotransmitters, hormones and growth factors, which are detected by specific receptors on the plasma membrane of the responding cell. Stimulation of a receptor initiates a cascade of biochemical processes that produce an intracellular signal, mediated by G proteins, which causes a change in the behavior of a cell, for example, secretion of an enzyme, contraction, growth or cell division.

As previously shown, both GAP-43 and G protein-coupled receptors activate G proteins by enhancing guanine nucleotide exchange, and there are sequence similarities in the G protein-interacting regions of the two proteins. This suggests that GAP-43 and receptors might act either antagonistically or synergistically to modulate G protein activity.

Studies with purified receptor Go and GAP-43

In order to demonstrate that GAP-43 enhances receptor activation of G proteins, the inventors conducted several studies to further investigate the interaction of GAP-43 with receptor-G-protein effector systems within cells. The following experiments show that GAP-43 and receptors act in concert to activate G-protein dependent systems. The experiments involved the incubation of phospholipid vesicles containing purified muscarinic m2 receptor and G_o, with various concentrations of GAP-43 and the receptor agonist, carbachol.

METHODS

The purification of GAP-43 from neonatal rat brain has been previously described (Strittmatter *et al., J. Biol. Chem. 266*:22465-22471 (1991)), and the concentration was determined by a dye-binding method (Biorad). Recombinant human muscarinic m2 receptors were isolated from baculovirus-infected insect cells and reconstituted with purified bovine brain G_o into phospholipid vesicles (Parker *et al., J. Biol. Chem. 266*:519-527 (1991)). GTPase activity at 30°C was measured in the presence of 0.22 nM receptor, 5 nM G_o, the indicated concentrations of GAP-43, 30 nM (α^{32}P)GTP, 1.5 μM GDP, 2 mM $MgCl_2$, and 1 mM EDTA with or without 100 μM carbachol (Higashijima *et al., J. Biol. Chem. 265*:14176-14186 (1990)). The effect of GAP-43 in these assays was most prominent at 30°C, in the presence of GDP.

The activation of G_o was monitored by the level of steady state GTPase activity (Figure 37). Carbachol alone doubled GTPase function, and saturating concentrations of GAP-43 cause a 45% increase. The presence of carbachol and GAP-43 resulted in higher levels of GTPase than either agent alone and the effects are more than additive, with a total increase of 175% from basal values. Thus, GAP-43 and receptor appear to act in concert, and do not

compete with one another. This data provides a molecular basis for the GAP-43 augmentation of receptor action within cells.

GAP-43 augments receptor-stimulated chloride channels in Xenopus oocytes

To examine the interaction of GAP-43 with G protein transduction systems within living cells, the inventors utilized the *X. laevis* oocyte. This cell has a well-characterized pathway from transmembrane receptor to G protein to phospholipase C to inositol 1,4,5-triphosphate (IP_3) to intracellular Ca^{++} release to chloride channel (Moriarty *et al.*, *G proteins*, Iyengar, R. and Birnbaumer, L. eds., New York: Academic Press, Inc., pp. 479-501 (1990)). Injection of activated bovine brain G_o stimulates this cascade (Moriarty *et al.*, *Nature 343*:79-82 (1990)).

METHODS

GAP-43 immunoblots were prepared as described (Strittmatter *et al.*, *J. Biol. Chem. 266*:22465-22471 (1991)), using fractions obtained by sonicating whole neonatal rat brain or oocytes in 10 mM Tris-HCl, pH 7.5, 1% SDS. Protein was determined by the BCA method (Pierce). Fractions from oocytes injected with GAP-43 were processed within two hours of injection. Uninjected oocytes exhibit no detectable GAP-43 on immunoblots (Figure 38). Stage V and VI oocytes were removed from anesthetized (0.15% tricaine immersion) female *X. laevis* (Xenopus I, Ann Arbor, Michigan, USA). The oocytes were defolliculated by incubation with 2 mg/ml collagenase (GIBCO). The cells were stored at 18°C in 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Na Hepes, pH 7.6 (ND-96 solution) with 1.8 mM $CaCl_2$, 2.5 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. $5HT_{1c}$ receptor mRNA was synthesized with T7 RNA polymerase from plasmid M1C2.3-p7 (plasmid generously provided by L. Yu, Indiana

University) and 40 nl of a 50 ng/μl solution was injected into oocytes 2-4 days prior to further experiments.

All experiments, except acetylcholine application, utilized $5HT_{1c}$ receptor RNA-injected oocytes. Oocyte current responses were monitored at a membrane potential of -60 mV with a two-electrode voltage clamp apparatus (Dagan TEV-200). The 150 μl bath was perfused with ND-96 containing 0.3 mM $CalCl_2$, and acetylcholine (1 μM) or serotonin (1 μM) were included in the perfusion for brief periods. GAP-43 was introduced by pressure injection through pipettes of 3 μ diameter, and the volume of injection was pre-calibrated from the size of falling drops of buffer expelled from pipettes of equal diameter with videomicroscopic observation. For the lower concentration of GAP-43, 10 nl of 20 μM GAP-43 in 100 mM KCl, 5 mM Na Hepes, 0.1 mM DTT, pH 7.5 was injected, resulting in a final estimated concentration of 0.2 μM from a 200 pmol injection into the 1000 nl oocyte. The higher concentration of GAP-43 was 200 μM in the pipette, yielding 2 μM in the cell from a 2 pmol injection.

RESULTS

As described, oocytes were injected with two different quantities of GAP-43. The consequent intracellular concentration from the lesser amount is about one-twelfth that of whole neonatal rat brain, and from the greater amount is equal to or slightly less than that in newborn rat brain. An exact comparison of active GAP-43 concentrations is difficult because palmitoylation levels may vary between the oocyte and the brain, and this reversible post-translational modification regulates GAP-43 stimulation of purified G_o (Sudo *et al., EMBO J. 11*:2095-2102 (1992)).

The ability of receptor agonists to induce an inward current flow in voltage-clamped oocytes was compared before and after injection with buffer or GAP-43 (Figure 38). Only one frog yielded oocytes with an endogenous

acetylcholine response. GAP-43 injection more than doubles the total current flow induced by acetylcholine in these cells. No oocytes from twelve other frogs exhibited a response to acetylcholine. Hence, the inventors expressed an exogenous receptor, the rat serotonin$_{1c}$ ($5HT_{1c}$) receptor, by injecting *in vitro* transcribed mRNA three days before analysis. The magnitude of the 5HT-induced current in these cells is not altered by buffer injection, but is increased two- to five-fold by the lower GAP-43 concentration and greater than ten-fold by the higher concentration of GAP-43 (Figure 38).

In addition to facilitating receptor responses, GAP-43 has a direct, transient stimulatory effect on Ca^{++}-mediated chloride channel opening under certain conditions (Figure 39). The lower concentration of GAP-43 causes an oscillating inward current of 10-250 nAmps which lasts for 3-10 minutes, when injected 4-10 minutes after a 5HT response, but the same GAP-43 concentration elicits no response from naive oocytes. Thus, 5HT and GAP-43 are cross-sensitizers in this system. This may be related to previous observations that shortly after receptor stimulation, even though current amplitude returns to baseline, waves of calcium continue to reverberate within the oocyte; and the response to IP_3 injection is exaggerated (Lechleiter *et al., Cell 69*:283-294 (1992); (Parker *et al., J. Neurosci. 9*:4068-4077 (1989)).

The higher GAP-43 concentrations produce a 5-30 minute period of oscillating inward current, even without prior receptor stimulation (Figure 39B). The peak amplitude varies among different oocytes, and ranges from 30 to 2500 nAmps. This prolonged response is similar to that occurring after stimulation of certain G protein-coupled receptors in the oocyte, and appears to be mediated via the same G protein cascade (Lechleiter *et al., EMBO J. 9*:4381-4390 (1990)). The reversal potential for the GAP-43 induced current is -21 to -24 mV (not shown), which is the reversal potential for chloride in the oocyte (Kusano *et al., J. Physiol. 328*:143-170 (1982); Barish, *J. Physiol. 342*:309-325 (1983)). The response to GAP-43 is abolished if the oocytes are

co-injected with EGTA, as predicted for a calcium-mediated event (Figure 39).

Injection of 4 pmol of IP_3 results in a large inward current followed by refractoriness to application of IP_3, receptor agonist, or activated G protein for at least one hour (Moriarty *et al., Nature 343*:79-82 (1990); Singer *et al., Pflugers Arch. 416*:7-16 (1990)). Prior IP_3 injection also blocks the response to subsequent high concentrations of GAP-43 for up to two hours (4 of 4 cells, not shown). If the oocyte is allowed to recover from IP_3 for more than six hours, the inward current response to GAP-43 is similar to that of control oocytes. GAP-43 primarily stimulates this system rather than preventing the desensitization process induced by IP_3, since desensitization for both IP_3 and receptor agonists occurs normally even if GAP-43 is injected before IP_3 (Figure 40).

GAP-43 augments G_i inhibition of adenylate cyclase in A431 cells.

Non-neuronal cells increase their propensity to form filopodia and decrease their rate of spreading when transfected with GAP-43, and it has been shown that this is dependent on the G protein-interacting domain of GAP-43. This predicts that G protein-dependent second messenger systems will be altered in GAP-43-expressing cells with a modified morphology.

The inventors investigated whether GAP-43 transfectants have different cAMP levels. In the basal state, cAMP levels are low and unaffected by GAP-43. When stimulated by forskolin, which directly activates adenylate cyclase, control cell lines accumulate 100 times more cAMP than in the basal state. GAP-43-expressing A431 cells increase cAMP levels only 50-fold in the presence of forskolin (Fig. 41). This appears to be due to increased G_i function in the GAP-43 cells, because receptor-mediated stimulation of G_i with lysophosphatidic acid (LPA) deceases the level of cAMP in control cells to the level seen in forskolin-treated, GAP-43-expressing cells. Because the GAP-43 cells contain activated G_i, addition of LPA has no significant inhibitory effect

on cAMP levels in the presence of forskolin. The effect of GAP-43 expression on the action of forskolin and LPA is identical to that reported for transfection with mutated G_i a subunits that are constitutively activated ((Wong *et al.*, *Nature 351*:63 (1991).

Prior studies on purified G protein interaction with GAP-43 showed that G_i, but not G_s, is activated by GAP-43. The studies above argue that the predominant action of GAP-43 in these A431 cells is on G_i, and not G_s since the forskolin-stimulated cAMP level is decreased by GAP-43. The inventors tested this more directly by measuring isoproterenol-stimulated cAMP levels. The GAP-43-expressing cells increase cAMP levels only 4.5 fold as compared to 8-fold in the control cells (Fig. 42). LPA decreases isoproterenol-stimulated cAMP levels nearly to basal values in both control and GAP-43-expressing cells. These findings suggest that GAP-43 does not augment isopreternol stimulation of G_s and adenylate cyclase, but does increase the level of active G_i. The change in isoproterenol stimulation caused by GAP-43 is similar to the decrease in PGE_1 stimulation of adenylate cyclase in 3T3 cells transfected with activated α_i subunits (Wong, *et al. Nature 351*:63 (1991)).

DISCUSSION

These data demonstrate that intracellular GAP-43 can both augment receptor activation of a G protein transduction cascade, and directly stimulate the same system. It is likely that the molecular mechanism involves GAP-43 stimulation of the α subunit of a G protein, an interaction previously demonstrated for the purified protein. GAP-43 facilitation of receptor agonist action occurs at low GAP-43 concentrations, and therefore, maybe the more prominent effect *in vivo*. GAP-43 can be considered an intracellular modulator of the sensitivity, or gain, of G protein-coupled receptor transduction. In general, regulation of G protein cascades occurs through transmembrane receptors for extracellular ligands, (Gilman, *A. Rev. Biochem.*

56:615-649 (1987)), so GAP-43 control of G protein function is a novel facet of this system. The recent description of phosducin as an inhibitor of heterotrimeric G proteins is another example of intracellular modulation of G protein activity (Bauer *et al., Nature 358*:73-76 (1992)).

The present study further supports the previous demonstration that GAP-43 alters growth cone motility by regulating G protein activity. The data also supports the previous demonstration, that GAP-43 can modulate cell shape and, in some settings, increase neurite extension, that G_o and GAP-43 are highly concentrated in the growth cone membrane, and that G protein activation state alters neurite extension. Although the inventors do not wish to be bound by any particular theory, the most likely explanation is that in the growth cones of developing and regenerating neurons, GAP-43 could have two actions, one as a direct intracellular regulator of second messenger systems, and a second as a means to increase the sensitivity to receptor stimulation. A means for increasing the sensitivity to receptor stimulation is particularly important in the growth cone, where very shallow gradients of extracellular molecules must be sensed by single filopodia and amplified enormously to alter the behavior of an entire growth cone and axon. The localization of GAP-43 to the neuronal growth cone may reflect a requirement for high level amplification of G protein-mediated signals derived from a single filopodial contact. Thus, GAP-43 can be said to increase the "gain" of a G protein-based signal transduction system.

In addition to the effect exerted within neuronal cells, this unexpected, additional capability of GAP-43 provides a means for modulating intracellular receptor-dependent systems which are present in non-neuronal cells. Intracellular receptor-dependent systems have been shown to have a central role in an increasing array of cellular activities, ranging from mating in yeast, to chemically-induced movement in slime molds, to vision, smell, hormone secretion, muscle contraction and cognition in humans. In particular, G

protein-based signal transduction systems have important roles in the liver (glycogen breakdown), fat cells (fat breakdown), ovarian follicles (synthesis of estrogen and progesterone), kidney cells (water conservation), heart muscle (control of heart rate and force of pumping), smooth muscle cells in blood vessels (contraction, blood pressure elevation), lung (bronchodilation), gastrointestinal tract (gastric motility regulation), and retinal cells (detection of visual signals). Thus, GAP-43 may be used to regulate the effects of a variety of receptor-dependent systems, such as those listed above, by modulating receptor stimulation of G proteins.

In view of this surprising discovery, one embodiment of the invention relates to a method for augmenting activation of a desired protein by a receptor, comprising introducing into an environment comprising the protein and receptor, an effective amount of an IRP, such as GAP-43. In this embodiment, administration of GAP-43 enables modulation of the duration and thereby, magnitude of receptor activation, and thereby, the resultant intracellular signal. In a particular aspect of this embodiment, augmentation results in amplification of receptor activation and the intracellular signal.

Proteins which are suitable for activation include G proteins, and preferably, G_o. Examples of receptors include those which activate G proteins in receptor-based systems, and in particular, adrenergic (α_1, β_1 and β_2), cholinergic (α_1, α_2, platelet α_2, β_1, and β_2), rhodopsin, acetylcholine, including muscarinic and nicotinic, and serotonin. Suitable environments for augmentation of receptor activation include, but are not limited to, cellular compoments of the various organs recited above.

A particular aspect of this embodiment provides a means for augmenting receptor-activation of a G protein, and preferably G_o, by introducing GAP-43 into an environment which consists of a peripheral or central neural cell. In this manner, GAP-43 modulates structural remodeling of the neuronal cell. It is appreciated by one of skill that the net effect of

structural remodeling may be either stimulation or inhibition of neuronal growth.

Impairment of the function of signal transduction systems contribute to a variety of disorders and diseases involving cellular function, such as cholera, whooping cough, cancer, heart failure, diabetes, and neurological disturbances. Therefore, in another embodiment, administration of GAP-43 would also provide a means for the treatment or prevention of such disorders, in which receptor stimulation of G proteins is impaired. Thus, the amplification of receptor activation effected by GAP-43 would compensate for the deficiency in receptor stimulation, and thus allow the proper functioning of the signal transduction system.

174

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 40, line 3.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
21 December 1989	HB 10316

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Hybridoma, ANTI-GAP-43 (H5)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☑ This sheet was received with the international application

Authorized officer

Nathaniel R. Hayden
PCT INTERNATIONAL SERVICES DIVISION

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 40, line 3.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
21 December 1989	HB 10316

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Hybridoma, ANTI-GAP-43 (H5)
In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☑ This sheet was received with the international application

Authorized officer
Nathaniel R. Hayden
PCT INTERNATIONAL SERVICES DIVISION

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

WHAT IS CLAIMED IS:

1. Recombinant mammalian GAP-43, or a functional derivative thereof.

2. The composition of claim 1, wherein said mammalian GAP-43 is rat GAP-43.

3. The composition of claim 1, wherein said mammalian GAP-43 is human GAP-43.

4. The composition of claim 2, wherein said rat GAP-43 has an amino acid sequence as shown in or substantially similar to that shown in Figure 2.

5. The composition of claim 3, wherein said human GAP-43 has an amino acid sequence as shown in or substantially similar to that shown in Figure 5A.

6. A polypeptide comprising an amino acid sequence as shown in or substantially similar to that shown in Figure 2, or a functional derivative thereof.

7. A polypeptide comprising an amino acid sequence as shown in or substantially similar to that shown in Figure 5A, or a functional derivative thereof.

8. cDNA comprising a nucleotide sequence as shown in or substantially similar to that shown in Figure 2, or a functional derivative thereof.

9. cDNA comprising a nucleotide sequence as shown in or substantially similar to that shown in Figure 5A, or a functional derivative thereof.

10. A DNA expression vector comprising the cDNA of claim 8.

11. A DNA expression vector comprising the cDNA of claim 9.

12. A host cell transfected with the vector of any of claims 10 or 11.

13. The host cell of claim 12, wherein said cell is selected from the group consisting of prokaryotic cells and eukaryotic cells.

14. GAP-43 produced by the cell of claim 13, or a functional derivative thereof.

15. A method of producing mammalian GAP-43 or a functional derivative thereof, comprising transfecting a prokaryotic or eukaryotic host cell with a vector comprising cDNA encoding mammalian GAP-43, culturing said host cell in a suitable medium under conditions permitting expression of said mammalian GAP-43, and separating said mammalian GAP-43 from said medium.

16. Hybridoma strain H5, having accession number ATCC HB 10316, or a functional or chemical derivative thereof.

17. A hybridoma producing a monoclonal antibody having substantially the specificity of the monoclonal antibody produced by hybridoma strain H5, having accession number ATCC HB 10316.

18. Monoclonal antibody MAb anti-GAP-43 (H5), produced by hybridoma strain H5, said hybridoma strain having accession number ATCC HB 10316.

19. A monoclonal antibody having substantially the specificity of MAb anti-GAP-43 (H5) or a functional or chemical derivative thereof, said MAb anti-GAP-43 (H5) produced by hybridoma strain H5, said hybridoma strain having accession number ATCC HB 10316.

20. The antibody of claim 19, wherein said antibody is detectably labeled.

21. The antibody of claim 19, wherein said antibody is therapeutically labeled.

22. A pharmaceutical composition comprising the composition of any of claims 1,2,3,4,5,6,7 or 14, together with a pharmaceutically acceptable carrier.

23. The composition of claim 22, additionally comprising one or more therapeutically effective agents.

24. A method of determining or detecting mammalian GAP-43 in a sample, comprising contacting a sample suspected of containing GAP-43 with the antibody of claim 20, incubating said sample with said antibody so as to allow the formation of a GAP-43 antibody complex, separating complexed antibody from uncomplexed antibody, and detecting the labeled complexed antibody.

