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A ROLE FOR MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

by

GITALI MUKHOPADHYAY

A dissertation submitted to the Graduate faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York.

1997

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

22nd Jan '97
Date

Chair of Examining Committee

January 23, 1997
Date

Horst Schulz
Executive Officer

Peter Lipke
Thomas Schmidt-Glenewinkel
James Salzer
David Colman.

Supervisory Committee

The City University of New York

Abstract

A ROLE FOR MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

by

Gitali Mukhopadhyay

Advisor: Professor Marie T. Filbin

Following nerve injury, axons in the CNS do not normally regenerate. It has been shown that CNS myelin inhibits neurite outgrowth. However the precise nature of the molecules responsible for this effect is not known. In the nervous system myelin-associated glycoprotein (MAG), a well characterized transmembrane glycoprotein has been shown to promote neurite outgrowth from newborn dorsal root ganglion (DRG) neurons. Here, we demonstrate that MAG expressed by Chinese hamster ovary (CHO) cells can inhibit neurite outgrowth from cerebellar neurons of all post-natal ages and PND3 (postnatal days) through adult DRG neurons. Both the large (L-MAG) and small (S-MAG) isoforms of MAG behave in this manner. In addition, it has been shown that MAG binds to sialoglycoproteins on the surface of neurons and inhibition/promotion by MAG depends directly or indirectly on this binding. To begin to map the sialic acid

binding site in MAG, arginine 118 (R118), a conserved amino acid among the family of molecules termed the Sialoadhesins, to which MAG belongs, was mutated to either alanine (R118A) or aspartic acid (R118D). We found that MAG mutated at R118, when expressed in CHO cells, still inhibited neurite outgrowth. Therefore, we suggest that sialic acid binding of MAG to neurons is necessary but insufficient to affect axonal regeneration. It has been reported that PNS myelin, unlike CNS myelin is permissive for neurite outgrowth. Using various concentrations of purified PNS myelin as a substrate, we demonstrate that this membrane is also inhibitory for axonal regeneration.

In conclusion, besides its putative role in the initiation of myelination, we have identified additional role for MAG as an inhibitor of axonal regeneration. This inhibitory effect of MAG may be responsible, in part, for the lack of CNS nerve regeneration *in vivo*.

ACKNOWLEDGMENTS

My work reached its desired end only because of the help and cooperation I have received from all sides. My husband Amarnath has been one of the key persons whose unrelenting support and sacrifice made it possible for me carry on my work and manage my home. My little son Shouvik seemed to provide inspirations through his baby talk which was also a source of enjoyment in difficult times.

My gratitude is due to Dr. Frank Walsh and Dr Patrick Doherty with whom I worked in London who also made my trip to London fruitful and enjoyable.

I would be failing my duty if I do not express my heartfelt gratitude to my all other colleagues, Kejia, Manher, Maria-Elena, Song, Ying-Jing, Wenhui, Marat and Yamina in the laboratory and also the professors, Dr. Peter Lipke, Dr David Colman, Dr James Salzer, Dr, Robert Lazarini and Dr, Thomas Glenewinckel those who extended their help every now and then in course of my work and helped me to reach my final goal.

My mentor Dr. Marie T Filbin has been the most instrumental person who has helped to fructify my research work. Her guidance, supervision and amiable nature has made me ever indebted to her.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter I

General introduction

1. Regeneration in the Nervous System

In mammals, regeneration of injured axons in the CNS is limited. In contrast, peripheral axons regenerate well (Ramon y Cajal 1928; Schwab and Thoenen, 1985; Sandrock and Matthew, 1987). However, CNS neurons are able to regenerate if the correct environment is provided. For instance, when a piece of peripheral (sciatic) nerve is grafted onto the lesion site of spinal cord, injured CNS axons grow through the graft (David and Aguayo, 1981). However, function was not restored because the axons stopped growing after re-entering the spinal cord. In contrast, if optic nerves were implanted into sciatic nerves they were almost completely avoided by regenerating peripheral axons (Aguayo et al., 1978). These *in vivo* experiments, nevertheless, show the importance of the environment in the repair of injury. Similarly from *in vitro* experiments it has been shown that when dissociated PNS neurons, i.e. sympathetic or sensory neurons, from newborn rat were cultured on adult rat sciatic and optic nerve explants, the neurons regenerated on the sciatic nerve but not on the optic nerve explants (Schwab and Thoenen, 1985). Neurite growth inhibitory molecules present in CNS tissue may contribute, in part, to the poor regenerative capacity of the CNS. These inhibitors are less abundant, or may be completely absent from PNS tissue.

2. CNS White Matter, but not Gray Matter, is an Inhibitor for Axonal Outgrowth

In order to investigate the putative inhibitory properties of CNS tissue, cryosections of various CNS tissue were used as a substrate for the growth of embryonic or newborn neurons (Watanabe and Murakami, 1990; Savio and Schwab, 1989). Major white matter tracts were found to be inhibitory for axonal growth. In contrast, cell-rich gray matter was permissive for the growth of axons. These observations suggest the presence of inhibitory molecules in the white matter of CNS tissue. Although CNS white matter also has neurite growth promoting

molecules, such as N-CAM, it is suggested that because of an excess of neurite growth inhibitors, the overall property of the white matter of CNS tissue is inhibitory.

There are four major components of white matter (myelin), axons, astrocytes and oligodendrocytes with their accompanying myelin. In an attempt to distinguish which specific component of CNS white matter is responsible for inhibition, myelination was blocked by injecting newborn rats with the antimitotic agent 5-azacytidine (Schwab and Savio, 1989). There was a decrease in the mature oligodendrocyte population and as a consequence there was reduced myelination. However, the astrocyte population was not affected by this drug treatment. White matter containing immature oligodendrocytes and less myelin, was compared to the white matter of control untreated animals for the ability to support neurite outgrowth from neuroblastoma cells. Many more neuroblastoma cells grew on the white matter which was partially deficient in myelin compared to that of the control myelin-rich white matter tissue. Hence, myelin of white matter of the CNS is not a preferable substrate for the growth of neurons.

Other evidence in support of an inhibitory role for CNS myelin was demonstrated by Schwab and his colleague in 1990 by an *in vivo* experiment (Savio and Schwab, 1990). They showed that in X-ray treated, myelin-deficient spinal cord, transected corticospinal tract (CST) fibers can regenerate longer distances than control myelinated CST fibers.

The next step was to identify more precisely the inhibitors of neurite outgrowth in CNS myelin (Caroni and Schwab, 1988a and 1988b). Schwab and his colleague established that the proteins, rather than the lipids, from CNS myelin membranes were responsible for the inhibition of the growth of neurites, because brief treatment of CNS myelin with trypsin can abolish its non-permissive property. Then they wanted to identify which specific proteins from CNS myelin were responsible for its non-permissiveness. The method they used to monitor non-permissiveness of CNS myelin was to measure the spreading of fibroblast cells on CNS myelin

(Caroni and Schwab, 1988a and 1988b). Taking the effect of total CNS myelin as 100% non-permissive, they found that when the proteins of CNS myelin were extracted from a SDS gel, and used as a substrate, non-permissiveness was about 20% of that of total CNS myelin. A similar degree of non-permissiveness was detected from proteins in that gel region which corresponds to the molecular weight of 35 and 250 kD. A reduced level of non-permissiveness of extracted CNS myelin proteins compared to intact myelin was attributed to interference from the SDS gel extraction buffer. Hence, it was concluded that the 35 and 250 kD proteins, designated as NI-35 and NI-250, accounted for most of the non-permissive activity of gel-extracted CNS myelin proteins. These two proteins have not yet been cloned but monoclonal antibodies designated as IN-1 and IN-2 have been raised against 250 kD protein fraction from rat CNS myelin (Caroni and Schwab, 1988a and 1988b).

The *in vitro* application of the antibody IN-1 to purified CNS myelin was found to ameliorate the inhibition of neurite outgrowth from SCG (superior cervical ganglion) neurons (Caroni and Schwab, 1989). Similarly, after injury, *in vivo* application of IN-1 combined with the growth factor neurotropin-3 (NT-3), resulted in 5-10% of CST axons regenerating 25-33 mm; a greater length, than seen with IN-1 alone, (5-11 mm) (Schnell et al., 1994). Because only 5-10% of axons regenerate it is possible that inhibitors, other than NI-35 and NI-250, are also present in CNS myelin. Schwab and his colleagues had also reported that corticospinal axons undergo regeneration and anatomical plasticity after application of IN-1 antibody (Bregman et al., 1995). This conclusion derives from the observation that 4 to 6 weeks after a spinal cord lesion, 80% of the IN-1 treated rats recovered contact-placing responses compared to the control horseradish perioxidase (HRP) treated animals. These contact placing responses are a functional assay to detect the recovery of sensorimotor reflex function which is known to be dependent upon the integrity of the corticospinal pathway.