25. A kit useful for the determination or detection of GAP-43, comprising carrier means being compartmentalized to receive in close confinement therein one or more container means, wherein one or more of said container means comprises detectably labeled antibody to GAP-43.

26. The kit of claim 25, wherein said antibody is selected from the group consisting of polyclonal and monoclonal.

27. A method of inducing expression of GAP-43 in cells, comprising exposing said cells to an effective amount of nerve growth factor.

28. The method of claim 27, wherein said cells are neural cells.

29. The method of claim 28, wherein said cells are exposed to said nerve growth factor *in situ*.

30. A method of enhancing expression of GAP-43 in cells, comprising introducing into said cells a DNA expression vector comprising cDNA encoding GAP-43.

31. The method of claim 30, wherein said vector is introduced into said cells by transfection, transduction, or direct microinjection.

32. A method of promoting structural remodeling in neural cells, comprising inducing GAP-43 expression in said cells by the method of any of claims 28, 29, 30 or 31.

33. A method of promoting healing of damaged neural tissue, comprising inducing GAP-43 expression in and around said damaged neural tissue.

34. The method of claim 33, wherein said neural damage is caused by infarction with ischemia, transient ischemia without infarction, hypoxia, anoxia, anoxic encephalopathy, hypoperfusion, or stroke.

35. A method of modulating structural remodeling in neuronal cells, comprising exposing said cells to an effective amount of one or more substances selected from the group consisting of nerve growth factor, steroid and their functional derivatives.

36. A method of modulating synaptic plasticity in neuronal cells, comprising exposing said cells to an effective amount of one or more substances selected from the group consisting of nerve growth factor, steroid and their functional derivatives.

37. A method of modulating the microenvironment of neuronal cells, comprising exposing said cells to an effective amount of one or more substances selected from the group consisting of nerve growth factor, steroid and their functional derivatives.

38. A method of inhibiting GAP-43 expression in mammalian neuronal cells, comprising exposing said cells

to an effective amount of one or more steroids.

39. A method of modulating GAP-43 expression in fully differentiated mammalian neuronal cells, comprising exposing said cells to an effective amount of one or more steroids.

40. The method of any of claims 35, 36, 37, 38 or 39, wherein said steroid is a corticosteroid.

41. The method of claim 41, wherein said corticosteroid is selected from the group consisting of mineralocorticoid and glucocorticoid.

42. The method of claim 41, wherein said mineralocorticoid is selected from the group consisting of dexamethasone, corticosterone, aldosterone and progesterone.

43. A method of augmenting steroidal inhibition of GAP-43 expression in mammalian neuronal cells exposed to steroids, comprising exposing said cells to an effective amount of cycloheximide.

44. The method of any of claims 30 or 31, wherein said cells are non-neuronal cells.

45. cDNA encoding a membrane-targeting peptide comprising the nucleotide sequence

atg ctg tgc tgt atg aga aga acc aaa cag

or a functional derivative thereof.

46. A membrane-targeting peptide comprising an amino acid sequence selected from the group consisting of

I. MET LEU CYS CYS MET ARG ARG THR LYS GLN;
II. MET LEU CYS CYS MET ARG ARG THR LYS;
III. MET LEU CYS CYS MET ARG ARG THR;
IV. MET LEU CYS CYS MET ARG ARG;
V. MET LEU CYS CYS MET ARG;
VI. MET LEU CYS CYS MET;
VII. MET LEU CYS CYS; and
VIII. functional derivatives thereof.

47. A DNA sequence encoding a membrane-targeting peptide comprising nucleotides encoding an amino acid sequence selected from the group consisting of

I. MET LEU CYS CYS MET ARG ARG THR LYS GLN;
II. MET LEU CYS CYS MET ARG ARG THR LYS;
III. MET LEU CYS CYS MET ARG ARG THR;
IV. MET LEU CYS CYS MET ARG ARG;
V. MET LEU CYS CYS MET ARG;
VI. MET LEU CYS CYS MET;
VII. MET LEU CYS CYS; and
VIII. functional derivatives thereof.

48. A structural gene or fragment thereof, comprising, at its amino-terminus end, in phase, nucleotides encoding a membrane-targeting peptide having the sequence of claim 46.

49. A protein or peptide comprising, at its amino-terminus end, a membrane-targeting peptide comprising the sequence of claim 46.

50. A method for directing a desired protein or peptide to the membrane of a cell, comprising

(a) ligating to the amino-terminus of said protein or peptide a membrane-targeting peptide comprising the amino acid sequence of claim 46; and

(b) introducing the resulting protein or peptide comprising said membrane-targeting domain into a cell;

wherein the resulting protein or peptide of step (b) is directed to said membrane of said cell by said membrane-targeting domain.

51. The method of claim 50, wherein said cell is selected from the group consisting of neuronal and non-neuronal cells.

52. The method of claim 51, wherein in said neuronal cell said resulting protein or peptide of step (b) is directed to the growth cone region of said cell.

53. An antibody directed against the peptide of claim 46 or against the protein or peptide of claim 49, or a functional or chemical derivative thereof, said antibody or its said derivative optionally detectably or therapeutically labeled.

54. The antibody of claim 53, wherein said antibody is a monoclonal antibody.

55. A monoclonal antibody having substantially the specificity of MAb anti-GAP-43 (H5) or a functional or chemical derivative thereof, said MAb anti-GAP-43 (H5) produced by hybridoma strain H5, said hybridoma strain having accession number ATCC HB 10316.

56. Hybridoma strain H5, having accession number ATCC HB 10316, or a functional or chemical derivative thereof.

57. A nucleotide sequence as shown in Figure 13 encoding genomic GAP-43, or a functional or chemical derivative thereof.

58. A nucleotide sequence as shown in Figure 14 encoding the GAP-43 promoter, or a functional or chemical derivative thereof.

59. A promoter substantially as shown in Figure 14, characterized in that it contains multiple start sites and a consensus Pit-1 binding site, but lacks a TATA box and consensus Sp-1 binding sites, and further characterized in that it comprises long homopurine-homopurimidine stretches capable of taking on triple stranded (H-DNA) conformation.

60. A structural gene or fragment thereof, comprising, at its amino terminus end, in phase, the nucleotide sequence of claims 56 or 57, or a functional or chemical derivative thereof.

61. A DNA expression vector comprising the structural gene of claim 58.

62. A host cell transfected with the vector of claim 59.

63. An Internal Regulatory Protein (IRP).

64. An IRP peptide comprising an amino acid sequence selected from the group consisting of:

I. MLCCMRRTKQVEKNDEDQKIEQDGV;
II. MLCCMRRTKQVEKNDEDQKIEQDG;
III. MLCCMRRTKQVEKNDEDQKIEQD;
IV. MLCCMRRTKQVEKNDEDQKIEQ;
V. MLCCMRRTKQVEKNDEDQKIE;
VI. MLCCMRRTKQVEKNDEDQKI;
VII. MLCCMRRTKQVEKNDEDQK;
VIII. MLCCMRRTKQVEKNDEDQ;
IX. MLCCMRRTKQVEKNDED;
X. MLCCMRRTKQVEKNDE;
XI. MLCCMRRTKQVEKND;
XII. MLCCMRRTKQVEKN;
XIII. MLCCMRRTKQVEK;
XIV. MLCCMRRTQVE;
XV. MLCCMRRTKQV;
XVI. MLCCMRRTKQ;
XVII. MLCCMRRTK;
XVIII. MLCCMRRT;
XIX. MLCCMRR;
XX. MLCCMR;
XXI. MLCCM;
XXII. MLCC; and
XXIII. functional derivatives thereof.

65. A nucleotide sequence encoding the IRP peptide of claim 64.

66. An IRP peptide having the consensus amino acid sequence

hydrophobic-leu-cys-cys-x-basic-basic

or a functional derivative thereof.

67. An IRP peptide comprising an N-terminal amino acid sequence consisting of MET LEU CYS CYS MET ARG ARG THR LYS GLN; or a functional derivative thereof.

68. The IRP peptide of claims 64, 66 or 67, wherein the cysteines are prone to palmitoylation.

69. The IRP peptide of claims 64, 66 or 67 wherein the cysteines are replaced by an amino acid selected from the group comprising THR, ASP or GLU.

70. A method of modulating the binding activity of a desired protein, comprising introducing into an environment comprising said desired protein and its binding substrate an effective amount of an IRP peptide

71. The method of claim 70, wherein the desired protein is a G protein.

72. The method of claim 71, wherein said G protein is G_o.

73. The method of claim 70, wherein said IRP peptide is the peptide of claims 64, 66 or 67.

74. The method of claim 70, wherein said environment is inside a living cell.

75. The method of claim 74, wherein said cell is a central or peripheral neural cell.

76. The method of claim 70, wherein said binding substrate is GTP.

77. An antibody directed against the IRP peptide of claim 64, 66, 67 or a functional or chemical derivative thereof, said antibody or its said derivative optionally detectably or therapeutically labeled.

78. The antibody of claim 77, wherein said antibody is a monoclonal antibody.

79. A pharmaceutical composition comprising the IRP peptide of claim 64, 66 or 67, together with a pharmaceutically acceptable carrier, and optionally comprising one or more therapeutically effective agents.

80. A pharmaceutical composition comprising the antibody of claim 77, together with a pharmaceutically acceptable carrier, and optionally comprising one or more therapeutically effective agents.

81. A method of modulating structural remodeling in a neural cell, comprising administering to said cell an effective amount of the composition of claim 79.

82. A method of modulating structural remodeling in a neural cell, comprising administering to said cell an effective amount of the composition of claim 80.

83. The method of claim 81 wherein the neuronal cell is central or peripheral.

84. The method of claim 81 wherein the modulation of structural remodeling results in stimulation of neuronal growth.

85. The method of claim 81 wherein the modulation of structural remodeling results in inhibition of neuronal growth.

86. A method of screening for a substance capable of modulating the binding activity of a desired protein, comprising introducing into an environment comprising said desired protein and its binding substrate a substance which it is desired to screen, and measuring the increase or decrease in substrate binding relative to substrate binding in the absence of said substance.

87. The method of claim 86, wherein said desired protein is a G protein.

88. The method of claim 87, wherein said G protein is G_o.

89. A neural growth-associated protein, characterized in that it binds specifically to GAP-43, has a molecular weight of 39,000, elutes in a single band with application of EDTA in 50 mM Tris buffer, and is non-reactive with polyclonal antibody to G_o.

90. A method of augmenting activation of a desired protein by a receptor, comprising introducing into an environment comprising said desired protein and receptor an effective amount of an IRP peptide.

91. The method of claim 90 wherein the desired protein is a G protein.

92. The method of claim 91 wherein said G protein is G_o.

93. The method of claim 90 wherein said IRP peptide is the peptide of claims 64, 66 or 67.

94. The method of claim 90 wherein said environment is inside a living cell.

95. The method of claim 94 wherein said cell is a central or peripheral neural cell.

FIGURE 1

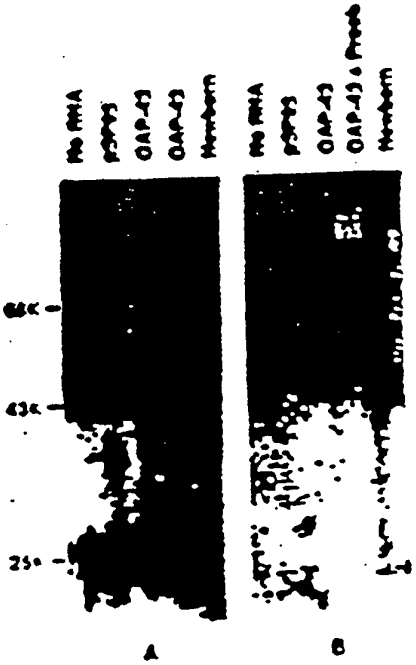

No RNA
pSP65
OAP-43
OAP-43
Newborn
No RNA
pSP65
OAP-43
OAP-43 + Peptide
Newborn
A
B

FIGURE 2

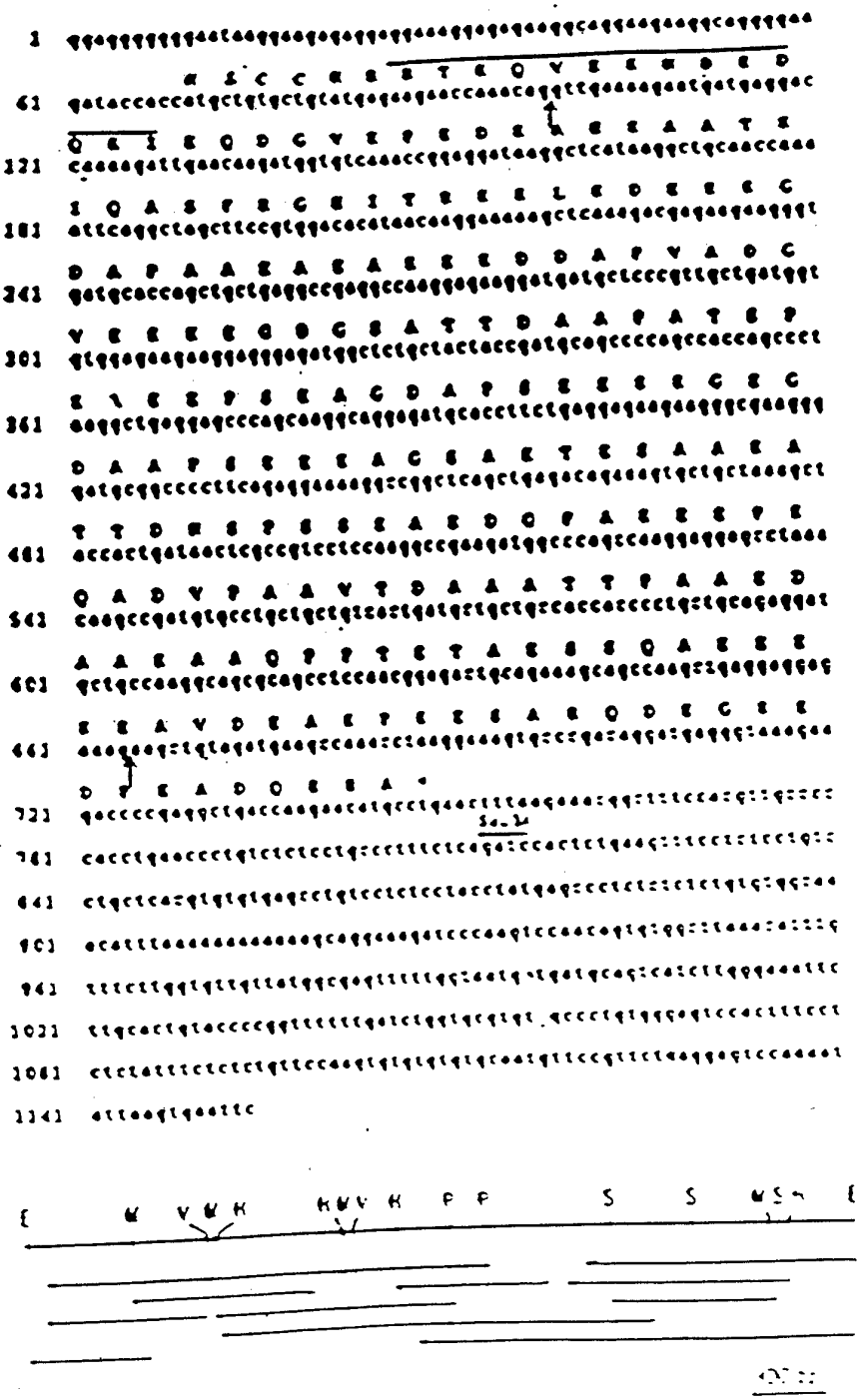


FIGURE 3

3/59

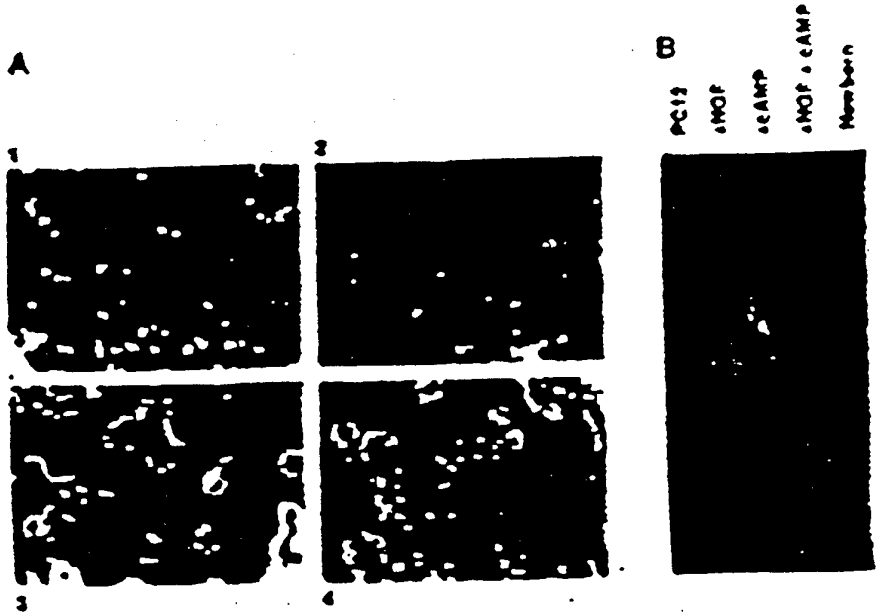


FIGURE 4

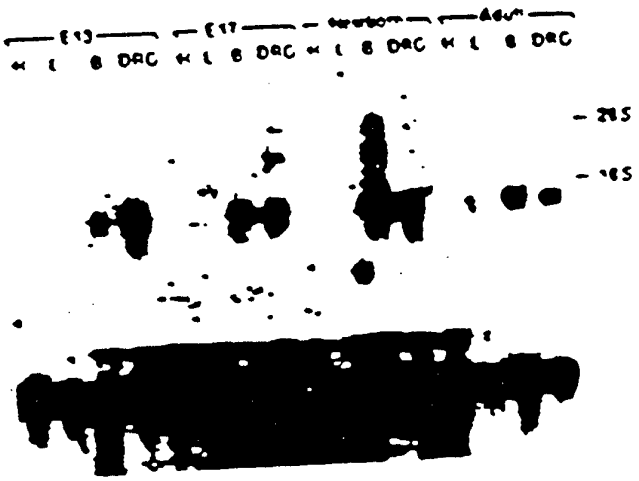


FIGURE 5

FIGURE 6

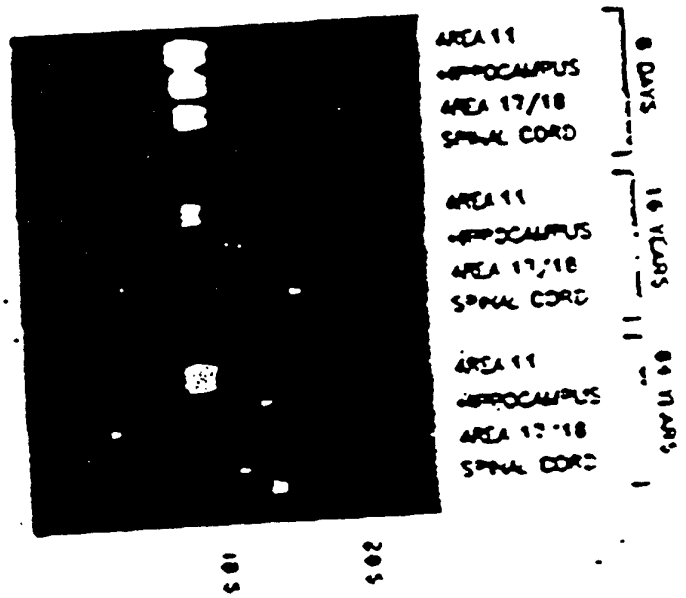

AREA 11
HIPPOCAMPUS
AREA 17/18
SPINAL CORD
AREA 11
HIPPOCAMPUS
AREA 17/18
SPINAL CORD
AREA 11
HIPPOCAMPUS
AREA 17/18
SPINAL CORD
DAYS
YEARS

FIGURE 7

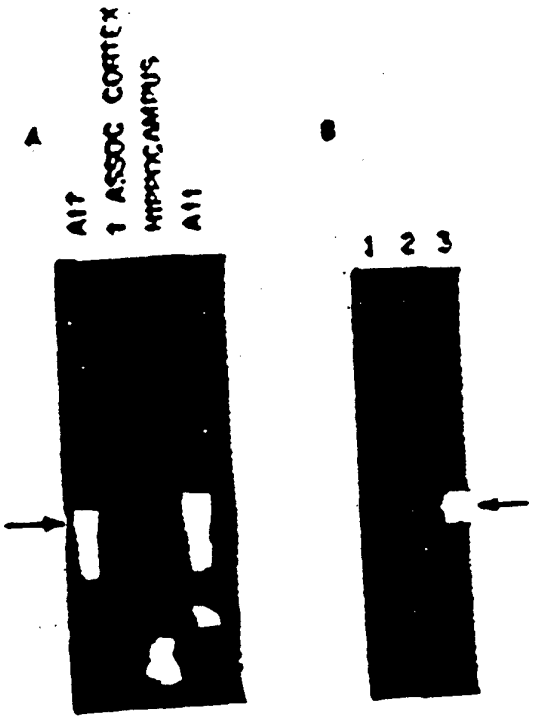


FIGURE 8

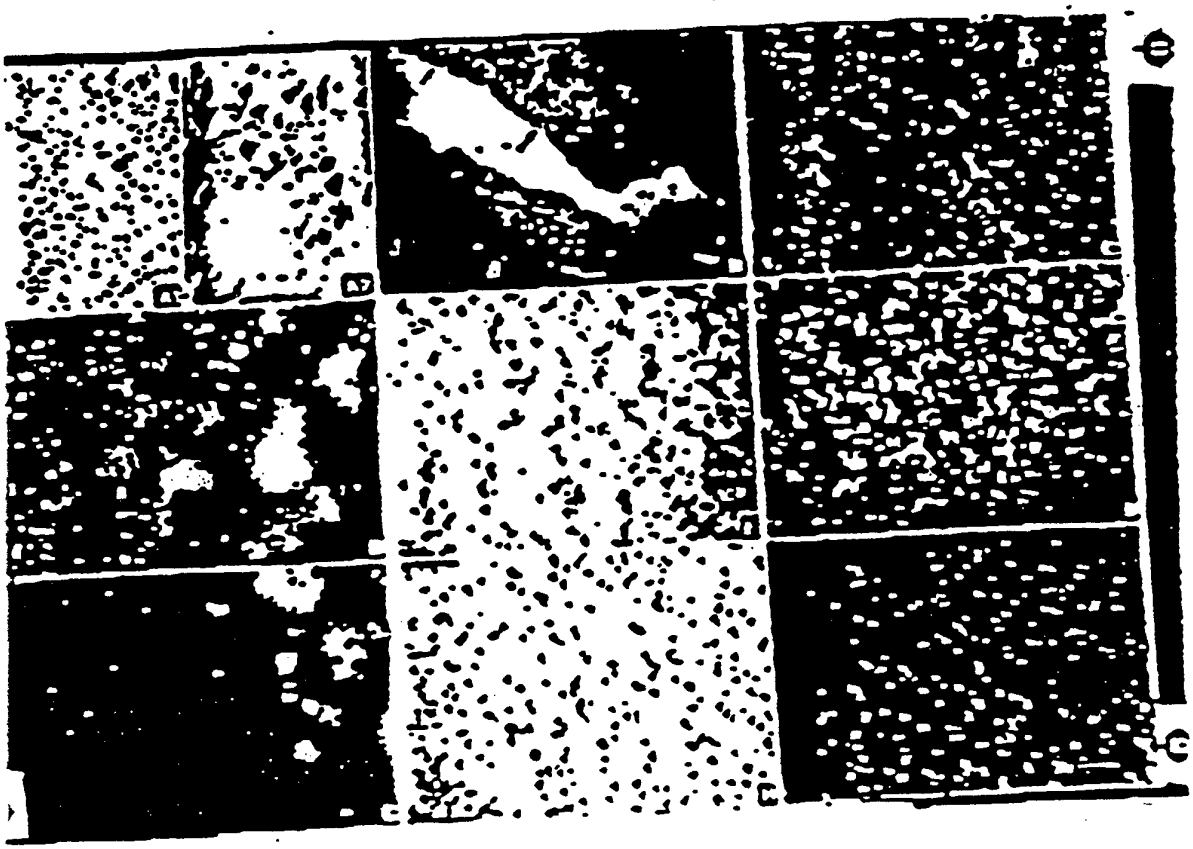


FIGURE 9

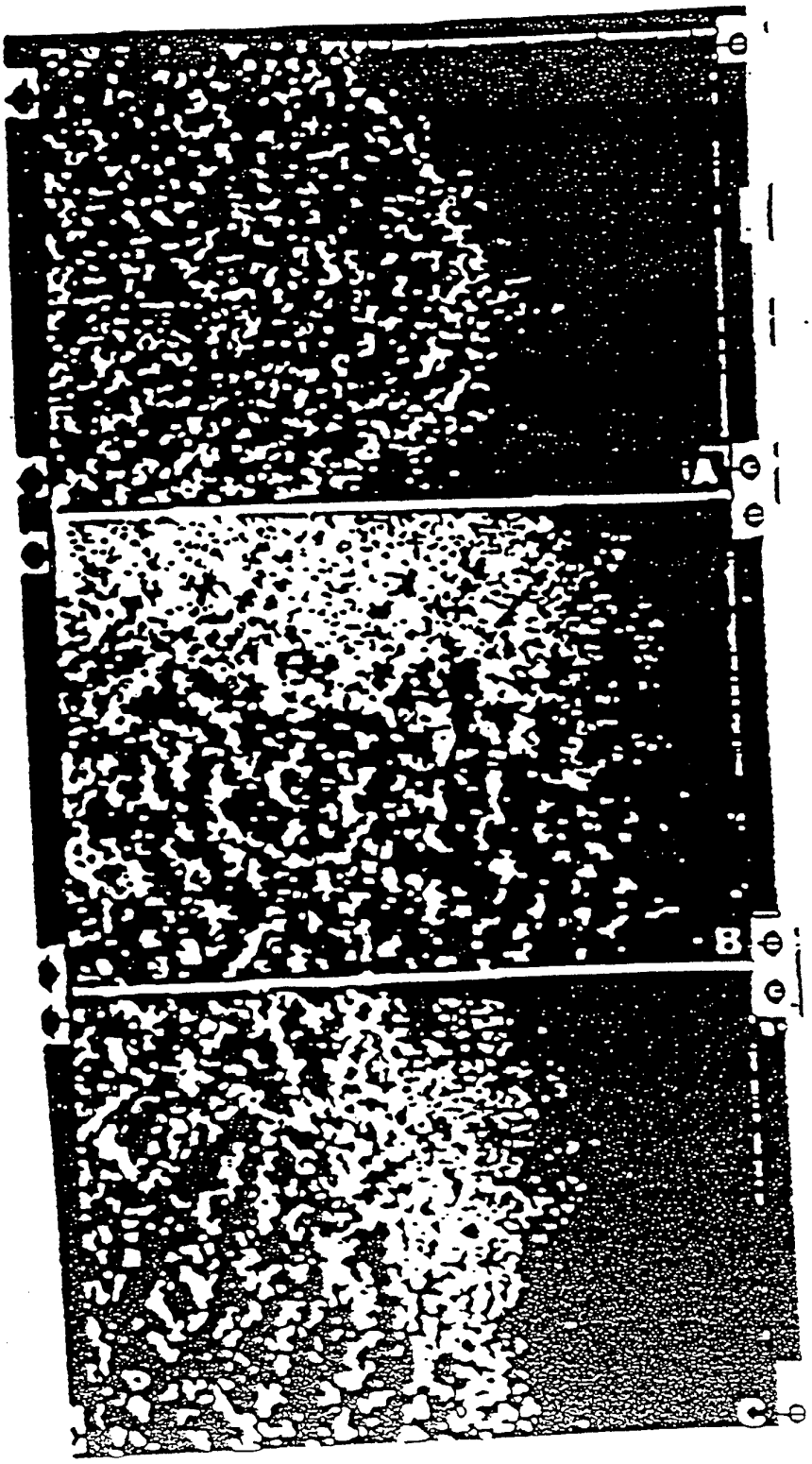
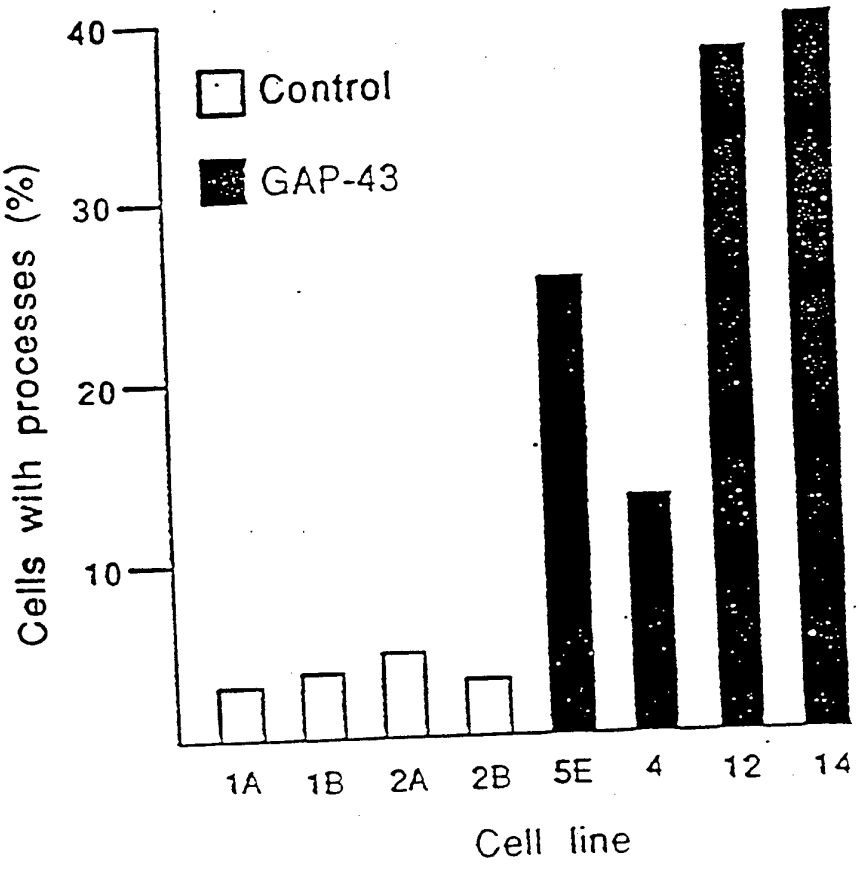


FIGURE 10


40
30
20
10
Cells with processes (%)
Control
GAP-43
1A
1B
2A
2B
5E
4
12
14
Cell line

MEMBRANE TARGETING OF GAP-43

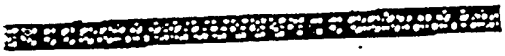
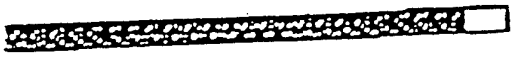
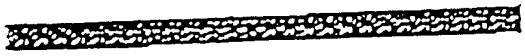
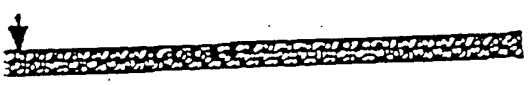
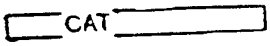
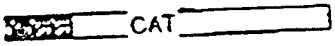
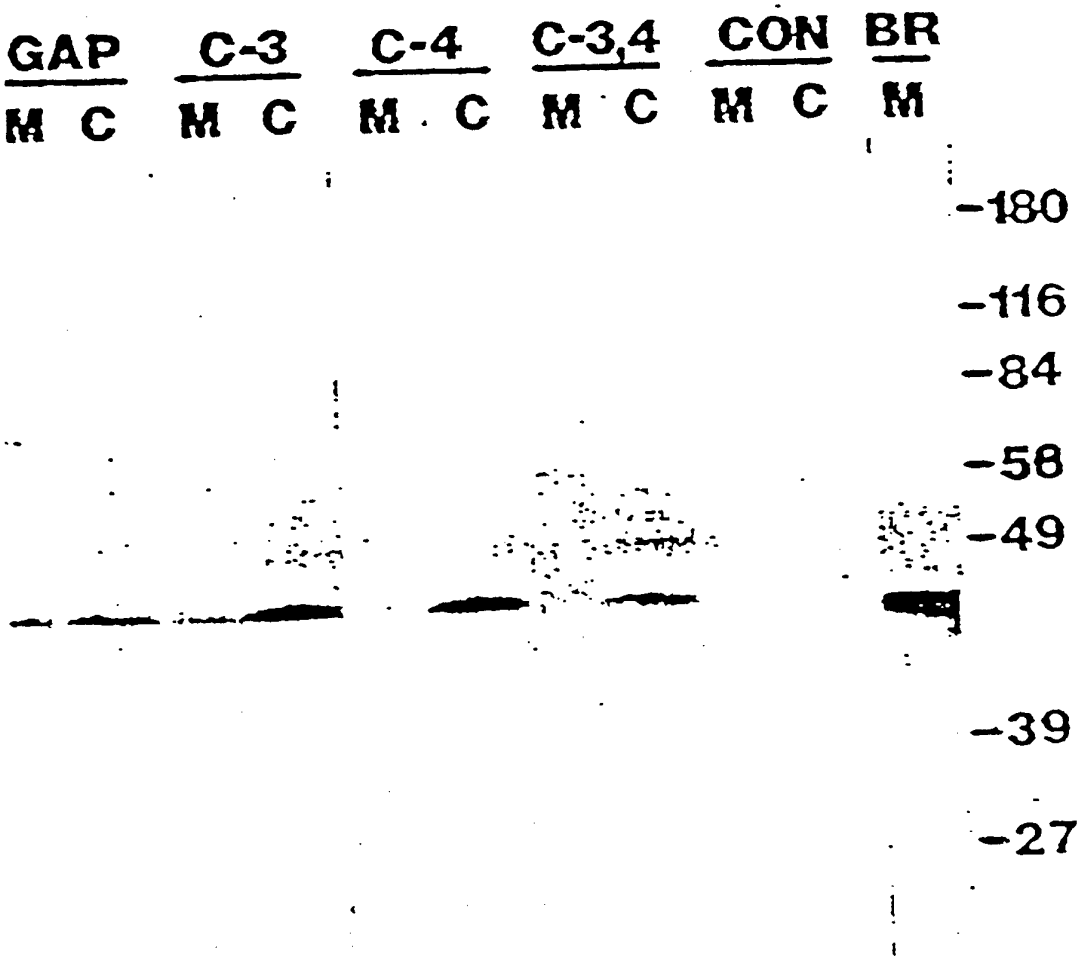
CONSTRUCTION		MEMBRANE
	GAP	+
	GAP(-intern.)	+
	GAPtag	+
	GAP(-1-4)	-
	GAP *C_3	+/-
	GAP *C_4	+/-
	GAP *$C_{3,4}$	-
	CAT	-
	GAP(1-10)CAT	+

FIGURE 11

FIGURE 12



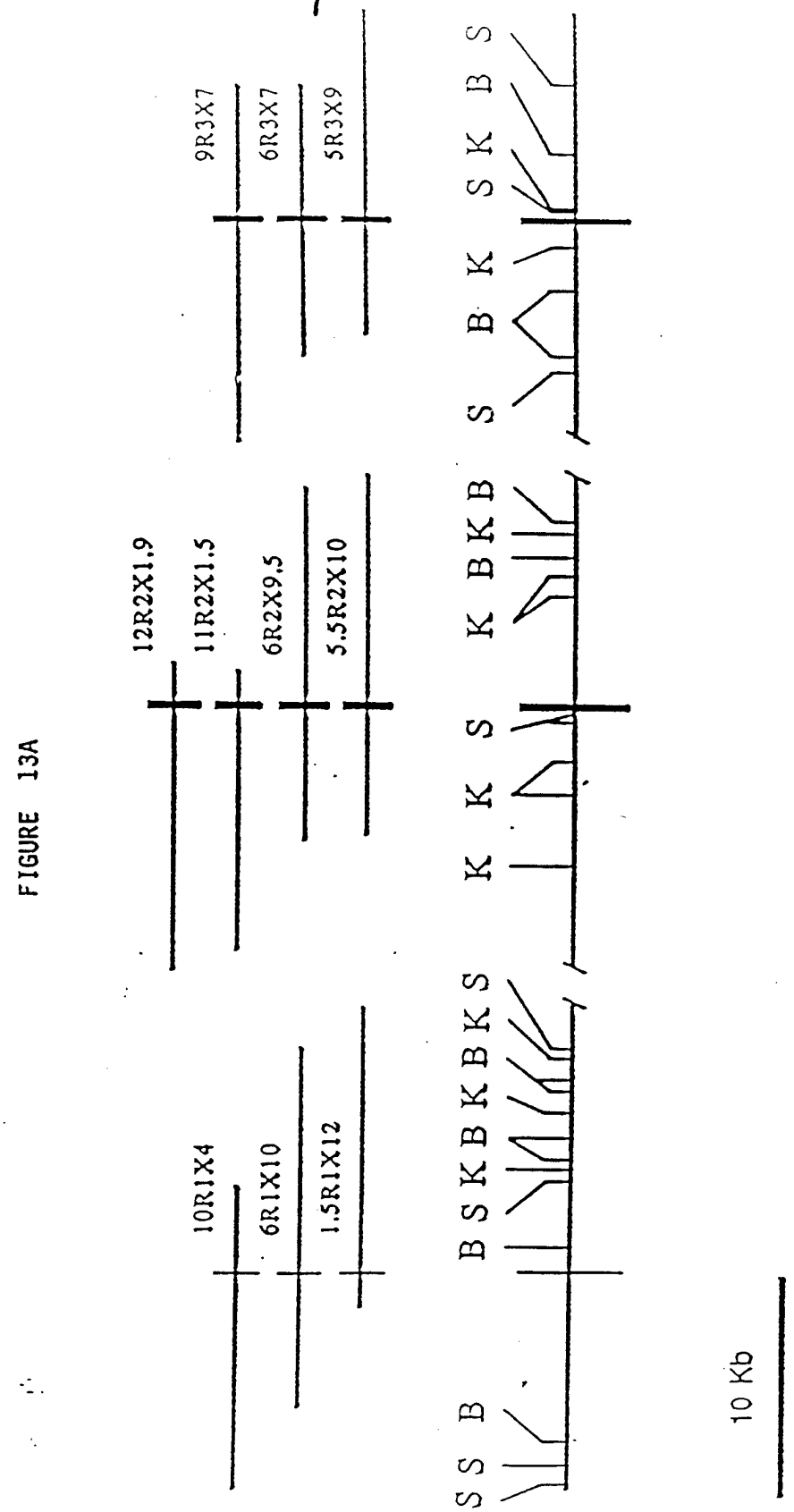

9R3X7
6R3X7
5R3X9
12R2X1.9
11R2X1.5
6R2X9.5
5.5R2X10
10R1X4
6R1X10
1.5R1X12
S S B B S K B K B B K S K K S K B K B S B K S K B S
10 Kb