3. Neurite Outgrowth of DRG Neurons on Lesioned CNS and PNS Tissue

Mammalian CNS tissue is generally believed to be a poor substrate for the regeneration of CNS or PNS neurons (Schwab and Thoenen, 1985; Caroni and Schwab, 1988b). However, David and co-workers showed that chick embryonic (E8-E10) DRG neurons extended longer neurites on pre-degenerated adult optic nerve than on non-degenerated optic nerve (David et al., 1990). They used adult rat optic nerves 4-6 months after transection. Such a long post-transectional waiting period was used because of the slow removal of myelin debris in Wallerian degeneration in the CNS. Therefore, they concluded that inhibitors which are present in myelin, are responsible for the lack of regeneration of DRG neurons.

Previously it has been shown that PNS tissue is permissive for nerve regeneration (Ramon y Cajal 1928). However, it was reported by Perry and his colleaques that in a strain of mutant mice, C57BL/O1a, in which myelin remains and is not rapidly removed after injury, regeneration of axons is very slow and occurs mostly along unmyelinated fiber tracts (Brown et al., 1992). In agreement with these findings, Bedi et al., 1992 reported that DRG neurons isolated from adult animals will extend neurites *in vitro* on PNS tissue which has previously been lesioned and permitted to undergo Wallerian degeneration *in vivo* (Bedi et al., 1992). In these degenerated nerves there is little or no myelin. Hence, all these studies emphasize the need to remove myelin which most likely contains inhibitors, before regeneration can take place.

4. The Function of Inhibitors of Regeneration in the Nervous System

The capacity of terminals of axons to sprout within the adult CNS has been studied after lesion. After partial lesion, uninjured nerve fibers can respond to the denervation of neighboring areas by developing new branches that innervate the denervated tissue. This phenomenon is known as collateral sprouting (Kapfhammer and Schwab, 1994). This collateral sprouting

depends on the presence of molecules from the surrounding environment of the nerve terminal. If the surrounding tissue has little or no inhibitors present, there will be increased collateral sprouting.

Schwab and his co-workers have shown a correlation between the plastic potential of axons of the CNS and the degree of myelination of surrounding tissue (Schwegler et al., 1995). It is known that growing neurites express an abundance of growth-associated protein (GAP-43). Therefore, the expression of GAP-43 was used as a marker for the growth of neurites, and consequently as an indicator of plasticity. Lesion-induced sprouting was shown in several CNS areas, namely the septum, the hippocampus, the olfactory bulb, cerebellum and the substantia gelatinosa in the spinal cord. It was found that all these areas of the CNS have little myelin and therefore express only low amounts of myelin-associated inhibitors. In contrast, evidence for lesion-induced sprouting in more heavily myelinating areas, such as fiber tracts, is very rare. Hence, it is suggested that neurite growth inhibitors in myelin are controlling the lesion-induced sprouting of CNS neurons.

5. Presence of Other Inhibitors and Bifunctional Molecules in the Nervous System.

A well system for study of the formation of specific neuronal connections in the CNS is the retinotectal projection. Within this retinotectal projection, nasal axons of retinal ganglion neurons project to the posterior tectum and temporal axons project to the anterior tectum. In a stripe assay (placing alternating stripes of posterior and anterior membranes), nasal and temporal axons are offered the choice of growing on either anterior or posterior tectal membranes (Walter et al., 1987a). Nasal axons can grow well on both anterior and posterior membranes. In contrast, temporal axons are repelled by posterior membranes and grow only on anterior membranes (Walter et al., 1987b). After incubation of the tectal membranes with phosphatidylinositol-

specific phosphatase C (PI-PLC) enzyme that specifically cleaves GPI-anchored proteins from the membrane surface (Low, 1989), the posterior tectal membrane lose their repellent activity for temporal axons, which then cross freely over the stripe membranes with no obvious preference for either of the membrane types (Walter et al., 1990b). Therefore, it was speculated that the possible candidate guidance molecules in the posterior membranes, which inhibit the growth of temporal axons, are GPI anchored. Recently RAGS, (repulsive axon guidance signal), a GPI anchored, 25 kD protein has been isolated by 2D gel from posterior tectal membrane of E6-E12 chicken embryo (Drescher et al., 1995). However, it has been shown by *in vitro* studies that RAGS expressed by COS cells, can induce collapse of growth cones of the axons of both temporal and nasal origin. Therefore, it is speculated that as well as RAGS other molecules are also involved *in vivo* for in inhibiting the growth of temporal axons towards posterior tectal membranes.

It has been shown that chondroitin sulfate proteoglycans (CSPGs) can inhibit N-CAM or Ng-CAM mediated neurite outgrowth (Milev et al., 1994). Neurocan (ID1) and phosphocan (3F8) are CSPGs which can bind to neurons via binding to N-CAM or Ng-CAM (Grumet et al., 1993). An antibody against N-CAM or Ng-CAM can abolish the binding of neurons to these CSPGs. By immunostaining it has been shown that neurocan, phosphocan, N-CAM and Ng-CAM all co-localize at high concentrations in the molecular layer and fiber tract. All these findings, support the view that CSPGs may modulate neuronal adhesion and neurite outgrowth during development by binding to neuronal cell adhesion molecules.

Another inhibitor J1-160/180 (janusin), is an ECM molecule, synthesized by oligodendrocytes (Erickson 1993). The growth of cerebellar neurites is inhibited when they encounter J1-160/180 *in vitro* (Pesheva, Spiess, and Schachner, 1989). However, it is not possible to conclude whether, J1-160/180 plays a significant role in regenerative failure, as no

experiments have been done to block its function *in vivo*. In addition, another molecule tenascin (also known as J1/tenascin, hexabrachion and cytotactin) is a large ECM molecule secreted by astrocytes. It is multimeric and the subunits contain both fibronectin type III (FNIII) and epidermal growth factor-like (EGF) repeat sequences. Recently, to characterize the function of different domains of tenascin, fragments of tenascin were generated by expressing different fragments of histidine tagged recombinant tenascin in bacteria and purifying them by nickel columns (Gotz et al., 1996). These different recombinant proteins were then assayed for their adhesive, antiadhesive (repulsive) and neurite outgrowth properties from E18 hippocampal neurons and PND6 cerebellar neurons. It was found that the FNIII domain has distinct neuronal cell attachment sites and neurite outgrowth promoting sites. Anti-adhesive properties of tenascin with the neurons are attributed to the EGF repeats and a distinct site in the FNIII domain.

Other identified inhibitors of neurite outgrowth are the family of molecules termed collapsins/semaphorins (Kolodkin et al., 1993). Collapsin-2 isolated from chick brain is expressed by developing spinal cord and optic tectum (Luo et al., 1995). Collapsin-2 was shown to collapse growth cones selectively from DRG neurites. The dorsal distribution of collapsin-2 in the early spinal cord suggests that it may play a role in patterning interneuron axon outgrowth by driving them towards the ventral midline. These interneurons connect sensory and corticospinal fibers with the anterior motoneurons of the spinal cord. Semaphorin 1/Faciclin IV (Kolodkin et al., 1992), a transmembrane protein expressed by epithelial cells in grasshopper limb bud, guides the growth cones of Til axons and also prevents these axons from defasciculating and inhibits branching. Semaphorin II is transiently expressed by a specific muscle of Drosophila during motoneuron outgrowth and regulates the formation of the synapse. Semaphorin III, can selectively function as a chemorepellent because experiments show that

among sensory DRG neurons semaphorin III repels the growth of NGF responsive DRG neurons, but has no effect on the growth of NT-3 responsive axons (Messersmith et al., 1995).