FIGURE 13A

FIGURE 13B

AACCAAACAGgtagagctaa... intron 1 >24 Kb ...ctccacagGTTGAAAAAGA
Gln (10) Val (11)

GAGGAGAAAGgtgagtacca... intron 2 >20 Kb ...tcttttcagAAGCTGTAGA
Glu (199) Glu (199)

AATCTGAATATTAATAAATCATGAGAGTAATCAActttggtgttctgtgttttctt

FIGURE 14

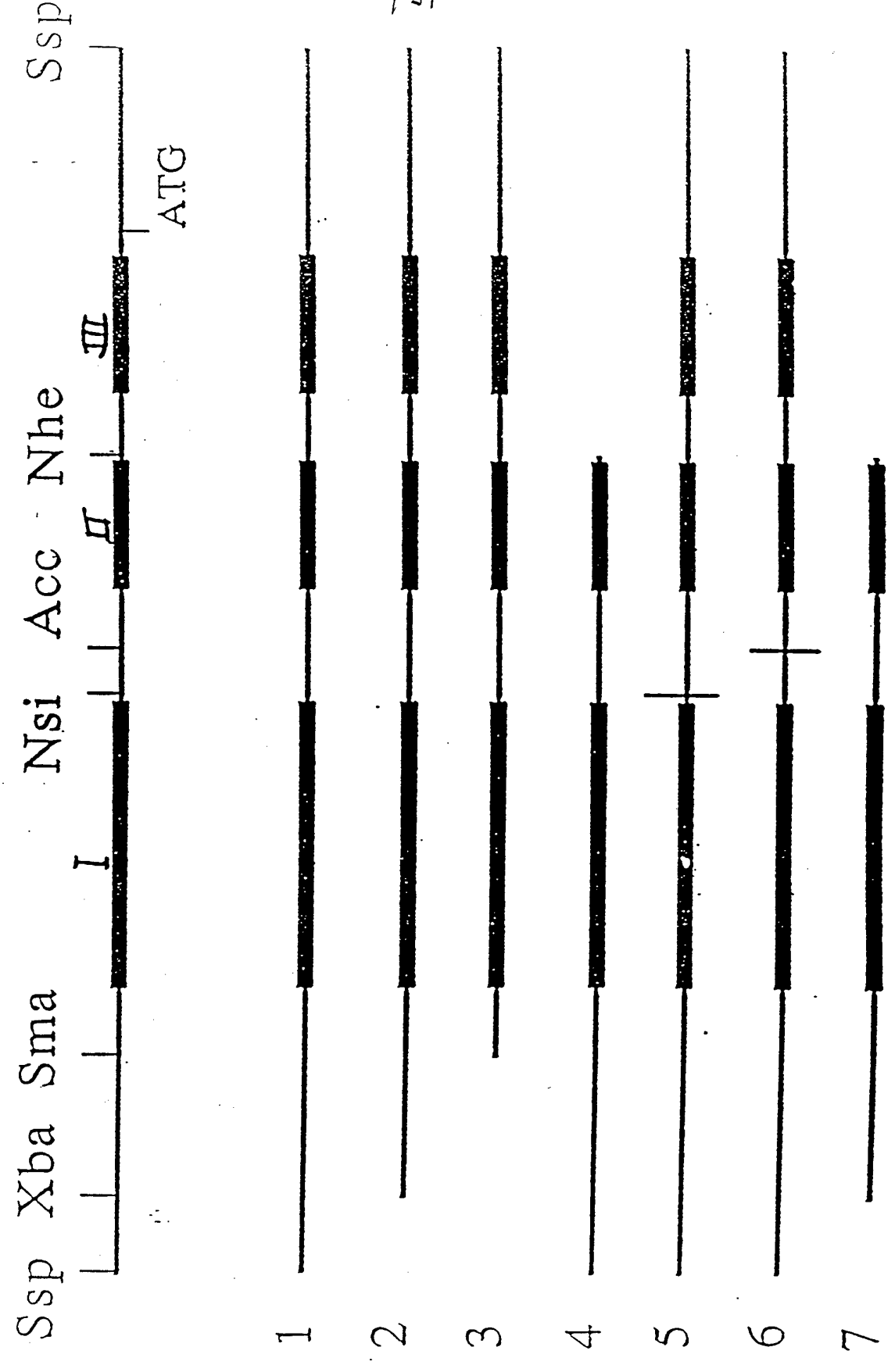


Figure 15

FIGURE 16

p38-A	IIHEDGFSGEDVK
$Alpha_o$ 11-23	IIHEDGFSGEDVK
p38-B	MEDTEPFSAELLSAMM
$Alpha_o$ 70-85	MEDTEPFSAELLSAMM
p38-C	IGAADYQPTEQDIL
$Alpha_o$ 119-132	IGAADYQPTEQDIL
p34-A	LIIWDSYTTNK
$Beta_1$ 79-89	LIIWDSYTTNK
$Beta_2$ 79-89	LIIWDSYTTNK
p34-B	TGQQT[TT/VG]T[T/A]FT[T/S]GHTGDVMSL
$Beta_1$ 173-190	TGQQTTTFTGHTGDVMSL
$Beta_2$ 173-190	TGQQTVGFAGHSCDVMSL
p34-C	IWN
$Beta_1$ 338-340	IWN
$Beta_2$ 338-340	IWN

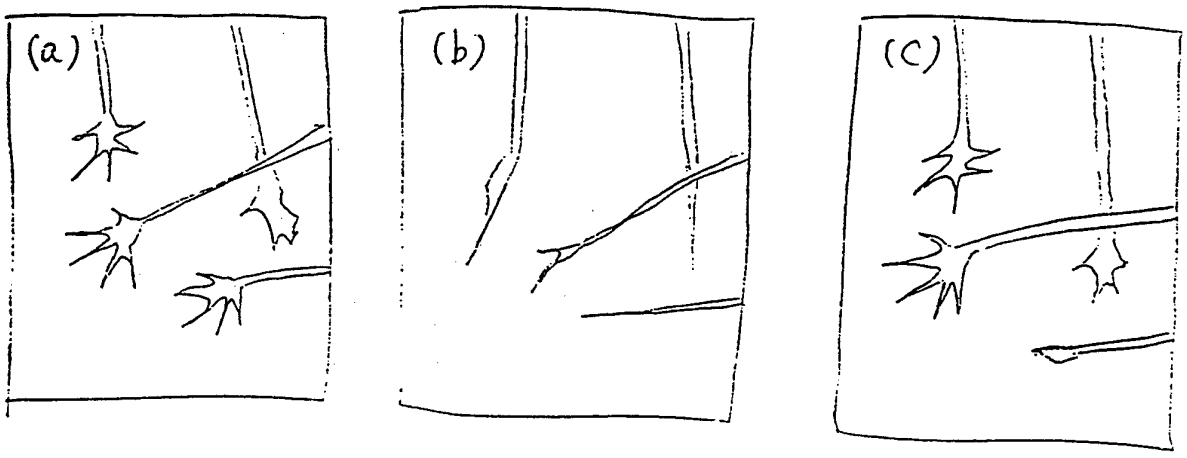


FIGURE 17

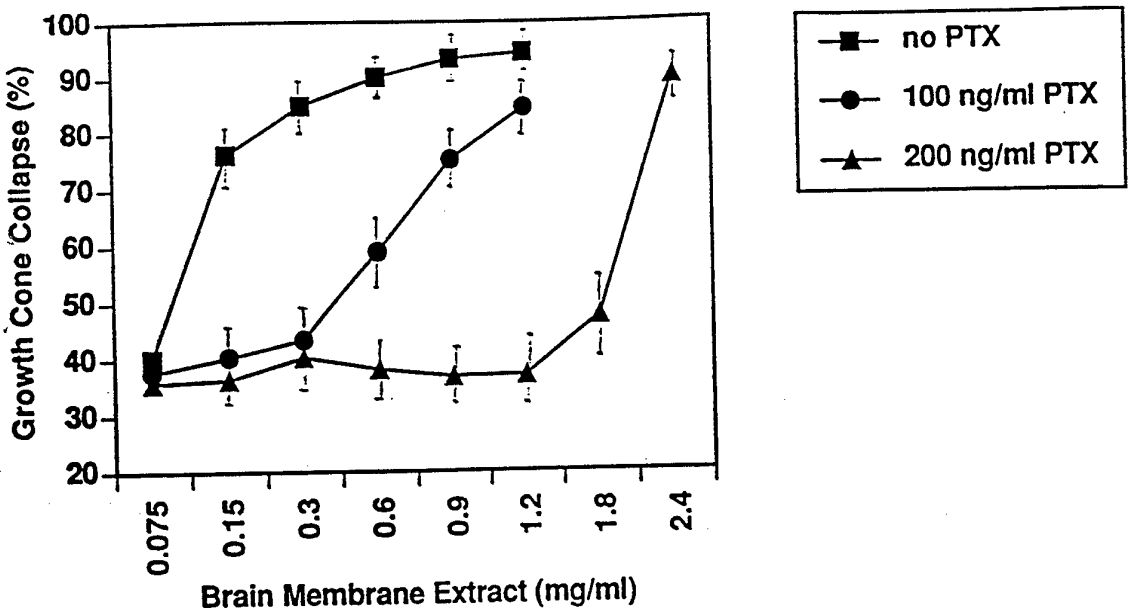

Growth Cone Collapse (%)
100
90
80
70
60
50
40
30
20
0.075
0.15
0.3
0.6
0.9
1.2
1.8
2.4
Brain Membrane Extract (mg/ml)
no PTX
100 ng/ml PTX
200 ng/ml PTX

Figure 18a

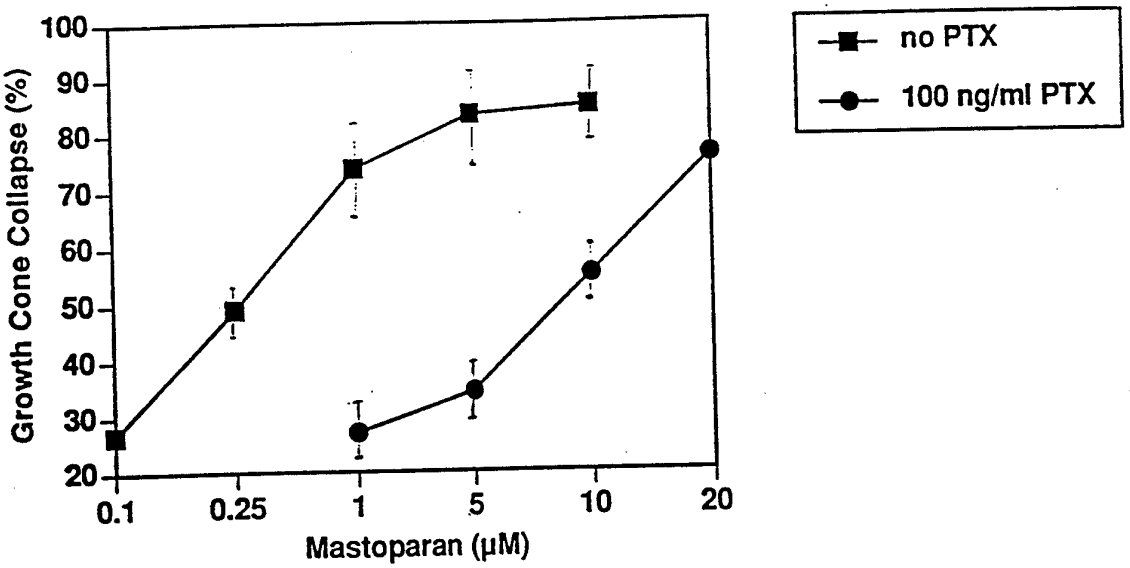

Growth Cone Collapse (%)
100
90
80
70
60
50
40
30
20
0.1
0.25
1
5
10
20
Mastoparan (μM)
no PTX
100 ng/ml PTX

FIGURE 18b

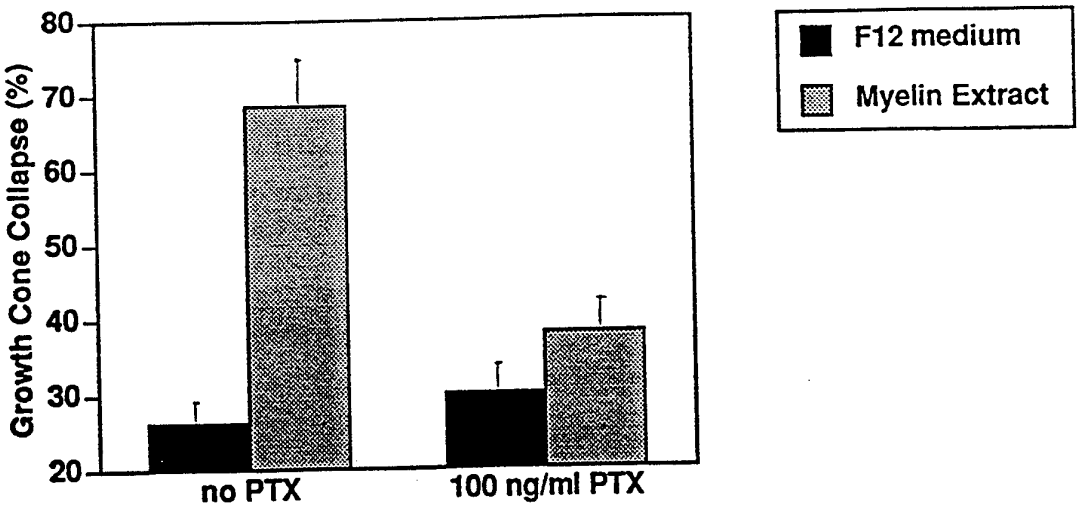

Growth Cone Collapse (%)
80
70
60
50
40
30
20
no PTX
100 ng/ml PTX
F12 medium
Myelin Extract

Figure 19

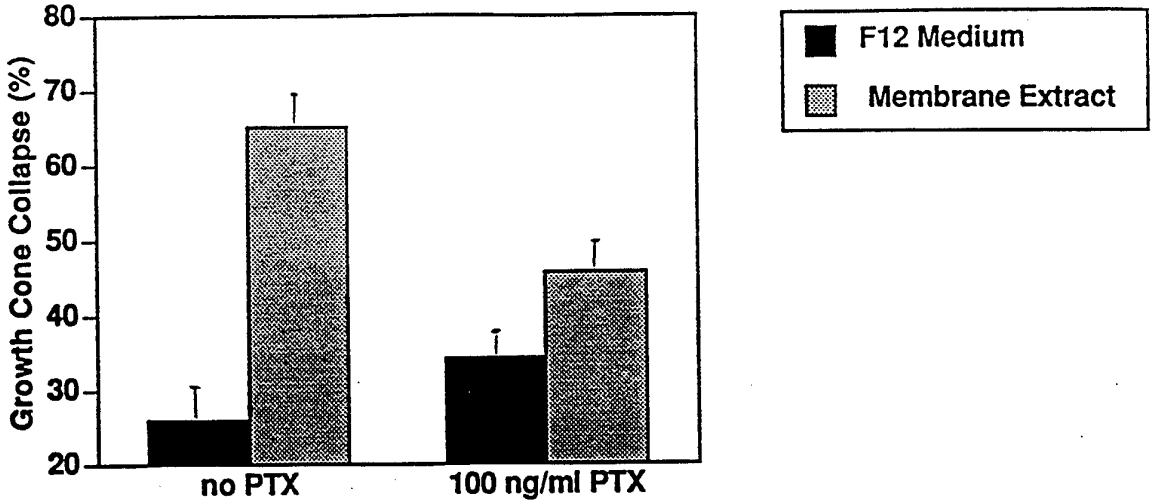

Growth Cone Collapse (%)
80
70
60
50
40
30
20
no PTX
100 ng/ml PTX
F12 Medium
Membrane Extract

Figure 20

Figure 21

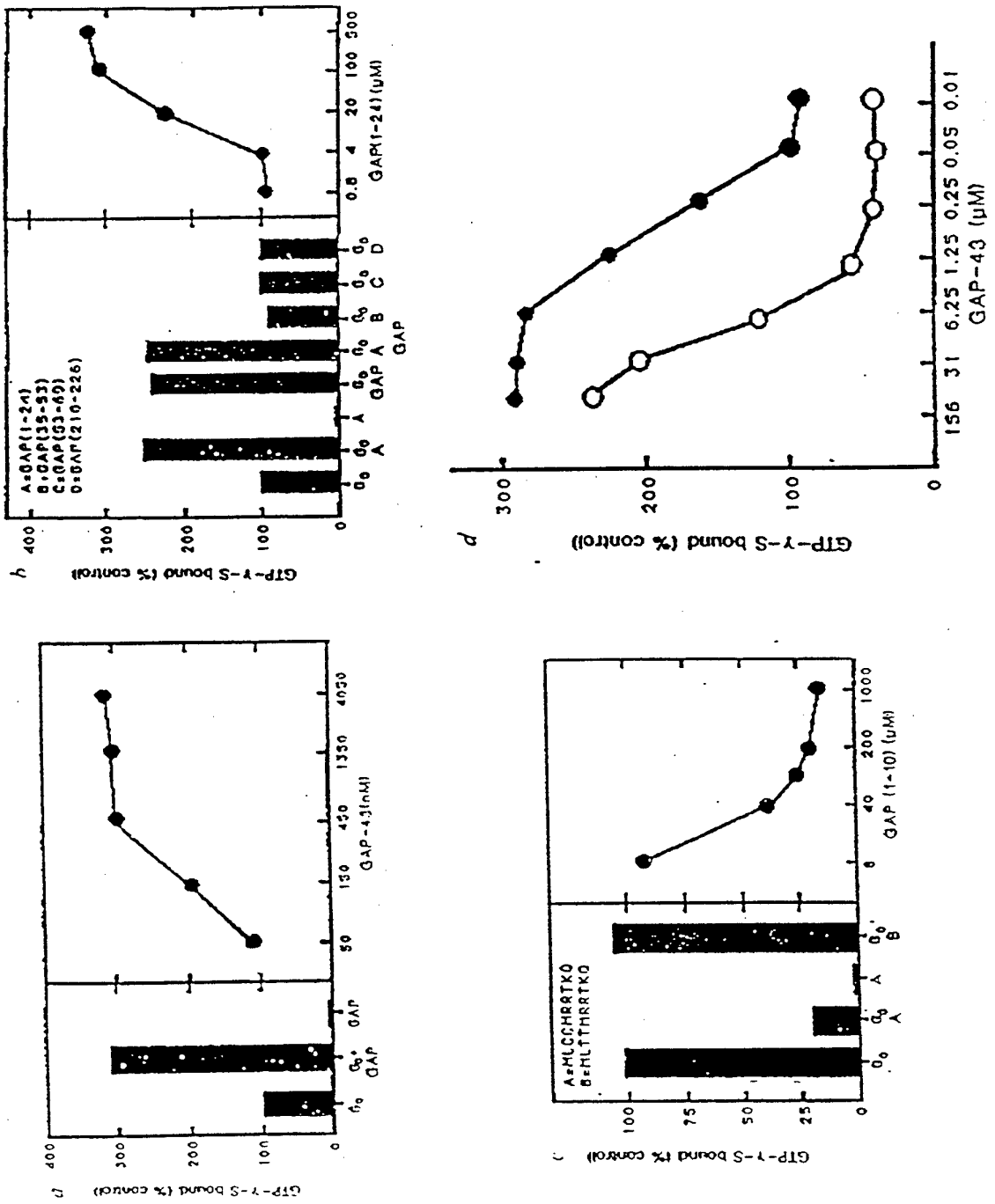
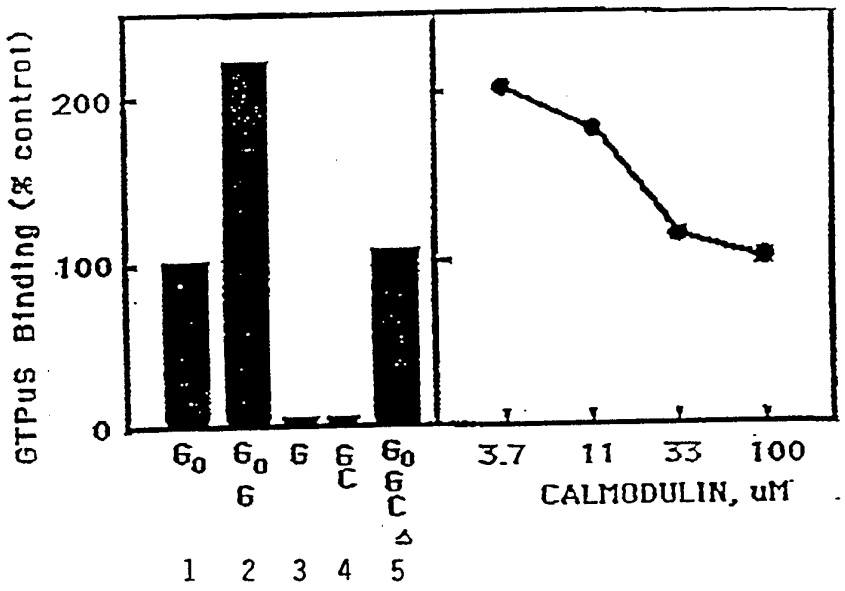

GTP-γ-S bound (% control)
A=GAP(1-24)
B=GAP(35-53)
C=GAP(53-60)
D=GAP(210-226)
GAP
GAP(1-24) (μM)
GAP-43 (μM)
GAP-43(nM)
GAP (1-10) (μM)
A=MLCCMRRTKQ
B=MLFTMRRTKQ

Figure 22

GAP-43, Human	M	L	C	C	M	-	R	R
GAP-43, Fish	M	L	C	C	I	-	R	R
B_1-adrenergic	L	L	C	C	A	-	R	R
B_2-adrenergic	L	L	C	L	-	-	R	R
Rhodopsin	T	I	C	C	G	-	K	N
Blue-opsin	M	V	I	C	G	-	K	A
A_1-adrenergic	I	L	G	C	Q	C	R	S
pA_2-adrenergic	I	L	C	R	G	D	K	R
Mus-ACh-1	L	L	C	R	W	-	K	R
Mus-ACh-2	L	M	C	H	Y	-	K	N
Mus-ACh-3	L	L	C	Q	C	D	K	K
Mus-ACh-4	L	L	C	Q	Y	-	R	N
Mus-ACh-5	L	L	C	R	W	-	K	K
5HT-1A	I	I	K	C	W	F	C	R
5HT-1C	L	R	C	D	Y	-	K	P
5HT-2	I	Q	C	Q	Y	-	K	E

Figure 23


GTPuS Binding (% control)
200
100
0
G_0
G_0 G
G
G C
G_0 G C Δ
1 2 3 4 5
3.7
11
33
100
CALMODULIN, uM

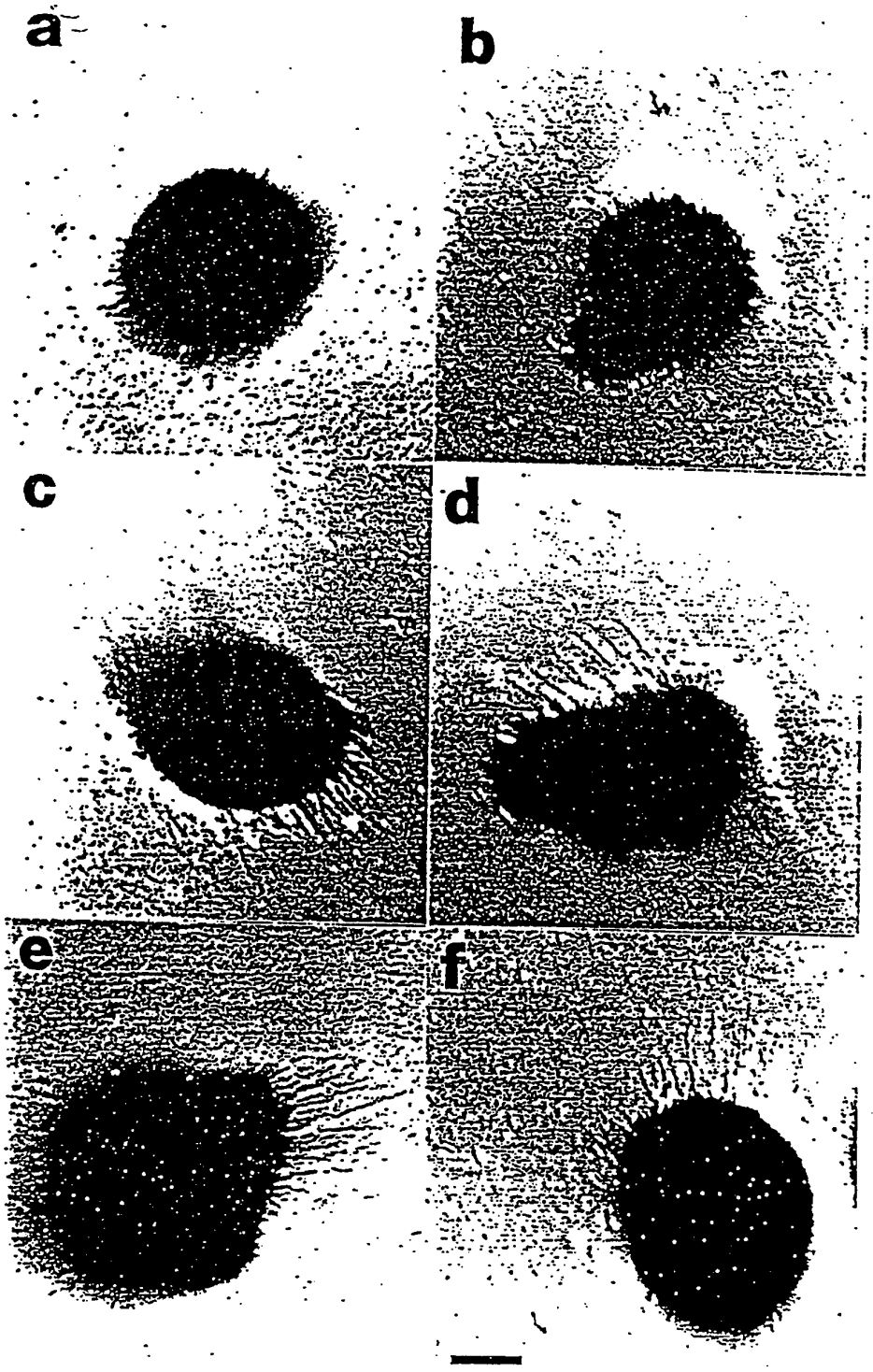


Figure 24(a)

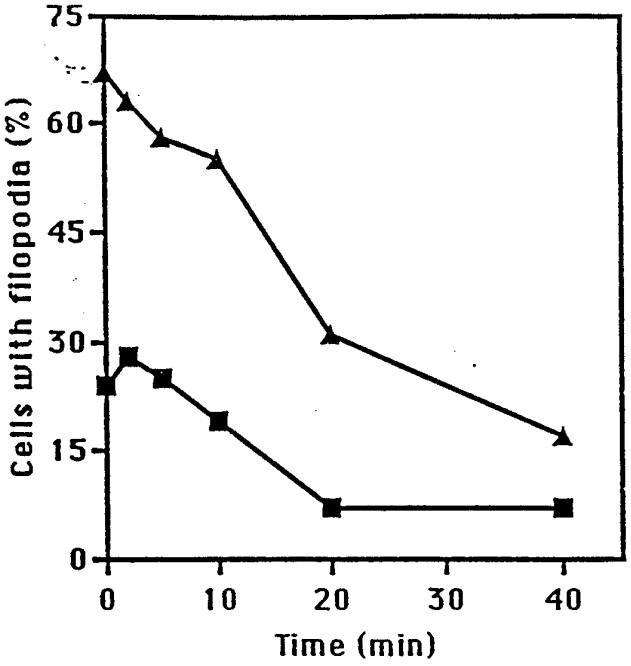


Figure 24(b)

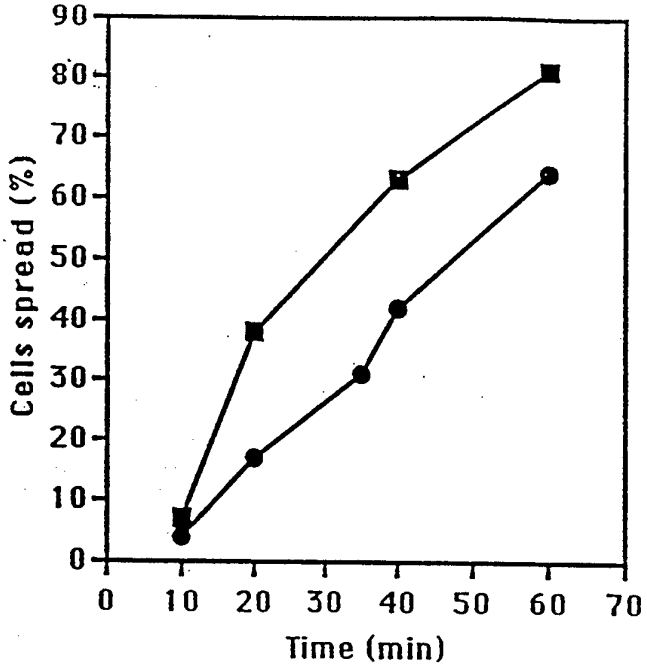

90
80
70
60
50
40
30
20
10
0
Cells spread (%)
0
10
20
30
40
50
60
70
Time (min)

Figure 25A

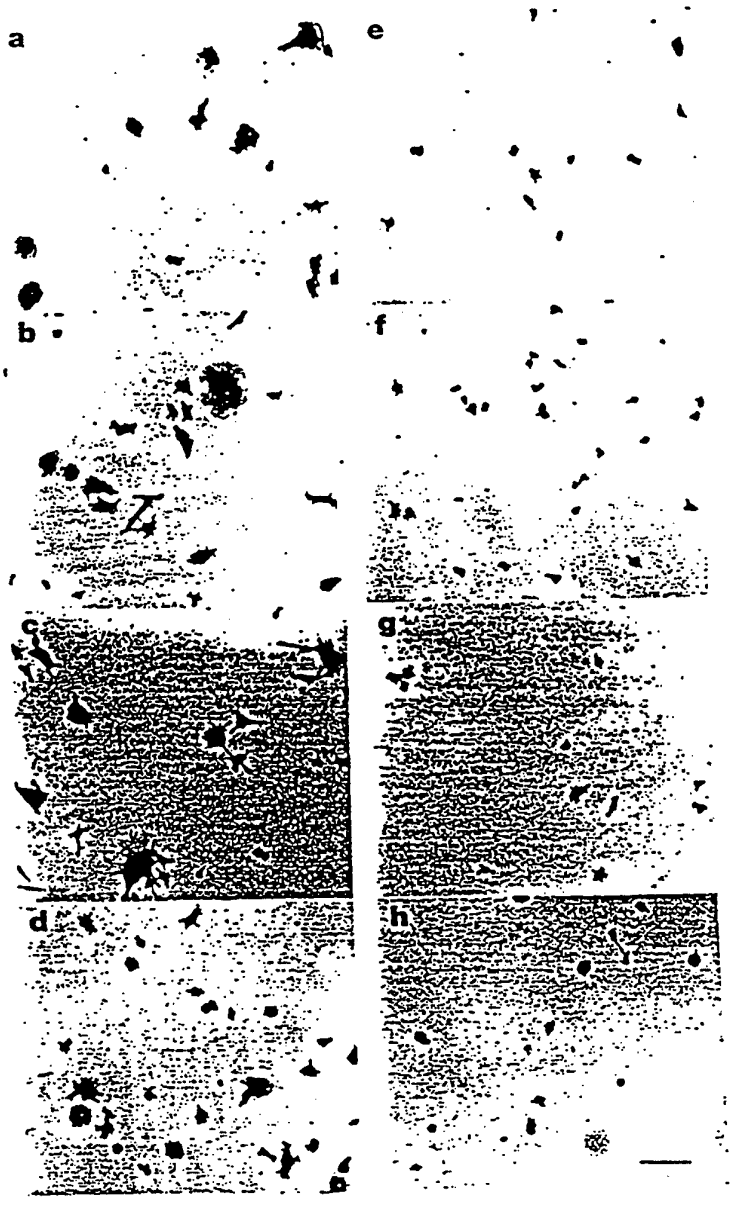


Figure 25(b)

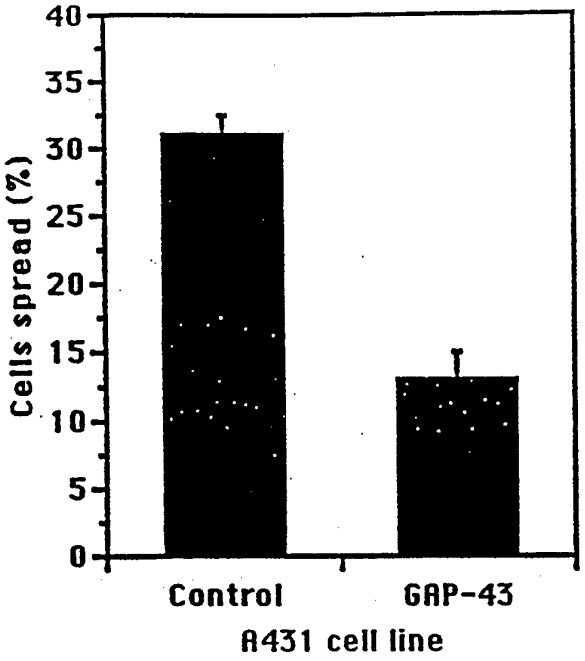

40
35
30
25
20
15
10
5
0
Cells spread (%)
Control
GAP-43
A431 cell line

Figure 25(c)

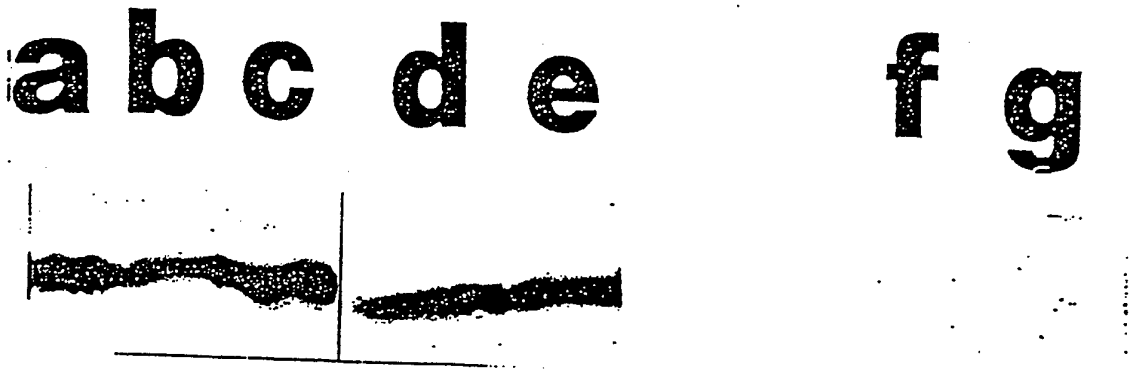


Figure 25(d)

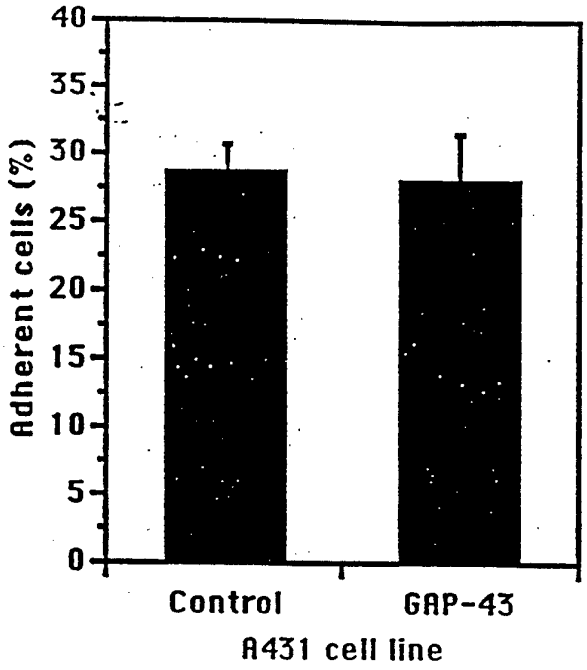

40
35
30
25
20
15
10
5
0
Adherent cells (%)
Control
GAP-43
A431 cell line

Figure 25(e)