Netrins are the first diffusible chemotropic factors to be identified in the vertebrate CNS (Serafini et al., 1994). Structurally, netrins are similar to the extracellular matrix molecule (ECM) molecule laminin. Floor plate cells at the ventral midline of the spinal cord express diffusible netrin-1, which is proposed to mediate long-range chemoattraction for commissural axons (Kennedy et al., 1994). An *in vitro* experiment was used to demonstrate the chemotropic effect of floor plate cell, in which commissural axons turn towards the aggregates of transfected COS cells in response to the secretion of netrin-1 by these cells. In contrast, netrin-1 can also behave as a chemorepellent for trochlear motor axons. COS cells secreting netrin-1 can repel the growth of trochlear motor axons (Colamarino et al., 1995).

In conclusion, the axon has a defined pathway to reach its final destination. It seems logical that on the way to its final target, other axons should not interfere. Therefore, a molecule which promotes the growth of the axon in a particular direction, and prohibits the other axons from crossing that area by inhibiting their growth are known as bifunctional molecule.

Now the question to be asked is, how does the same molecule provide attractive and repulsive signals for neurite outgrowth? One explanation is that each effect is mediated by a different receptor. Alternatively, the receptor may be the same but the downstream signaling molecules may be different.

6. Background and History of MAG

Quarles et al in 1972 first reported the presence of a glycoprotein of molecular weight 100 kD in highly purified myelin fractions from developing rat brain (Quarles et al., 1973). When this purified myelin was run on 5% polyacrylamide gels and stained with periodic acid-

schiff reagent, an indicator for glycoproteins, a band appeared around a molecular weight of 100 kD, which is now known as myelin-associated glycoprotein (MAG).

Initially, in 1983 Sutcliffe et al made a cDNA library from rat brain (Milner and Sutcliffe, 1983). Then they determined the tissue distribution of some randomly selected cDNA clones by Northern analysis. One of these clones was IB236, which is expressed only but low levels by brain tissue. They also determined the corresponding amino acid sequence of IB236 protein from the nucleotide sequence of IB236 clone and made different synthetic peptides to raise polyclonal antibodies against IB236 protein. By screening rat brain extracts with this polyclonal antibody, they found that a 100 kD protein is recognized by this antibody and this protein is a membrane protein because it requires detergent for solubilization (Malfroy et al., 1985). Later, Arquint et al independently made a cDNA library from rat brain and screened that library with two monoclonal antibodies GenS1 and GenS3 which are directed against different epitopes on human MAG (Arquint et al., 1987). Similarly, Salzer et al made a cDNA library from postnatal day (PND) 20 rat brain and screened that library for MAG cDNA with a polyclonal antibody directed against rat MAG (Salzer et al., 1987). Both groups (Arquint et al., 1987; Salzer et al., 1987) showed that their sequences of a cDNA clone were identical to the cDNA sequence of IB236 isolated previously by Sutcliffe (Milner and Sutcliffe, 1983). Later Sutcliffe and his colleagues also confirmed this finding ((Lai et al., 1987).

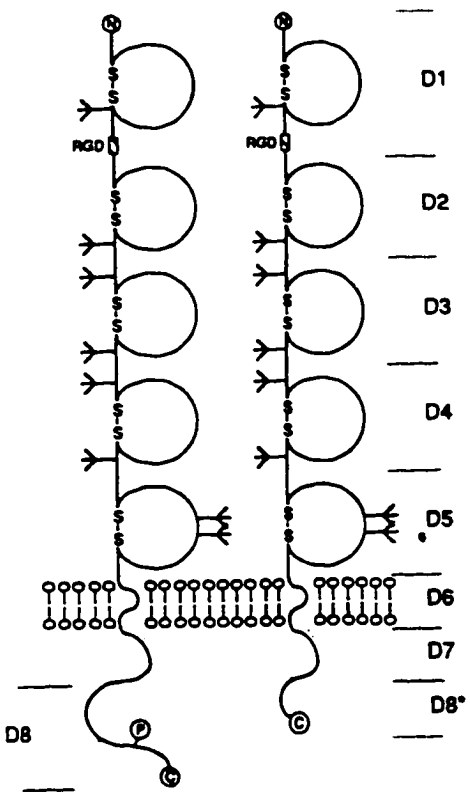
7. Chemical Structure of MAG

MAG in rat brain exists in two forms resulting from alternative splicing of the primary mRNA transcript. The protein structure of the two different forms of MAG is shown schematically in Fig. 1. The two spliced forms differ only in the length of the cytoplasmic C-terminal regions of the molecule (Lai et al., 1987).

MAG has five Ig like domains (Salzer et al., 1987), one of which is a V-like (variable) domain (domain-1, membrane distal domain) and four are C2-like of Ig domains. A V-domain consists of 9β strands, designated A, B, C, C', C'', D, E, F,and G, making up two β-sheets, the GFCC'C'' sheet and ABED sheet (Williams and Barclay, 1988). C2 domains have features intermediate between variable (V) and constant (C) Ig domains, because their sequence is more homologous to V-domains but like C-domains, C2 domains have similar spacing of cysteine residues and have 7β strands (Williams and Barclay, 1988). MAG has 14 cysteines in its ectodomain distributed within its five Ig-like domains (Pedraza et al., 1990). Domains 3 and 4 each contains two cysteines to form disulfide bond. Domains 1 and 2 each contain three cysteines and domain 5 has four cysteines. MAG does not have any unconjugated cysteines because an alkylating reagent iodocaetamide failed to detect any free sulfhydryl groups from nonreduced MAG. It was predicted by Salzer and his co-workers that additional cysteines of domain 1 and 2 are linked to each other by disulfide bridge. This conclusion derives from studies in which domains 1 and 2 were found to coprecipitate with the antibody directed against only domain 1 under nonreducing conditions (Pedraza et al., 1990). MAG has an Arg-Gly-Asp (RGD) tripeptide sequence and which was originally proposed to be in the hinge region between domains one and two (Fig. 1) (Lai et al., 1987; Salzer et al., 1987). The RGD sequence of MAG may be important because this RGD tripeptide sequence is known to be a common binding site for some integrins (Ruoslahti and Pierschbacher, 1987). However, after cleaving MAG protein with cyanogen bromide (which cleaves after methionine residue) and co-precipitating specific fragments with antibodies, Salzer and his co-worker concluded that the RGD sequence falls in the interior of domain one, in the Fβ strand (Pedraza et al., 1990). It was also found that a RGD antibody reacts well with SDS denatured MAG but not with native MAG. Hence the proposed location of the RGD sequence, buried within the first domain, is consistent with the inability of the anti-RGD antibody to recognize MAG in its native conformation. Later, Schachner and her

group found that a synthetic peptide against the RGD sequence failed to block adhesion of liposome-incorporated MAG with axons which is in agreement with the prediction of Salzer and his group for the limited functional role of RGD sequence in MAG (Sadoul et al., 1990).

Figure 1: Schematic diagram of large and small forms of the IB236/MAG protein. Five immunoglobulin-like domains are indicated as disulfide-bonded loops and positions of carbohydrate side chains by branched structure. The Arg-Gly-Asp (RGD) sequence may be either in a loop that protrudes from "binding pocket" of domain 1 or in a linker region separating D1 from D2. Transmembrane domain D6 anchors the molecule in the cellular membrane. The two forms differ in the structure of their C termini by 44 amino acids. (Modified from Lai et al.: Proc. Natl. Acad. Sci. USA. 84:4337-4341)



The amino acid sequence of MAG has eight potential sites for N-linked glycosylation in the extracellular domains, and the high carbohydrate content (30%) in MAG suggests that all eight of these potential sites are used (Quarles 1984). The carbohydrate composition of rat brain MAG is a N-linked glycoprotein containing mannose, N-acetylglucosamine, galactose, fucose, and sialic acid. Nearly one-fifth of the total carbohydrate is sulfated that reacts with monoclonal antibodies which recognize HNK-1 sugars carried by such molecules as (N-CAM, L1 and J1 of the nervous system that function in cell-cell interaction (Kruse et al., 1984). Except for rodent, MAG in many species reacts with HNK-1 antibodies.

8. Localization

Immunocytochemical studies show that MAG is localized to the inner rim of the myelin sheath, the periaxonal membrane, both in the CNS and PNS, and that it is absent from compact myelin (Trapp and Quarles., 1982; Trapp et al., 1984; Martini and Schachner., 1986). This selective periaxonal localization suggests that MAG could be involved in forming and maintaining the junction between the myelin forming oligodendrocyte or Schwann cell and the axon. In addition to the periaxonal membrane, immunocytochemistry revealed that MAG is also present in Schmidt-Lanterman incisures, paranodal loops, and outer mesaxons of PNS myelin sheaths (Sternberger et al., 1979).