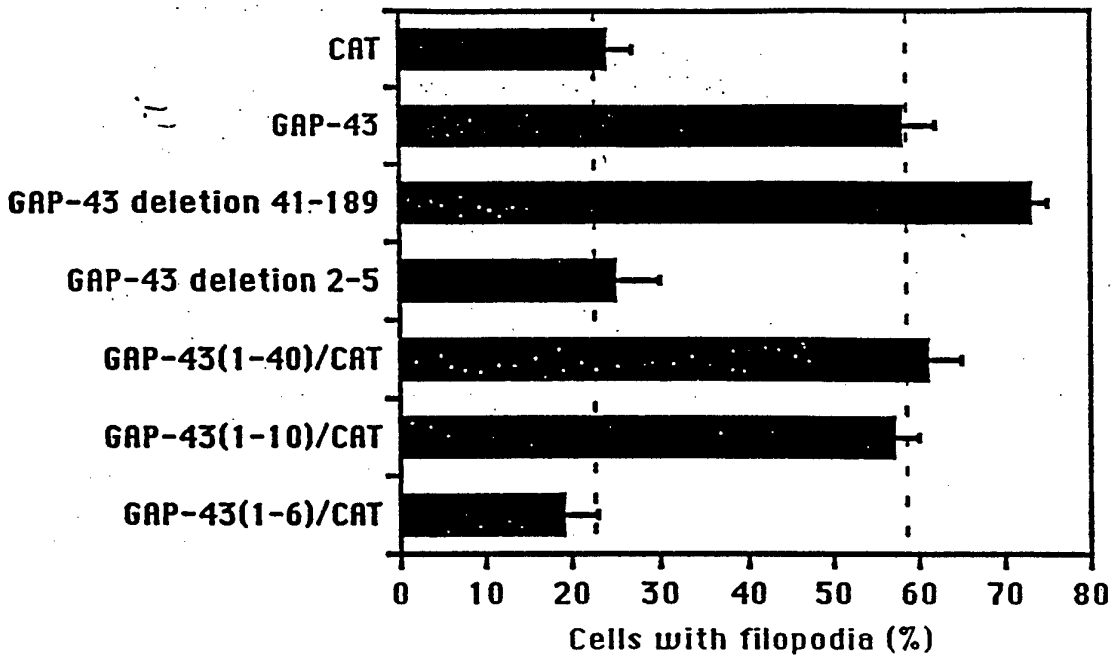

CAT
GAP-43
GAP-43 deletion 41-189
GAP-43 deletion 2-5
GAP-43(1-40)/CAT
GAP-43(1-10)/CAT
GAP-43(1-6)/CAT
0
10
20
30
40
50
60
70
80
Cells with filopodia (%)

Figure 26(a)

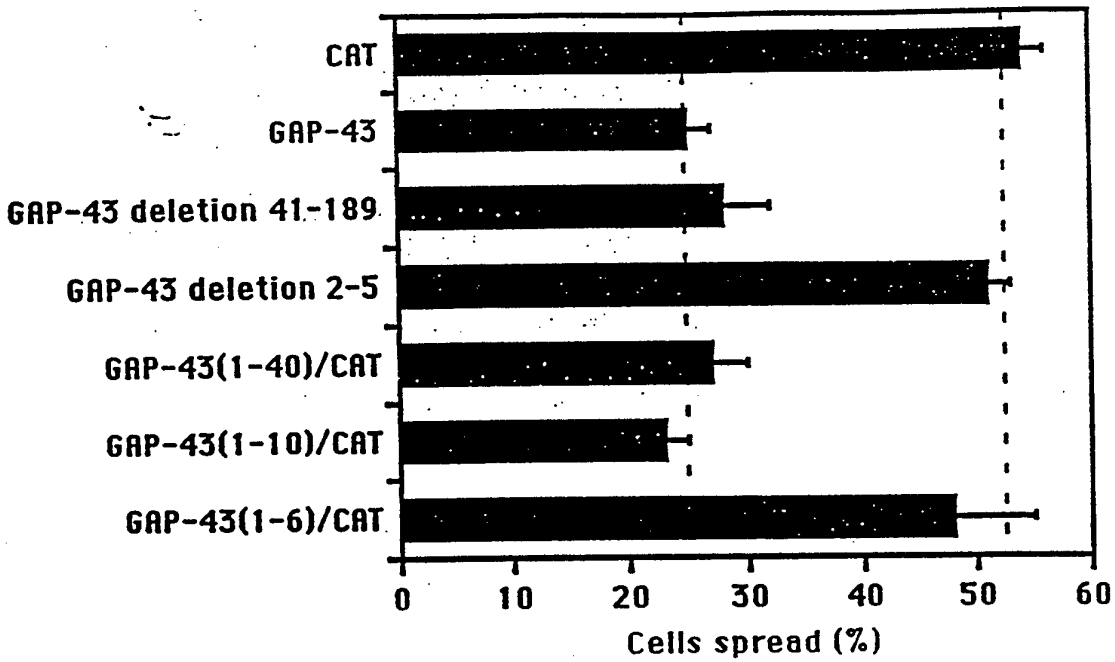

CAT
GAP-43
GAP-43 deletion 41-189
GAP-43 deletion 2-5
GAP-43(1-40)/CAT
GAP-43(1-10)/CAT
GAP-43(1-6)/CAT
0
10
20
30
40
50
60
Cells spread (%)

Figure 26(b)

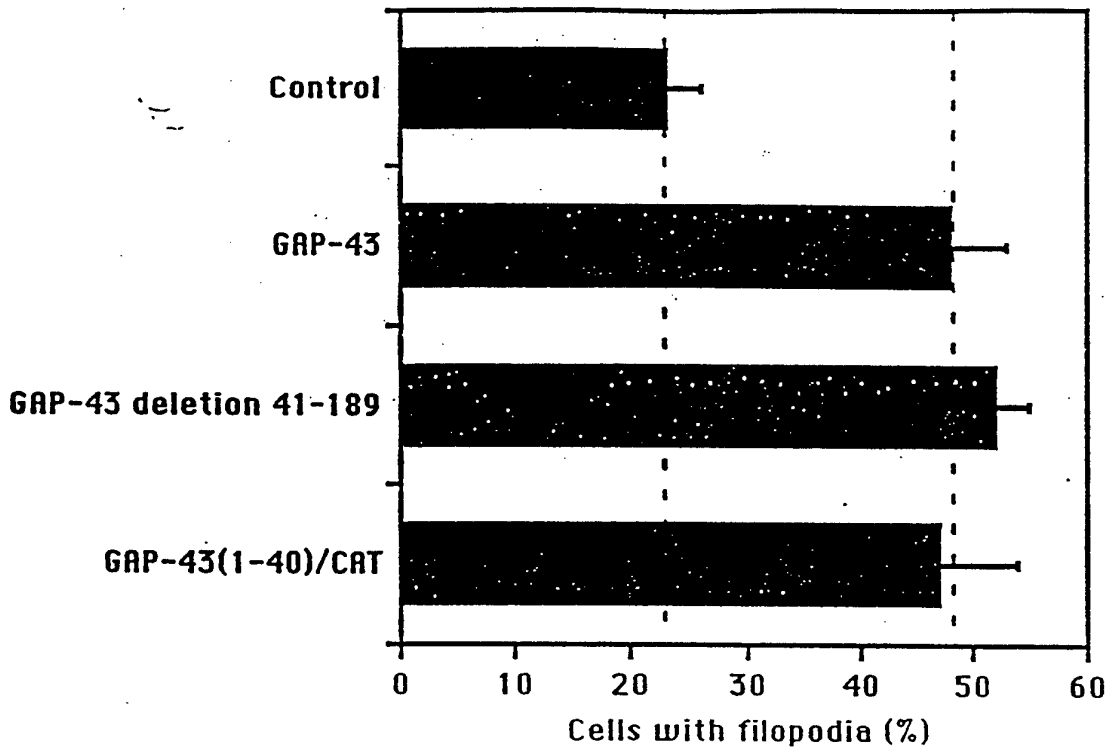

Control
GAP-43
GAP-43 deletion 41-189
GAP-43(1-40)/CAT
0
10
20
30
40
50
60
Cells with filopodia (%)

Figure 26(c)

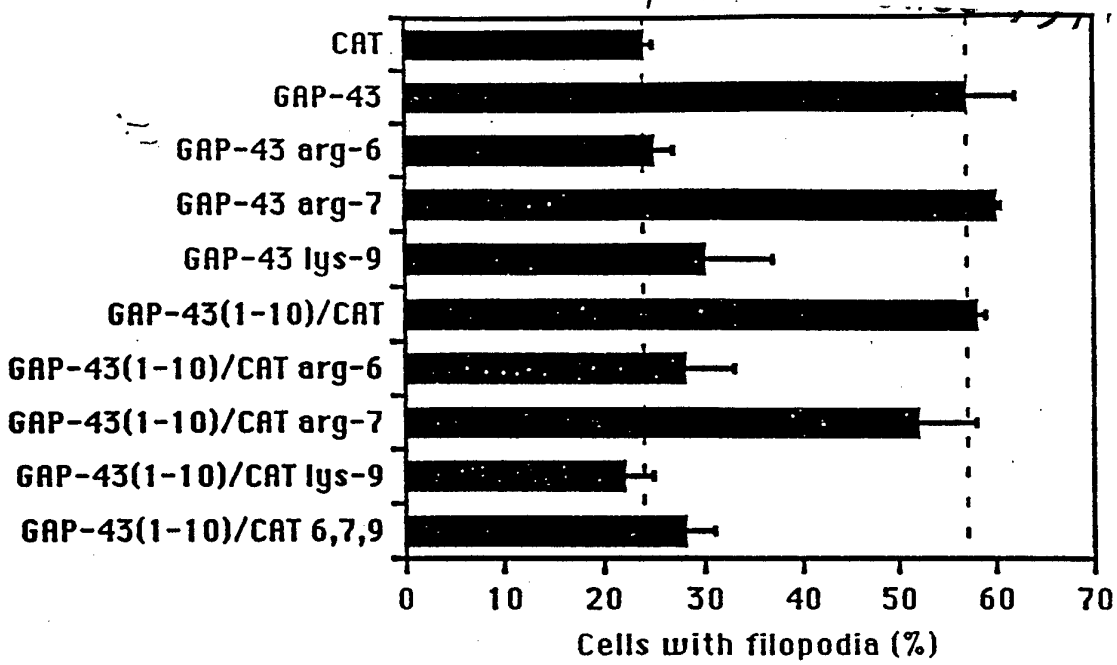

CAT
GAP-43
GAP-43 arg-6
GAP-43 arg-7
GAP-43 lys-9
GAP-43(1-10)/CAT
GAP-43(1-10)/CAT arg-6
GAP-43(1-10)/CAT arg-7
GAP-43(1-10)/CAT lys-9
GAP-43(1-10)/CAT 6,7,9
0
10
20
30
40
50
60
70
Cells with filopodia (%)

Figure 27(a)

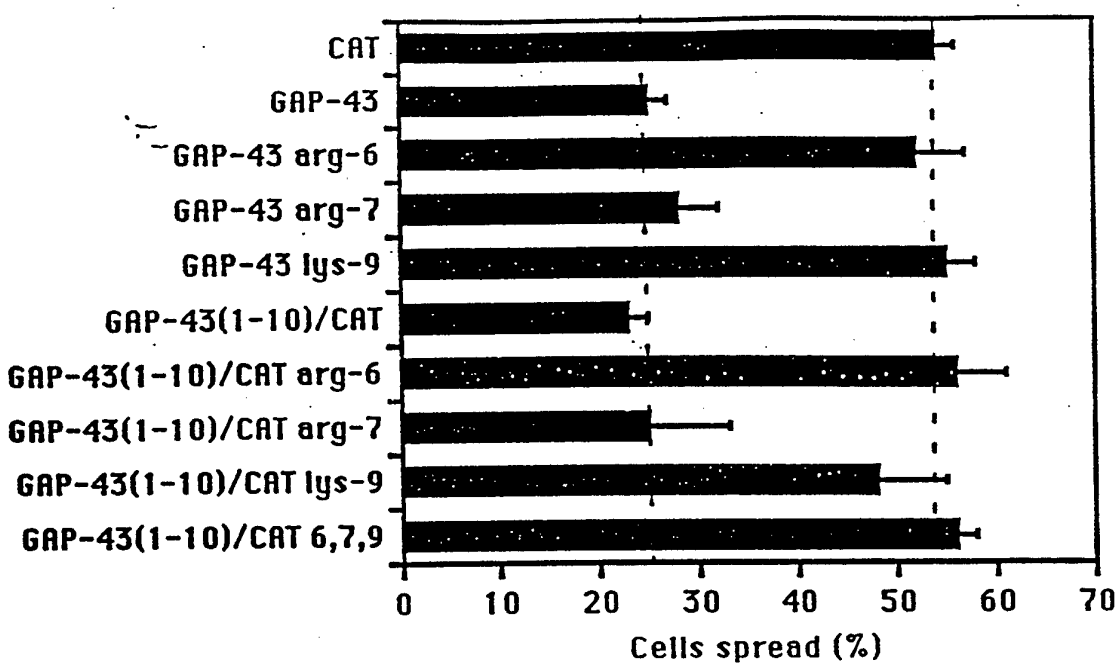

CAT
GAP-43
GAP-43 arg-6
GAP-43 arg-7
GAP-43 lys-9
GAP-43(1-10)/CAT
GAP-43(1-10)/CAT arg-6
GAP-43(1-10)/CAT arg-7
GAP-43(1-10)/CAT lys-9
GAP-43(1-10)/CAT 6,7,9
0
10
20
30
40
50
60
70
Cells spread (%)

Figure 27(b)

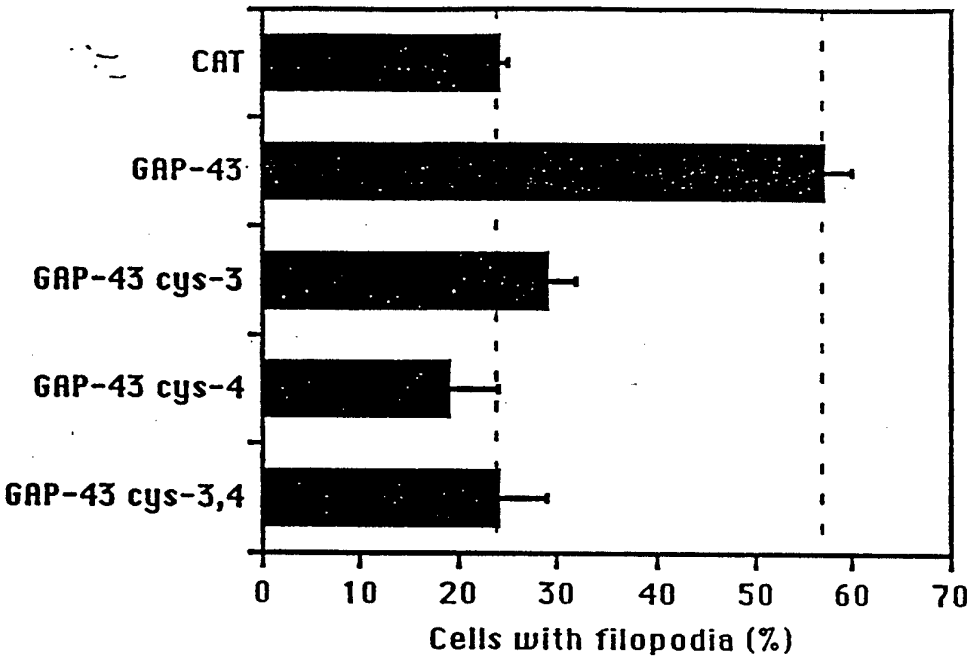

CAT
GAP-43
GAP-43 cys-3
GAP-43 cys-4
GAP-43 cys-3,4
0
10
20
30
40
50
60
70
Cells with filopodia (%)

Figure 28(a)

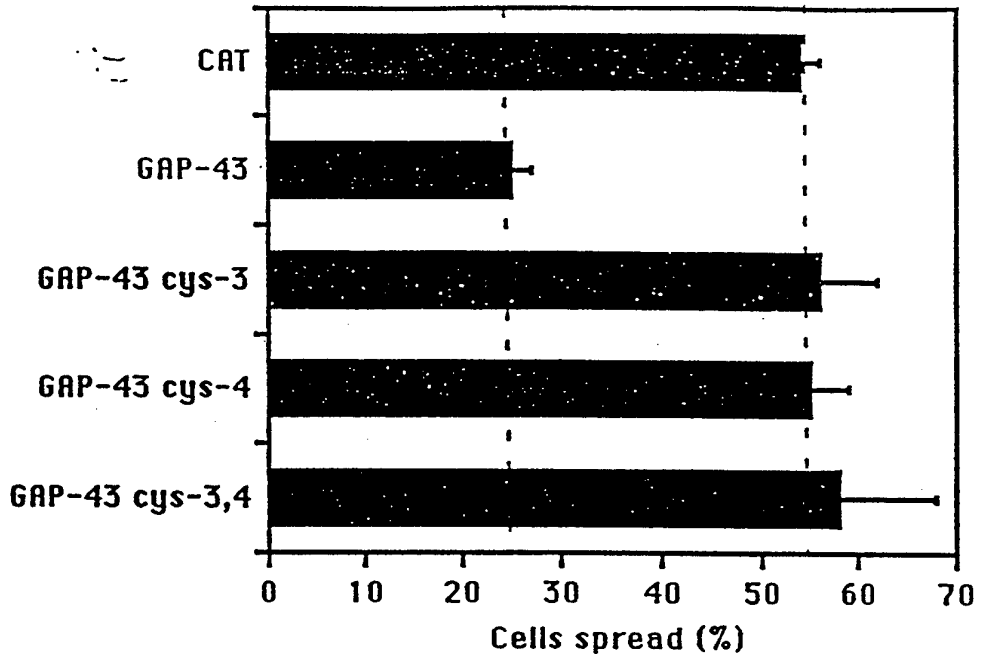

CAT
GAP-43
GAP-43 cys-3
GAP-43 cys-4
GAP-43 cys-3,4
0
10
20
30
40
50
60
70
Cells spread (%)

Figure 28(b)