9. Developmental Expression of MAG

The expression of L-MAG and S-MAG is developmentally regulated. L-MAG was shown to be expressed earlier than S-MAG, at the early stages of myelination of the CNS and PNS (Frail et al., 1985). Although in the CNS and PNS of adult animals S-MAG is the major form, L-MAG is also present (Inuzuka et al., 1991). However, a group in Canada reported that

L-MAG is not present in the adult PNS, but that only S-MAG is present (Frail et al., 1985). This apparent contradiction has yet to be resolved.

10. MAG Binds to a Sialic Acid Component on the Neuronal Surface

The Sialoadhesins are a distinct sub-group of the Ig superfamily, comprising to date sialoadhesin, CD22, MAG, CD33 and the Schwann cell myelin protein (SMP). Experiments show that CD33, CD22, sialoadhesin and MAG can bind to sialic acid (Freeman et al., 1996; Kelm et al., 1994). The ability of SMP to bind sialic acid has yet to be reported. MAG shares 40 to 50% amino acid sequence similarity in its four N-terminal domains with the four N-terminal domains of CD22 and sialoadhesin (Kelm et al., 1994). Furthermore, it was shown that CD22, sialoadhesin and MAG can recognize different linkages of sialic acid (Table 1)

Previous work has shown that the V-set domain of CD22 and sialoadhesin is both necessary and sufficient for sialic acid-dependent binding (Nath et al., 1995). In Fig. 17, the V-set domains of sialoadhesin family members (sialoadhesin, CD22, and MAG) were aligned with the V-set domain of CD8α (Vinson et al., 1996), whose crystal structure is known (Leathy et al., 1992). The amino acid arginine 118 (Arg118), of MAG in the F strand, which corresponds to Arg97 in sialoadhesin and Arg130 in CD22 (Fig.17), is conserved amongst all members of the Sialoadhesin family. By mutational analysis, it was shown that Arg97 in sialoadhesin (Vinson et al., 1996) and Arg150 in CD22 (Merwe et al., 1996) is important for sialic acid-dependent binding. Therefore, Arg118 in MAG is likely to be a key residue in mediating sialic acid-dependent binding of MAG to the neurons.

Table 1. Sialylated Glycans Found Commonly on Oligosaccharides Linked to Asparagine (N) or Serine/Threonine (O) on Glycoproteins.

Structure	Abbreviation	Sialyltransferase
Neu5Acα2→3Galβ1→3GalNAC	(3-O)	Galβ1→3GalNAc α2→3 sialyltransferase
Neu5Acα2→3Galβ1→3(4)GlcNAc	(3-N)	Galβ1→3(4)GlcNAc α2→3 sialyltransferase
Neu5Acα2→6Galβ1→4GlcNAc	(6-N)	Galβ1→4GlcNAc α2→6 sialyltransferase

11. The Function of MAG

The myelin sheath originates from the oligodendrocyte or Schwann cell plasma membrane (Webster et al., 1973; Raine et al., 1968). In CNS myelin, oligodendrocytes do myelinate more than one axon (Bunge 1964; Bunge and Glass, 1965). In contrast, Schwann cells in the PNS only myelinate one axon (Webster et al., 1973). Schwann cells which are committed to form myelin express MAG. On the basis of the early expression of MAG in Schwann cells (Martini and Schachner, 1986; Owens and Bunge, 1989) and its presence in the periaxonal membrane (Trapp and Quarles, 1982), it has been proposed that MAG plays a role in the initiation of myelination, in maintaining contact between the myelinating cell and the axon, and in maintaining the cytoplasmic collar of the myelinated axons.

To investigate the role of MAG in the initiation of myelination, MAG was prematurely expressed in Schwann cells (Owens et al., 1990). To do this, Schwann cells infected with retrovirus harbouring L-MAG cDNA, were cocultured with sensory neurons. Control, uninfected Schwann cells expressed MAG after 7 days coculture for with sensory neurons. Infected Schwann cells expressed MAG at all times irrespective of the presence of neurons. It was found that infected Schwann cells, which were expressing MAG earlier than usual, had segregated and ensheathed many more large axons compared with the uninfected, control Schwann cells. This observation suggested a role for MAG in the initiation of myelination.

It was also determined if myelination is affected when MAG expression is prevented. This was done by infecting Schwann cells with a recombinant retrovirus expressing MAG antisense RNA (Owens and Bunge, 1991). These infected Schwann cells were cocultured with sensory neurons but failed to segregate larger axons and initiate a myelin spiral. Therefore, in the absence of MAG, myelination does not proceed. In contrast, control Schwann cells infected with sense RNA, expressed MAG and formed normal compact myelin.

Two different groups independently knocked out the MAG gene in mice by creating a null mutation in the mag locus (Montag et al., 1994; Li et al., 1994). Both groups found that the amount and the compaction of myelin of these mutant mice was normal. However, the most frequent morphological phenotype reported was the reduction or absence of the characteristic cytoplasmic space between the first and second layers of myelin in the CNS. A similar morphological phenotypic abnormality was reported in the PNS. In addition, Roder and his colleagues reported that there were no apparent gross behavioral abnormalities in the mutant mice but that their fine motor coordination abilities were significantly affected (Li et al., 1994). The other group, Schachner and her colleagues reported that N-CAM was over-expressed and that some axons of the CNS were surrounded by two or more myelin sheaths (Montag et al., 1994). It was reported that in MAG-/- mice older than 8 months of age demyelination and axonal degeneration were apparent in the PNS (Fruttiger, et al., 1995). They found that out of 100 randomly selected femoral quadriceps muscle nerves from both wild type and mutant type, about 30 selected fibers from MAG-/- mice showed degenerative abnormalities. In contrast, only about 3 fibers from MAG+/+ mice showed degenerative abnormalities. Mutant mice had Schwann cells with distorted myelin sheaths or myelin debris, which indicated demyelination and onion bulb formation indicating remyelination. Schwann cells from mutant mice were either associated with degenerating or normal appearing axons. The degenerated axons were characterized by unusual small diameter, or axoplasm containing numerous vesicles and/or cellular debris.

In conclusion, although MAG may play an important role in the initiation of myelination, other molecules may carry out this function in its absence. One of the candidate molecules is N-CAM, which may be able to compensate for the function of MAG in the early stages of myelination but not in the later stages. To reconcile the findings *in vivo* with those in

culture, it is likely that this compensatory mechanism can only occur in the intact animal and not in tissue culture.

As in MAG-knock out mice, a similar compensatory situation is observed in N-CAM knockout mice (Tomasiewicz et al., 1993; Cremer et al., 1994). N-CAM has various functions during development including the growth, guidance and bundling of axons (Rutishauser and Edelman, 1990). Therefore, it is difficult to imagine the normal formation of the nervous system without the presence of N-CAM. However, in the absence of N-CAM, animals mice appear healthy and fertile, except for a few phenotypic morphological changes, a 36% decline in size of the olfactory bulb, a 10% reduction in overall brain weight and they show deficits in spatial learning when tested in the Morris water maze, a memory test, to assay the function of the hippocampus (Cremer et al., 1994).

Goals of this Thesis

In the adult CNS nervous system injured axons fail to regenerate over long distances (David and Aquayo, 1981). Poor axonal regeneration in the CNS has been related to the presence of inhibitory molecules for axonal growth. It has been shown by an vitro assay that MAG promotes neurite outgrowth from newborn DRG neurons (Johnson et al., 1989). The length of newborn DRG neurites are double in length on MAG transfected fibroblast cells compared to an control fibroblast cells. There are many neurite outgrowth promoting molecules in the nervous system but only a relatively few inhibitory molecules have been identified so far, such as tenascin (Gotz et al., 1996,), collapsins/semaphorins (Kolodkin et al., 1993), chondroitin sulfate proteoglycane (Milev et al., 1994; Grumet et al., 1993) and NI-35 and NI-250 (Caroni and Schwab, 1988a and 1988b).

In conclusion, like other Ig members such as L1 (Williams et al., 1992), N-CAM (Doherty et al., 1990), and TAG1 (Furley et al., 1990), MAG has neurite outgrowth promoting activity. In this respect except for newborn DRG (Johnson et al., 1989) and embryonic retinal ganglion cells (RGC) (Salzer et al., 1990) the effect of MAG on other types of neurons from newborn or older mice has never been tested. Therefore, experiments have been designed to address a number of questions. 1) How does MAG influence the growth of cerebellar or DRG neurons of different ages. 2) Do L-MAG and S-MAG behave in a similar manner? 3) Does MAG in PNS myelin affect neurite outgrowth? 4) It is known that MAG binds to cerebellar and to DRG neurons in a sialic acid-dependent manner (DeBellard et al., 1996). We will determine whether the sialic acid binding domain in MAG is separate from the inhibition/promotion domain or if they overlap.

Chapter II

General methods

1. Cell maintenance

CHO cells deficient in the dihydrofolate reductase (*dhfr*) gene (Urlaub and Chasin, 1980) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), proline (40 mg/ml) at 37^0 C in 5% CO_2. Untransfected cells, thymidine (0.73 mg/ml), glycine (7.5 mg/ml) and hypoxanthine (4.1 mg/ml) were added. For transfected cells, dialyzed FCS was used, hypoxanthine was omitted and 100 nM $CdCl_2$ was added.

2. Ligation of L-MAG and S-MAG cDNAs into a pSJL plasmid

The plasmid pSJL (Fig. 1) used for the expression of MAG -cDNA has been described previously (Lee and Nathans, 1988; Filbin and Tennekoon, 1990). The L-MAG-cDNA (obtained from Dr John Roder) were initially at PECE vector (Johnson et al., 1989). LMAG cDNA was cut from PECE vector by BglII and XbaI. S-MAG cDNA was cut from p-Bluescript vector by Asp718 and XbaI. Both cut cDNAs were run through 1% soft agarose and were electroeluted from the gel by Bio Rad Prep A Gene Kit. Purified L-MAG cDNA, with ends BglII and XbaI and S-MAG cDNA, with ends with Asp718 and XbaI were blunt ended by Klenow enzyme and finally ligated into pSJL plasmid at a unique site XhoI cloning site downstream from the metallothionine promoter and upstream from the poly(A) tail of the SV40 T antigen gene. The plasmid pSJL also contained the mini genes for G418 resistance and *dhfr* (dihydrofolate reductase enzyme).

3. Transformation of pSJL-LMAG/S-MAG construct to bacteria HB101

HB101 bacteria were electroporated with pSJL-LMAG and pSJL-SMAG. After electroporation bacterial cells were grown overnight on LB (Luria broth, Gibco BRL) plates supplemented with carbenicillin (Sigma). Bacteria harboring pSJL-LMAG or the pSJL-SMAG

construct were grown in single colonies on an LB plate supplemented with carbenicillin. Carbenicillin resistant bacterial colonies were picked and grown overnight at 37^0 C while shaking. Plasmids were isolated from several bacterial colonies by minipreparation, the orientation of MAG cDNA in the plasmid was confirmed by the orientation of L-MAG cDNA in pSJL plasmid after digestion with the restriction enzyme SalI. The sizes of DNA fragments are 1.87 kb, 3.94 kb, 5.4 kb for the sense and 1.87 kb, 3.09 kb, 6.33 kb for the antisense orientation of L-MAG in the pSJL plasmid. For S-MAG cDNA the sizes of DNA fragment are 1.87 kb, 4 kb and 5.4 kb for sense and 1.87 kb, 3.09 kb and 6.24 kb for antisense orientation of S-MAG within pSJL plasmid.

4. Transfection

CHO cells were transfected with 1-2 μg of DNA/10-cm plate by calcium phosphate precipitation (Graham and Van der Eb, 1973) followed by a glycerol shock (Frost and Williams, 1978). The cells were passed (1:2) the next day, and 3 days after transfection, 400 μg/ml of active G418 was added to the culture medium. Colonies appeared after ~3 weeks, and a number were picked, expanded, and single cell-cloned by limiting dilution. Several clones for wild type MAG, S-MAG or mutated MAG were grown and analyzed for protein expression in the Western blot.

5. Gene amplification

Cells which had multiple copies of the *dhfr* gene were selected by growing the cells in increasing concentrations (0.05-1 μM) of MTX. Cells were plated at 5 X 10^5 cells per 10 cm dish, and those surviving after 2-3 weeks at each concentration of MTX were allowed to multiply before being replated on the higher concentration of MTX .

6. Immunodetection of MAG immobilized on polyvinylidene difluoride (PVDF) membrane

Cells (80%-90% confluent) were lysed in 0.5M Tris-HCl (7.5) containing 2% SDS, 4% β-mercaptoethanol, and the following anti-proteases:1ug/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymotrypsin, 1 μg/ml pepstatin, and 1 μg/ml phenylmethylsulfonyl fluoride. The lysate was homogenized by passage through a 23 gauge syringe and centrifuged in a microfuge at maximum speed for 10 min. The supernatant fraction was removed, and protein was measured with a Bio-Rad kit before addition of β mercaptoethanol. The lysates were incubated at 95^0 C for 3 min, after which they were subjected to SDS -polyacrylamide gel electrophoresis in an 8% acrylamide gel (Laemmli 1970). The proteins were transferred to PVDF, immunostained (Filbin and Poduslo, 1986) with a monoclonal antibody to rat MAG (1:100; from Dr. Richard Quarles) and incubated overnight at 4^0 C; second antibody was alkaline phosphatase-conjugated goat anti-mouse (1:1000; Sigma). The substrate was 5-bromo-4-chloro-3-indolylphosphate, and the chromogen was nitroblue tetrazolium (Kirkegaard and Perry Laboratory) used according to the manufacturer's instructions.

7. Deglycosylation of MAG with PNGase F

Cells (80%-90% confluent) were lysed in 50 mM Tris (pH 8.6) containing 25 mM EDTA, 0.1% SDS , 0.5% NP40, 4% β-mercaptoethanol, and the same anti-proteases as listed above. The lysate was homogenized by passing through a 23 gauge syringe and spun for 10 min at maximum speed in a microfuge. The supernatent was incubated at 95^0 C for 10 min, allowed to cool, and incubated with PNGase F (41U/ml of lysate; New England Biolab) for 1 hr at 37^0 C. Proteins were then precipitated with ice-cold acetone and subjected to Western blotting and immunodetection for MAG as described above.

8. Indirect immunoflurorescence of intact cells

Cells were grown on glass coverslips coated with poly -L-lysine (Sigma) and blocked with 3% normal goat serum in DMEM for 1 hr at room temperature. They were then incubated with a monoclonal antibody against chicken MAG (5 μg/ml; Boehringer Mannheim) for 3 hr at 4^0 C, then fixed in 4% formaldehyde for 10 min at room temperature. The cells were then washed three times with DMEM, blocked for 30 min with 3% normal goat serum in DMEM, incubated with phycoprobe-conjugated goat anti-mouse IgG (1:50; Biomeda) for 1 hr at room temperature, and washed three times with DMEM and once with dH_2O before being mounted with Gel-mount and viewed with a Zeiss fluoresence microscope.

9. Quantitation of MAG expressed at the cell surface

An ELISA was carried out as previously described (Doherty et al., 1990a), modified as follows. Between 2000 and 3000 cells per well were plated in a 96 well ELISA plate and allowed to attach for 2 days. The cells were rinsed twice with phosphate-buffered saline (PBS), fixed for 30 min with 4% paraformaldehyde, then rinsed with PBS. The cells were blocked for 30 min with 3% normal goat serum in DMEM ,then incubated overnight at 4C with a monoclonal antibody against rat MAG (1:100) in DMEM containing 1% -normal goat serum. The cells were rinsed, again blocked, and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (1:500;Sigma). The cells were rinsed with PBS and then water. Color was developed by the addition of 50 μl of 0.2%(w/v) o-phenylenediamine (Sigma) and 0.025 $\%H_2O_2$ in citrate buffer (pH 5.0) to each well. The reaction was terminated after 25 min by the addition of 50 μl of 4.5 M H_2SO_4, and the optical density at 490 nm was determined with a 96 well plate reader. All incubations were at room temperature unless stated otherwise. Controls consisted of control transfected cells incubated

with MAG antibodies. For each experiment, 40 wells were assayed for each cell line, at least three times. The average number of cells per well was estimated by counting cells using a Coulter counter, after removal with trypsin, from 5 separate wells for each 96 well plate. Results were standardized to absorbance units per cell.