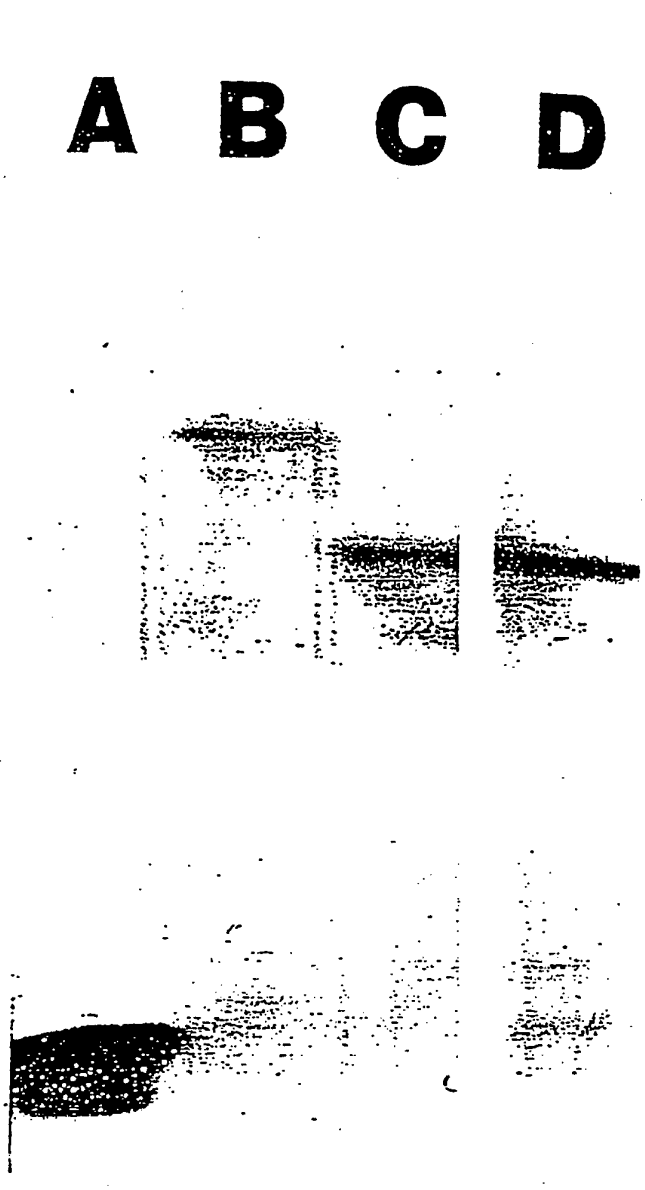


Figure 29

Figure 30

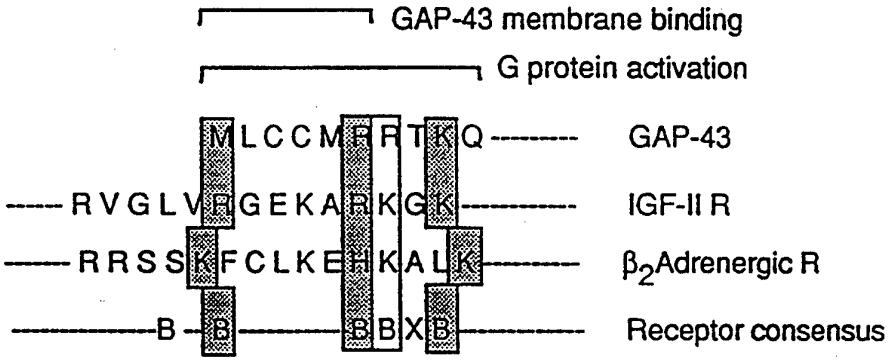

GAP-43 membrane binding
G protein activation
M L C C M R R T K Q
GAP-43
R V G L V R G E K A R K G K
IGF-II R
R R S S K F C L K E H K A L K
β2Adrenergic R
B B B B X B
Receptor consensus

Figure 31

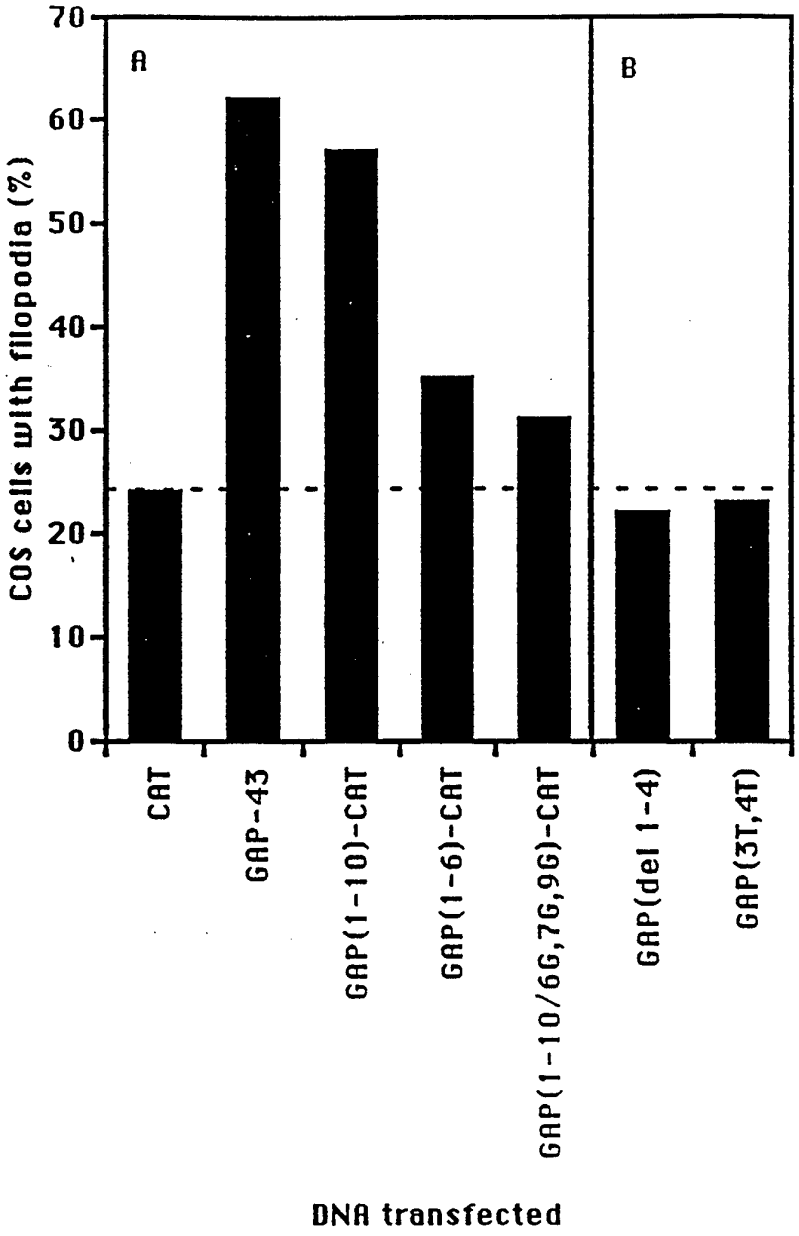


Figure 32

Filopodial formation in COS cells transiently transfected with expression vectors encoding CAT, GAP-43 or GAP-CAT fusion proteins.

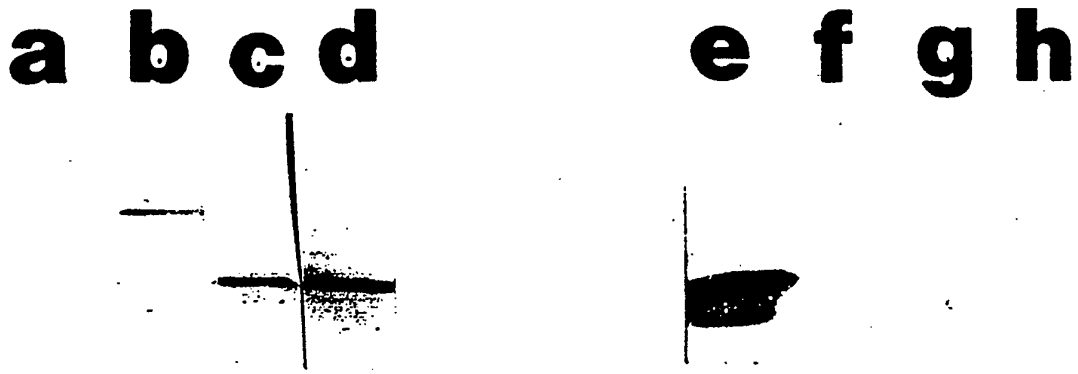


FIGURE 33

CAT immunoblot analysis of COS cell fractionsafter transfection with CAT and GAP-CAT fusion proteins. Lanes a-d are membrane fractions and lanes e-h are from soluble fractions. Lanes a and e are from the same CAT transfection; b and f from GAP(1-40)-CAT; c and g from GAP(1-10)-CAT; and d and h from GAP(1-6)-CAT.

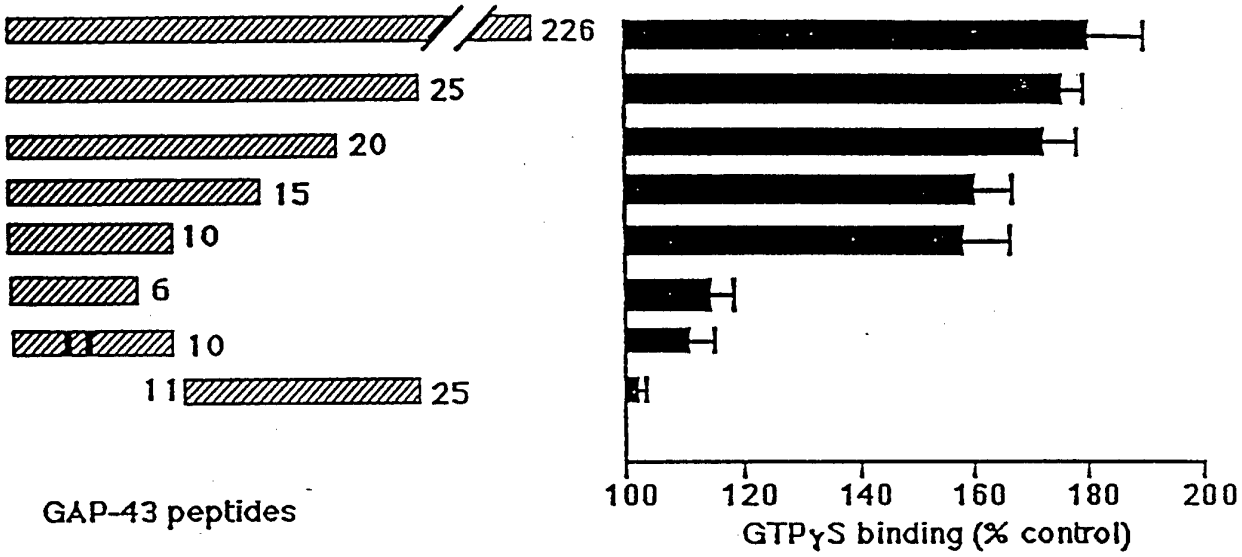


Figure 34

GAP-43 peptides (100 μM) stimulate GTPγS binding to G_o.

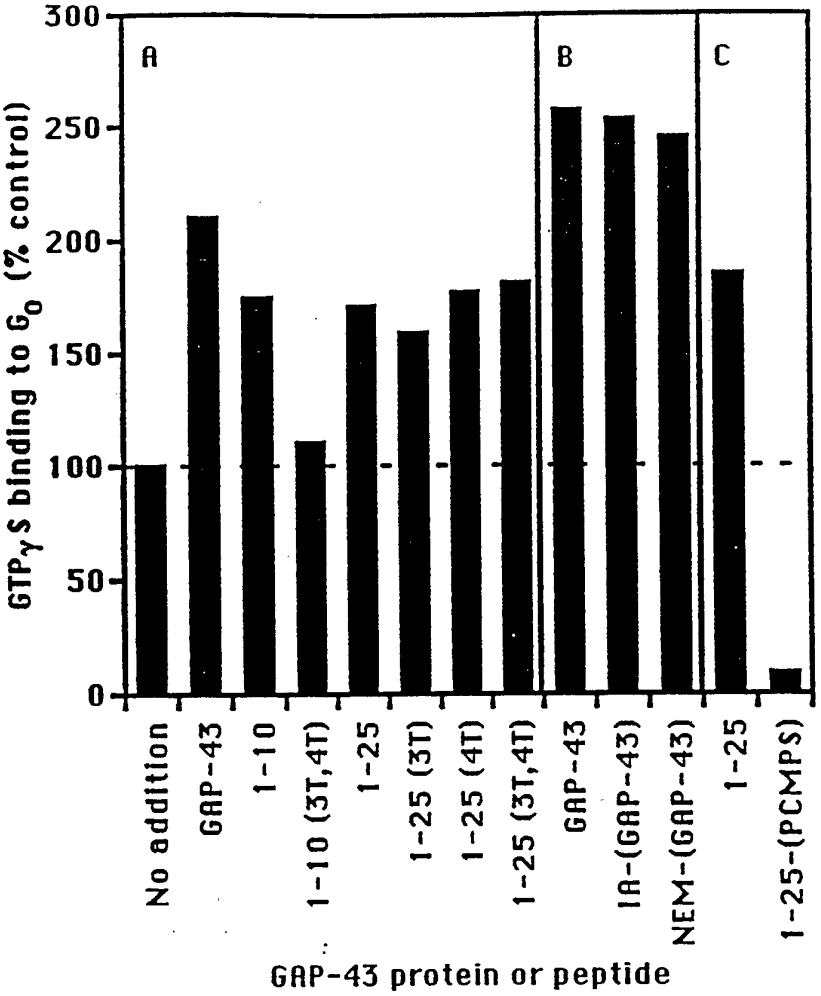


Figure 35

GTPγS binding to G_o in the presence of various GAP-43 peptides.

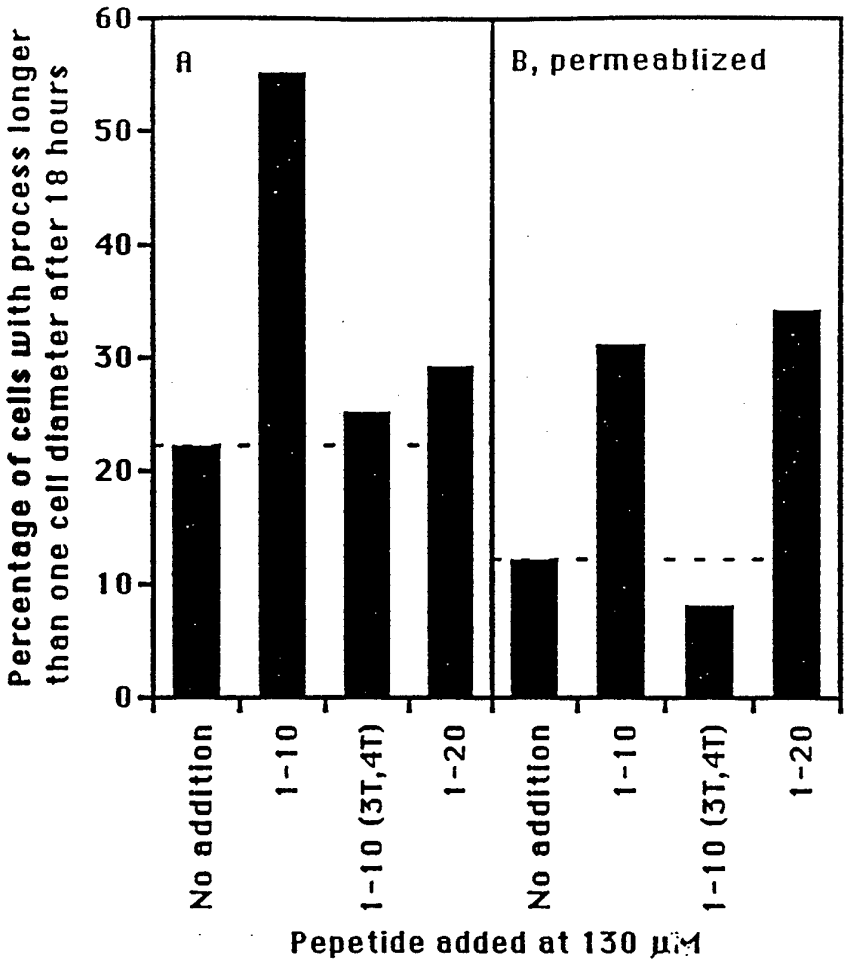


Figure 36

Neurite outgrowth from control and briefly permeabilized N1E-115 cells in the presence of the indicated GAP-43 peptides.

Figure 37(a)

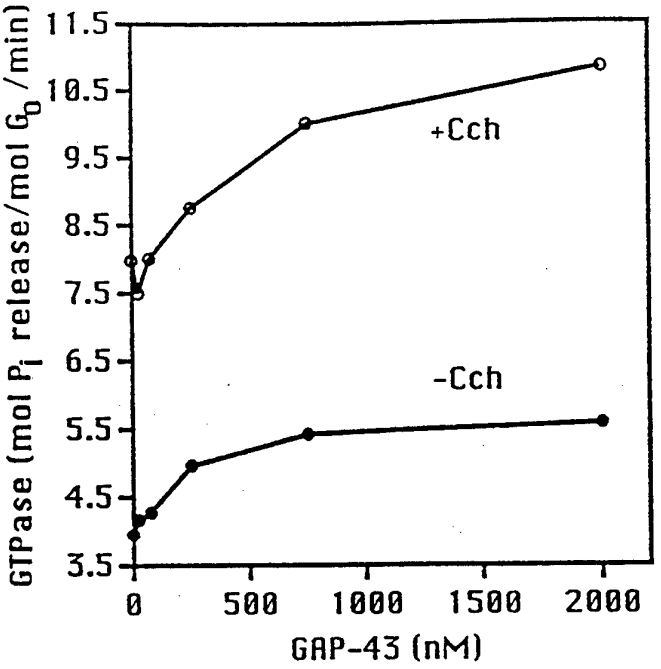

GTPase (mol P_i release/mol G_o /min)
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
+Cch
-Cch
0
500
1000
1500
2000
GAP-43 (nM)

Figure 37(b)

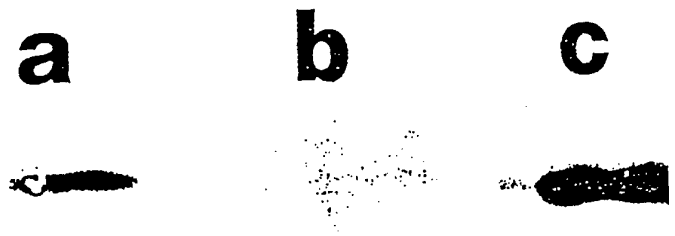


Figure 38(a)

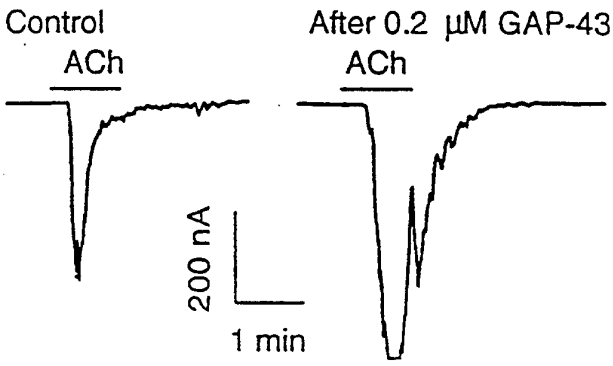


Figure 38(b)

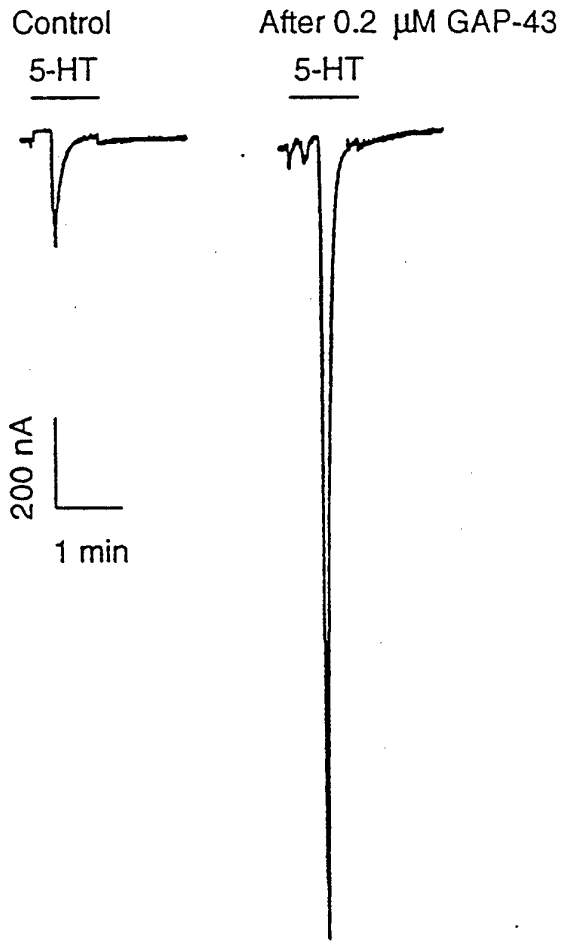


Figure 38(c)