10. Isolation of Cerebellar and DRG neurons

Neurons were isolated as previously described (Doherty et al.,1990a, 1990b; Kleitman et al., 1991). Briefly, for animals up to 7 days of age, the cerebellar and DRG neurons were removed from two animals. Like tissue was combined, placed in 4ml of 0.025% trypsin in PBS, triturated, and incubated for a further 10 min at 37^0C. Trypsinization was stopped by the addition of 4 ml of DMEM containing 10% FCS, and cells were centrifuged at 800 rpm for 6 min. The cells were resuspended to a single -cell suspension in 2 ml SATO (Doherty et al.,1990a;, 1990b; Kleitman et al., 1991) containing 2% FCS using a 19 gauge syringe. For adult neurons, ganglia were removed from one animal and incubated in 4 ml of DMEM containing 10% horse serum and 0.125% collagenase A, for 3 hr at 37^0 C. The cells were triturated with a 19 gauge syringe, then centrifuged at 800 rpm for 6 min and resuspended in a single-cell suspension with a 19 gauge syringe. Cells were counted with a Coulter counter.

11. Neurite Outgrowth on transfected CHO cells

Confluent monolayers of control and MAG-expressed cells were established over a 24 hr period in individual chambers of a 8 well tissue culture slide (Lab-TEK). Cocultures were established as described previously (Doherty et al., 1990a, 1990b, 1991) by adding ~3000 cerebellar neurons from PND 1, 4 or 7 rats to the monolayer cultures or ~3000 DRG neurons from PND1 or adult rats. Culture medium was SATO containing 2% FCS. Where indicated, a

rabbit polyclonal antibody raised to a chimeric protein, consisting of the extracellular domain of MAG fused to Fc portion of Ig or preimmune serum at a concentration of 3 μg/ml, were included for the entire coculture period. After periods of time, as indicated the cocultures were fixed for 30 min with 4% paraformaldehyde, permeabilized with methanol for 2 min at -20^{0}C, and washed three times with PBS-bovine serum albumin. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated with a rabbit polyclonal antibody against GAP43 (1:2000; from R. Curtis and G. Wilkin). Cells were washed three times with PBS-bovine serum albumin, then incubated for 30 min at room temperature with biotinylated donkey anti-rabbit Ig,(1:500; Amersham), washed three times, then incubated with streptavidin-conjugated Texas red (1:500; Amersham). After three more washes , the slides were mounted in Permfluor (Baxter) and viewed with a Zeiss fluorescent microscope. The length of the longest neurite for each GAP43-positive neuron was determined by computer-assisted digitized image analysis (Doherty et al ., 1990a, 1990b, 1991).

12. Site directed mutagenesis

L-MAG cDNA (obtained from Dr. John Roder) was cut out from the vector pECE with Asp718 and XbaI restriction enzymes (Boehringer Mannheim), gel purified and blunt ended by Klenow enzyme (Gibco-BRL). The blunt ended L-MAG cDNA was ligated to dephosphorylated pBluescript which was previously digested with enzymes HindIII and XbaI (NE-Biolab) enzymes. The orientation of the MAG cDNA in the plasmid was confirmed by restriction enzymes DraIII and XhoI. L-MAG cDNA which has antisense orientation within pBluescript was chosen for site directed mutagenesis (Chameleon TM double stranded site directed mutagenesis Kit from Stratagene).

The plasmid p-Bluescript with antisense L-MAG cDNA was simultaneously annealed with oligonucleotide primers, selection (XmaI, provided by stratagene) and mutagenic (either Ala-118 or Asp-118) primers (Fig. 3). Both selection and mutagenic primers are 32 mer, PAGE (polyacrylamide gel electrophoresis) purified and phosphorylated at the 5'end (Fig. 3). On the first day, the double-stranded target plasmid DNA was heat denatured and the two oligonucleotide primers (one selection and other mutagenic, either Ala or Asp) were simultaneously annealed to the one strand of p-Bluescript-L-MAG. Both selection (XmnI) and mutagenic primers (either Ala or Asp) encoding for the mutation were extended around the plasmid using a nucleotide mix and an enzyme mix containing native T7 DNA polymerase and T4 DNA ligase supplied by the company. The newly created strand then contained the mutation but no longer contained the unique restriction site XmnI. However, several populations of plasmids then existed, those that have incorporated the primers and those that have not incorporated the primers. All of these plasmid DNAs were subsequently digested with the restriction enzyme XmnI, while the mutated plasmid remained undigested. The extended and digested DNA was then transformed into a repair-deficient mutS Escherichia Coli strain (XLmutS competent cells), which could not distinguish the original unmutated strand from the newly created strand containing the desired mutation. The mutS-deficient strain randomly selected one of these strands as "correct' and changed the other strand to be complementary to the chosen strand. Since selection of the correct strand was random, half of the plasmids contained the desired mutation and the other half converted back to the original plasmids. Because the transformation efficiency of the circular plasmids is exponentially greater than linear plasmids, mutants were favored. These transformed bacterial population were then grown in liquid culture overnight. The following day, plasmid DNA was isolated and again restriction

digested with XmaI corresponding to the selection primer. The resultant DNA digestion was then transformed into Epicurian Coli XL1-Blue competent cells.

To demonstrate the effectiveness of the Chameleon double-stranded, site-specific mutagenesis kit, the p-Whitescript TM plasmid was used as the control plasmid to test the efficiency of the mutant plasmid generation. the pWhitescript plasmid contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the B-galactosidase gene of the pBluescript plasmid (corresponding to amino acid 9 of the protein). Epicurian Coli XL1-Blue competent cells transformed with these plasmids appeared white on LB-ampicillin agar plates, containing IPTG and X-gal, because B-galactosidase activity was obliterated. Annealing of a 'blue' mutagenic primer to denatured pWhitescript plasmid resulted in a point mutation used to convert the stop codon of the pWhitescript plasmid (TAA) back to the glutamine-encoding codon (CAA). Sequence of both mutated L-MAG-Asp and L-MAG Ala cDNAs were determined by sequencing (Fig. 18). The Ala-primer introduced a cutting site for restriction enzyme SgrA1 to R118A, hence Ala mutation was further confirmed by enzyme SgrAI.

Figure 2: Diagram of the plasmid (pSJL) containing MAG-cDNA and used for transfection. MAG cDNA were subcloned into pSJL at XhoI site downstream from a mouse metallothionein promoter and upstream from a poly (A) tail of the SV40 small t-antigen gene. pSJL also contains a G418 resistant gene as a selection marker and a dihydrofolate reductase (*dhfr*) gene functioning for gene amplification.

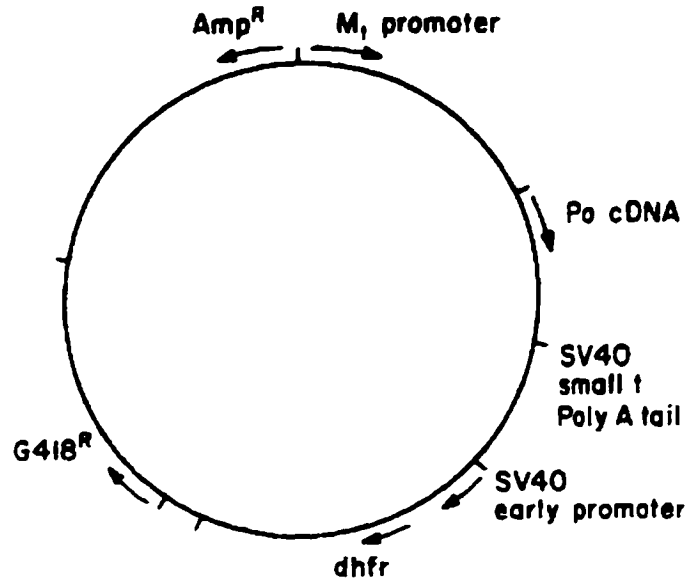


Figure. 3 Sequence of Primers

Sense primer#Ala

$^{5'}$**p**GGGAAATACTATTTCGCCGGTGACCTGGGC$^{3'}$

Sense primer#Asp

$^{5'}$**p**GGGAAATACTATTTCGACGGTGACCTGGGC$^{3'}$

13. Myelin preparation

Myelin was prepared according to Colman et al (Colman et al., 1982). Fresh sciatic nerves of two to three rats PND22 were homogenized in 0.25 M sucrose, 10 mM Hepes pH 7.4, 0.5 M $MgCl_2$, 3 mM DTT and 100 u/ml of Aprotinin in a polytron homogenizer on ice setting #4 twice for 30 second. The homogenate was spun at 2000 rpm for 2 min at 40C (HB Rotor #4). The supernatent was transferred to a cleaned tube. The concentrations of sucrose and KCl were adjusted to 1.4M and 0.25M respectively by adding sucrose buffer. The obtained homogenate (9 ml) was layered with 1 ml and 0.25 ml of 0.85 M and 0.25 M sucrose solution respectively. The sample was centrifuged at 40,000 rpm for 20 hr at 40 °C in a Beckman SW 41 rotor. Myelin formed at the 0.25-0.85M interface was removed with a pasteur pipette and homogenized on ice in 20 ml of 10mM hepes pH 7.4 and 100 U/ml Aprotinin then centrifuged at 40,000 rpm at 4^0 C for 4 hr, the final volume was weighed and then dissolved with 50:50 distilled water (W/V), the preparation was used the same day.

14. Neurite outgrowth on PNS myelin

Freshly prepared PNS myelin were dried (under tissue culture hood) overnight onto polylysine (PLY) coated chambers of an 8 well tissue culture slides (lab TEK) and 50,000 cerebellar or DRG neurons from PND1 or 2 rats will be added to each well. Culture medium was SATO containing 2% fetal calf serum. Neurons will be grown for 24 hr, After periods time, as indicated, neurons will be stained with the polyclonal antibody GAP43 as described before.

15. Desialylation of Neurons

Single cell suspensions of cerebellar or DRG neurons were washed and resuspended in PBS, approximately 2×10^6 cells were incubated with 50 mU of Vibrio cholera sialidase (VCS, Calbiochem) in a final volume of 0.5 ml, for 2 hr at 37^0 C. the neurons were washed with PBS, and resuspended in SATO containing 2% FCS for the neurite outgrowth experiments.

Chapter III

L-MAG is an inhibitor of neurite outgrowth from cerebellar neurons of all post-natal ages and adult DRG neurons

Introduction

Apart from its role in myelination, MAG has been shown to promote neurite outgrowth from newborn DRG neurons (Johnson et al., 1989). The length of neurites from DRG neurons grown on MAG transfected fibroblasts was double the length of those grown on control fibroblasts. It has been shown that MAG is also involved in neuron-oligodendrocyte adhesion and in oligodendrocyte-oligodendrocyte adhesion, but not in oligodendrocyte-astrocyte adhesion (Poltorak et al., 1987). The adhesion experiments were carried out by plating a monolayer of oligodendrocytes (target cells) into 96-well microtiter plates. Fluorescein-diacetate-labeled single cell suspensions of either neurons or oligodentrocytes (probe cells) were added to the monolayer of target cells. Adhering cells were scored by eye under a fluorescence microscope. Addition of the monoclonal MAG antibody 513 to the target cells was shown to interfere with adhesion of oligodendrocytes and oligodendrocytes to neurons. Binding of oligodendrocyte-oligodendrocyte could be attributed to a homophilic or heterophilic interaction of MAG. However, to-date, only heterophilic adhesion of MAG has been reported. Bell and his co-worker have shown that L-MAG-expressing fibroblast cells can bind to parental fibroblast cells which are not expressing MAG (Afar et al., 1991). Binding of MAG to neurons was also shown by other experiments, by Poltorak et al and Johnson et al by incorporating MAG protein into fluorescent liposomes (Poltorak et al., 1987; Johnson et al., 1989). Both forms of MAG are shown to bind to DRG neurons or spinal neurons. This binding was completely blocked by the monoclonal MAG antibody 513. As MAG is not expressed by neurons, binding of MAG to the neuronal surface must be a heterophilic interaction.

To extent the studies with DRG neurons, here, the effect of MAG on neurite outgrowth from another type of neuron, cerebellar neuron, will be studied. In addition, to assess if the

effect of MAG on neurite outgrowth changes during development, cerebellar and DRG neurons of different ages will be assessed.

Results

1. Expression of MAG by Chinese Hamster Ovary Cells

L-MAG cDNA was subcloned into a suitable plasmid, namely pSJL (Fig. 2), The transfection of MAG-cDNA containing plasmid into Chinese hamster ovary (CHO) cell lines was carried out by calcium phosphate (Graham and Van der Eb, 1973) followed by glycerol shock (Frost and Williams, 1978). After G418 selection, the dihydrofolate reductase (dhfr)/methotrexate (MTX) strategy of gene amplification was carried out as previously described (Filbin and Tennekoon, 1990, 1993). After selecting with MTX for cells with multiple copies of transfected cDNA, a number of individual clones expressing different amounts of MAG as determined by Western blot, were chosen for further study.

2. Characterization of MAG-Expressing CHO Cells

Four different cell lines were characterized as follows. Fig. 4A shows a Western blot, immunostained for MAG, of the cell lysates from four individual MAG-expressing cell lines (termed MAG1, MAG2, MAG3 and MAG4) and control CHO cells also grown in MTX. MAG expressed by all these cell lines (Fig. 4A, lanes c-f) was the same molecular weight as MAG from sciatic nerve (Fig. 4A, lane g), i.e., approximately 100 kD. This suggests that the proteins are glycosylated to the same extent as they are *in vivo* (Quarles et al., 1983). To confirm that L-MAG was glycosylated, cell lysates were treated with the enzyme N-glycosidase F (PNGase F), which removes all of the N-linked carbohydrates. As shown in Fig. 4B for MAG cells, the molecular weight of MAG decreased to about 70 kD after treatment with PNGase F. There is some microheterogeneity in the sugar composition of MAG expressed by CHO cell, as a number of minor bands are apparent (Fig. 4B) that disappear after deglycosylation.

From this Western blot it is apparent that these four cell lines express different amounts of MAG, with MAG1 expressing the most and MAG4 the least (Fig. 4A, compare lanes c and g). Sciatic nerve from adult rat contained approximately equivalent amounts of MAG (per milligram of total protein) as the cell line termed MAG2 (Fig. 4A, lane d and g, respectively). However, if these cell lines are to be tested for the effect of MAG on neurite outgrowth, it must first be established that MAG is reaching the cell surface. Furthermore, to compare the effect of different MAG-expressing cell lines on neurite outgrowth, MAG at the surface of the individual cell lines must be quantitated relative to each other. Accordingly, surface expression of MAG was assayed by immunofluorescent staining of live, intact cells using an anti-MAG monoclonal antibody and quantitated by an enzyme-linked immunosorbant assay (ELISA) of live cells. For all four cell lines, MAG can be easily detected at the surface where it is evenly distributed. Fig. 5a shows surface expression of MAG by the MAG1 cells. A similar pattern of staining, but with various degree of intensity, was obtained for the other three MAG-expressing cell lines (data not shown). There was no staining of the control tranfected cells (Fig. 5b).

The relative abundance of MAG at the cell surface of the four cell lines was determined by an ELISA of intact cells. As predicted from the Western blot analysis and the surface immunofluorescence, the MAG4 cells expressed the least amount of MAG at the surface, whereas MAG1 cells expressed the most, (about three times more), while MAG2 and MAG3 expressed intermediate amounts (Fig. 6).

3. Neurite Outgrowth from Cerebellar Neurons of Different Ages on MAG-Expressing and Control CHO Cells

Initially, rat cerebellar neurons isolated at PND1 were cultured on tranfected CHO cells before being fixed, and the average length of GAP43-positive neurites was determined. There

was substantial neurite outgrowth from PND1 cerebellar neurons when they were cultured on the control transfected cells (Fig. 7a). In contrast, when the same neurons were grown on MAG-expressing cells, neurite outgrowth was reduced by 70% (Fig. 7b). To determine whether the ability of MAG to inhibit neurite outgrowth also changed with age, outgrowth of cerebellar neurons from PND1, 4, and 7 animals was compared. A similar inhibition (70%) was observed for all ages tested (Fig. 8b). Furthermore, when the same cerebellar neurons were grown on transfected CHO cells expressing an abundance of another myelin protein, Po (Filbin and Tennekoon, 1990), which is also an adhesion molecule (Filbin et al., 1990) and a member of the Ig superfamily, their ability to extend neurites was not affected and the neurite length was as on control transfected cells (data not shown).