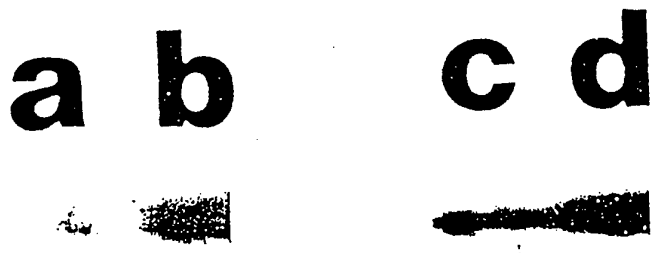


Figure 38(d)

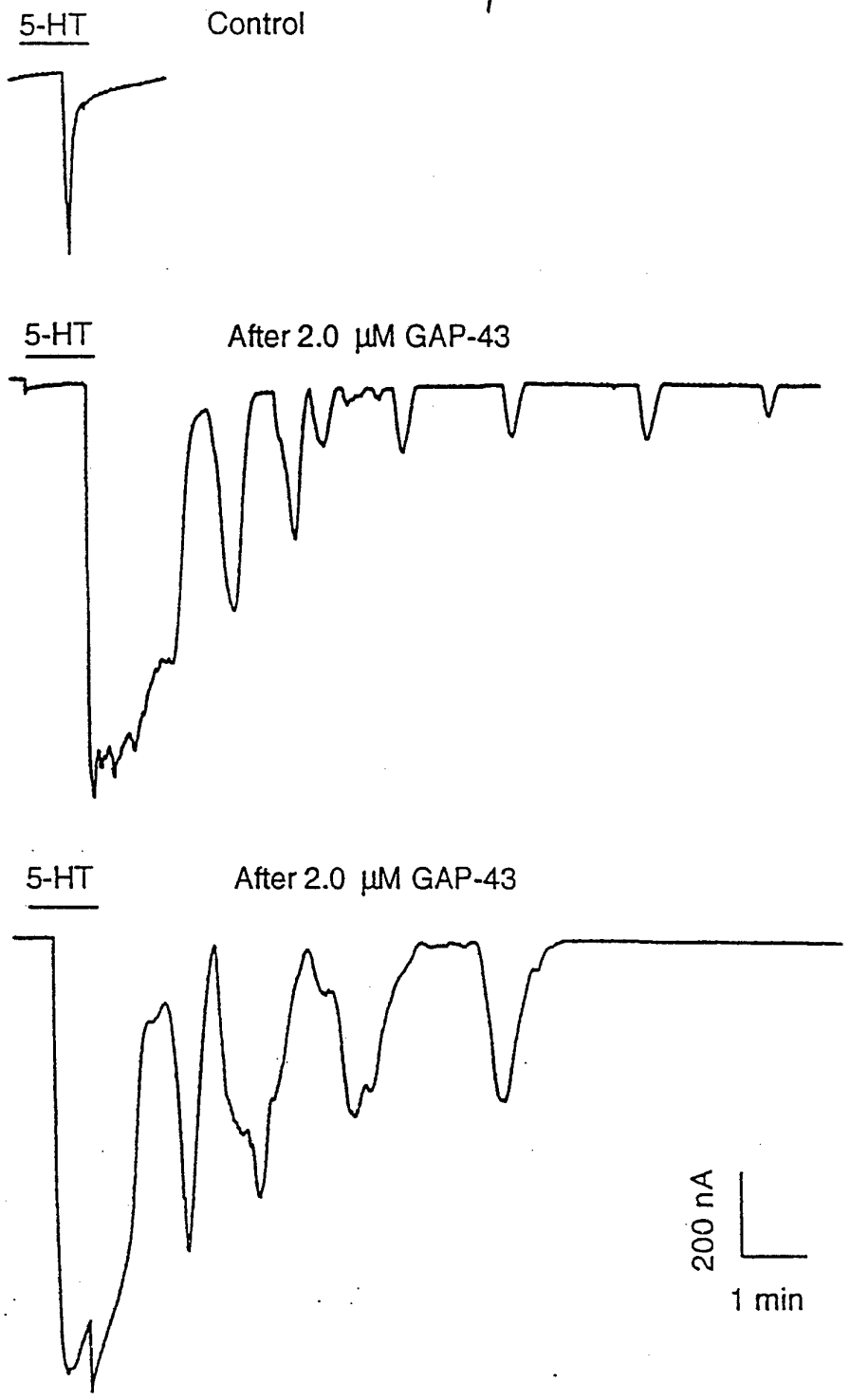

5-HT
Control
5-HT
After 2.0 µM GAP-43
5-HT
After 2.0 µM GAP-43
200 nA
1 min

Figure 38(e)

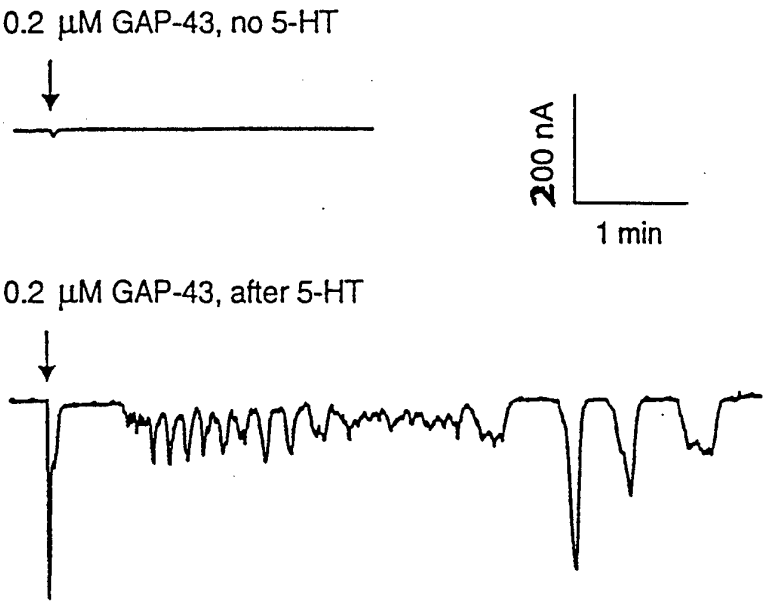


Figure 39 (a)

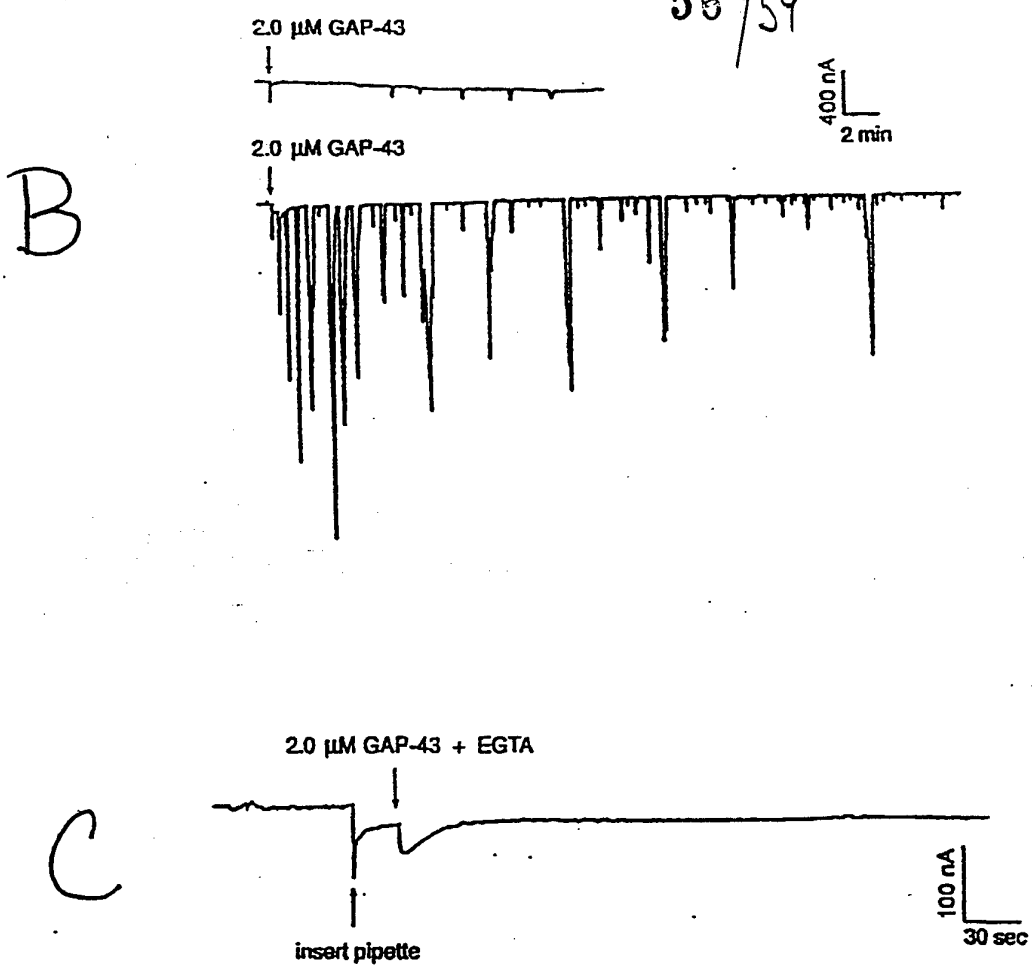

B
2.0 µM GAP-43
400 nA
2 min
2.0 µM GAP-43
C
2.0 µM GAP-43 + EGTA
100 nA
30 sec
insert pipette

Figure 39

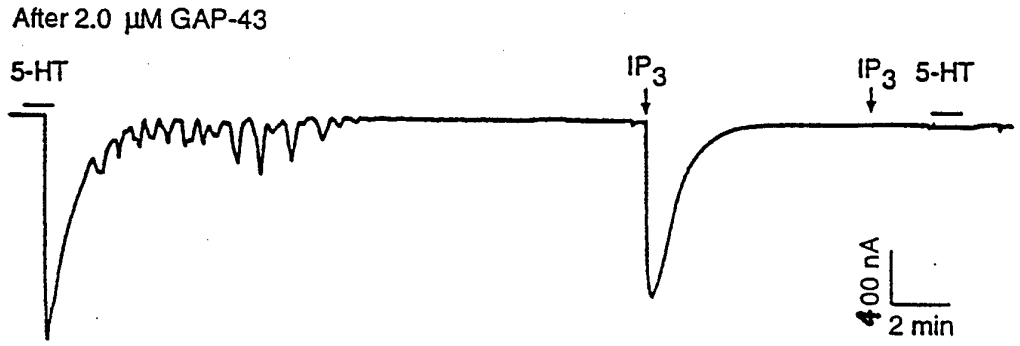


Figure 40.

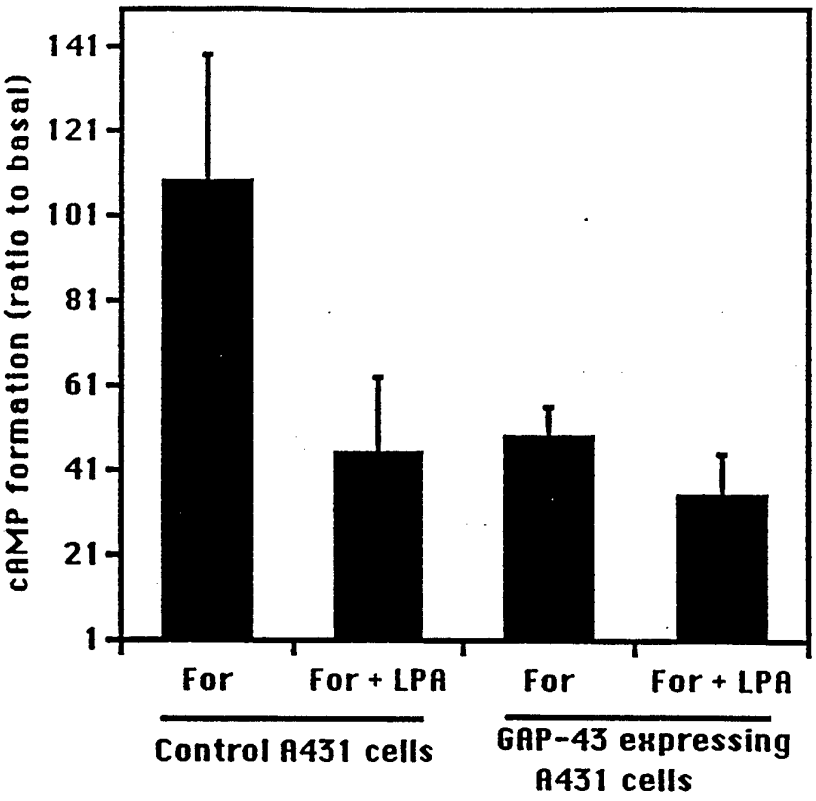

cAMP formation (ratio to basal)
141
121
101
81
61
41
21
1
For
For + LPA
For
For + LPA
Control A431 cells
GAP-43 expressing A431 cells

Figure 41

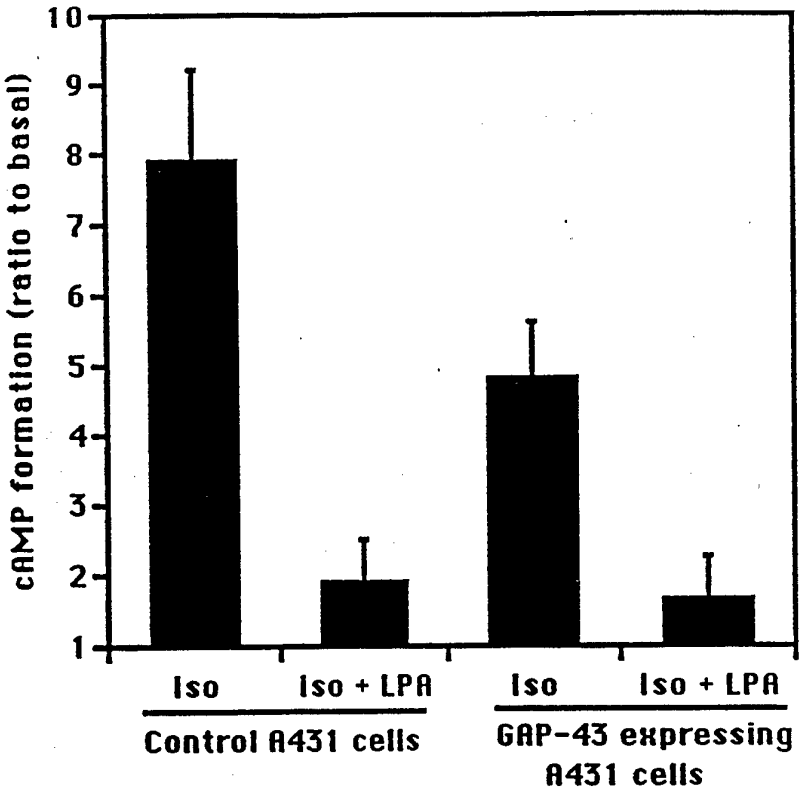

cAMP formation (ratio to basal)
10
9
8
7
6
5
4
3
2
1
Iso
Iso + LPA
Iso
Iso + LPA
Control A431 cells
GAP-43 expressing A431 cells

Figure 42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07643

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 7/00, 13/00; A61K 37/00
US CL :530/300, 350; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence databases, Medline, Biosis, CA, WPI, APS
Search terms: GAP-43, growth associated protein, neuron, sequence, recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Brain Research, Volume 565, issued 1991, S.M. Schuh, "Production of the neuronal growth-associated protein GAP-43 in a bacterial expression system", pages 85-93, see entire document.	1, 2, 4, 6, 14, 46,49,63,64,66-68 3,5,7,22,23,50-52, 69,79,89
X	Nature, Volume 341, issued 28 September 1989, M.X. Zuber et al., "A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43", pages 345-348, see entire document.	50-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 November 1993	Date of mailing of the international search report NOV 15 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JACQUELINE G. KRIKORIAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07643

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 49, issued 19 June 1987, G.S. Basi et al., "Primary Structure and transcriptional regulation of GAP-43, a protein associated with nerve growth", pages 785-791, see entire document.	1-7, 14,22,23,46,49,63,64,50-52, 66-69, 79,89
Y	Nature, Volume 344, issued 26 April 1990, S.M. Strittmatter et al., "Go is a major growth cone protein subject to regulation by GAP-43", pages 836-841, see entire document.	89
X Y	Neuron, Volume 1, issued April 1988, K.S. Kosik et al., "Human GAP-43: its deduced amino acid sequence and chromosomal localization in mouse and human", pages 127-132, see entire document.	1,3,5,7,14, 46,49,63,64,66-68 22,23,50-52, 69,79,89
Y	The Journal of Cell Biology, Volume 108, issued February 1989, J.H.P. Skene et al., "Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43", pages 613-624, see entire document.	1-7, 14,22,23,46,49,63,64,50-52, 66-69, 79,89
A	Trends in Neuroscience, Volume 10, Number 12, issued 1987, L.I. Benowitz et al., "A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity", pagees 527-532, see entire document.	1-7, 14,22,23,46,49,63,64,50-52, 66-69, 79,89

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-7, 14, 22, 23, 46, 49, 50-52, 63, 64, 66-69, 79 and 89, drawn to a first composition, recombinant GAP-43, an internal regulatory protein (IRP), and peptides thereof, and the first method of use, classified in Class/subclass 530/350 and 530/300, and 435/7.21.

II. Claims 8-13, 15, 45, 47, 48, 57-62, and 65, drawn to a second composition, DNA encoding GAP-43 or IRP, and an expression vector and host cell containing said DNA, classified in Class/subclass 536/23.1, 435/320.1, and 435/240.2.

III. Claims 16-21, 24-26, 53-56, 77, 78, and 80, drawn to a third composition, an antibody to GAP-43 or an IRP peptide, a hybridoma cell line that produces said antibody, and the method of use, classified in Class/subclass 530/388.1 435/7.1, and 435/240.27.

IV. Claims 27-29, 30, 31, and 38-44, drawn to methods for inducing, enhancing, inhibiting, or modulating expression of GAP-43 in cells, classified in Class/subclass 514/2, 435/240.2, 435/172.3, and 435/69.1.

V. Claims 32-37 and 81-85, drawn to methods for promoting structural remodeling, healing, synaptic plasticity in neuronal cells, and for modulating the microenvironment of neuronal cells, classified in Class/subclass 435/240.2, 435/172.3, 514/2, 424/85.8, and 424/180.

VI. Claims 70-76 and 86-88, drawn to a method for modulating the binding activity of a protein, and a method of screening for a substance capable of modulating the binding activity of a protein, classified in Class/subclass 435/29.

VII. Claims 90-95, drawn to a method for augmenting activation of a protein by a receptor, classified in Class/subclass 435/69.1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07643

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7,14,22,23,46,49,50-52,63,64,66-69,79, and 89

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.