4. Neurite Outgrowth from Cerebellar Neurons on Different MAG-Expressing CHO Cells

It is possible that the inhibitory effect of MAG on neurite extension from cerebellar neurons is an artifact of a particular MAG-expressing transfected CHO cell line. To ensure that this is not the case, the ability of all four of the MAG-expressing CHO cell lines to inhibit neurite outgrowth described in Fig. 4 and Fig. 5 was tested. It was found that the ability of PND4 cerebellar neurons to extend neurites was reduced to about 70%, compared with the control transfected cells, when they were cultured on any one of the MAG-expressing cell lines; a similar degree of inhibition was observed when the time of coculture was either 16.5 or 24.5 h (Fig. 8a). Clearly, the inhibitory effect of MAG on neurite outgrowth was not limited to any one cell line. Furthermore, because these four cell lines each express different amounts of MAG at the cell surface (Fig. 6) and because they all inhibit neurite outgrowth to the same extent, it can be assumed that the maximum inhibitory effect on neurite outgrowth was affected by the cells expressing the lowest amount of MAG, the MAG4 cells. Of note, the plating efficiency of

neurons of any age was the same for all the cell lines tested, regardless of whether they expressed MAG (data not shown).

5. The Effect of MAG Antibodies on the Inhibition of Neurite Outgrowth by MAG-Expressing Cells.

To demonstrate the specificity of MAG's inhibition of neurite outgrowth, a polyclonal antibody was tested for its ability to reverse the effects. This polyclonal antibody was raised to the extracellular domain of MAG which was fused with Fc domain of IgG and affinity purified (Gift from Dr. Paul Crocker). In the coculture, at a concentration of 3 μg/ml this polyclonal antibody reversed the inhibition by about 50%. Preimmune serum at the same concentration had no effect (Table 2). Furthermore, this MAG polyclonal antibody had no effect on the length of neurites from neurons grown on control CHO cells (Table 2). This demonstrates the specificity of the inhibition of neurite outgrowth by MAG.

6. Neurite Outgrowth from DRG Neurons of Different Ages on MAG-Expressing and Control CHO cells

It has been shown by others that MAG can promote neurite outgrowth from DRG neurons isolated from newborn animals (Johnson et al., 1989). We obtained almost similar results with DRG neurons from PND1 rats. After 12 h of coculture of neurons on MAG-expressing cells neurites were twice as long as those cultured on control cells (Fig. 9). In contrast to PND1 DRG neurons, the extension of neurites of adult DRG neurons on MAG-expressing cells was inhibited by 40%, when compared to control cells (Fig. 9 and 10). To investigate when the switch from promotion to inhibition of neurite outgrowth takes place for DRG neurons, the neurite outgrowth assay was carried out using DRG neurons from PND1, 2, 3,

4, 5, 7, 10 and adult animals. It was found that the transition from MAG promoting to inhibiting neurite outgrowth was sharp, occurring between PND2 and PND3 (Fig. 11).

Figure 4A: Western Blot Analysis of Different CHO Cell Lines Immunostained for MAG

Immunodetection of MAG (arrowhead on the right) in cell lysates of control transfected CHO cells (lane b), MAG-expressing clonal CHO cell lines (MAG1 to MAG4, lane c-f, respectively), and rat sciatic nerve (lane g). Each lane was loaded with 150 μg of total protein. Proteins were separated by polyacrylamide gel electrophoresis (8%), transferred to PVDF, and immunostained for MAG with a monoclonal rat anti-MAG antibody (1:100), followed by alkaline phosphatase-conjugated, goat anti-mouse (1:1000). The substrate was 5-bromo-4-chloro-3-indoylphosphate, and nitroblue tetrazolium was the choromogen. Molecular weight standards (lane a), 198, 120, 88, and 70 kD.

Figure 4B: Immunodetection of MAG Expressed by MAG1 Cells after Deglycosylation with PNGase F

One-half of the cell lysate from an 80% confluent 10 cm tissue culture dish of MAG 1 cells treated with PNGase F (lane C) for 1 hr at 37°C and immunostained for MAG as described earlier. The other half of the lysate was incubated in a similar fashion but without enzyme and treated in the same way (lane b). Control transfected lysate was incubated with enzyme and treated in the same way (lane a). Arrowheads refer to glycosylated and deglycosylated MAG.

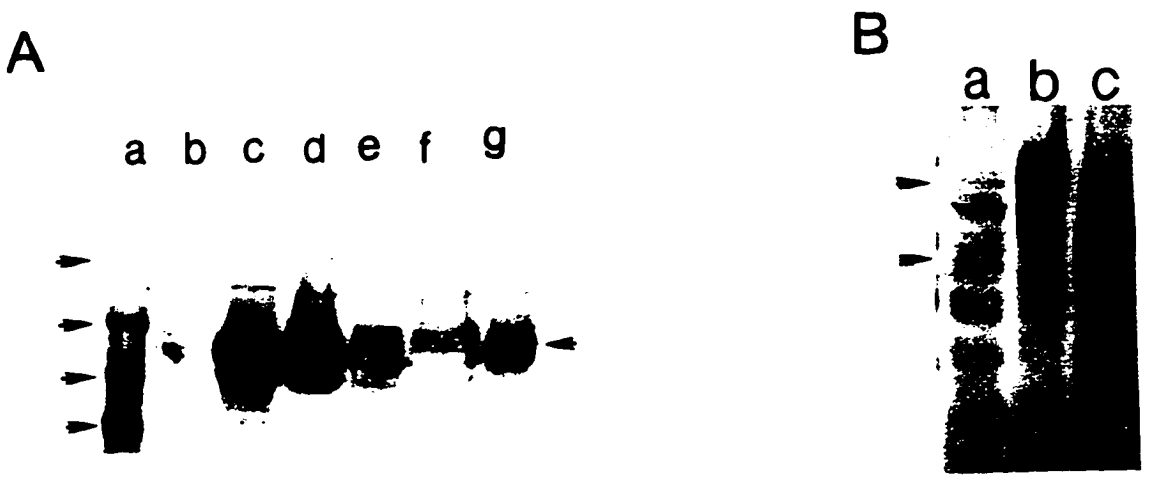


Figure 5: Surface Detection of MAG on Transfected CHO cells by Immunofluorescent Staining

Live CHO cells, either MAG1 cells (a) or control transfected cells (b) were incubated with a monoclonal antibody to chick MAG (5 μg/ml), then fixed with 4% paraformaldehyde and incubated with phycoprobe-conjugated, goat anti-mouse IgG (1:50)

a

b

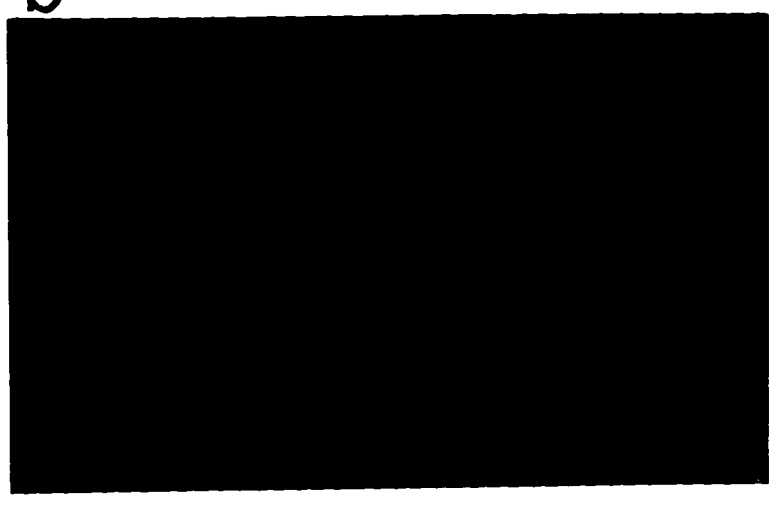


Figure 6: Quantitation of MAG Expressed at the Surface of Transfected CHO Cells

The relative amount of MAG expressed at the surface was quantitated by ELISA of fixed, unpermeabilized MAG1 (column 2), MAG2 (column 3), MAG3 (column 4), MAG4 (column 5), and control transfected (column 1) cells. Results are expressed in relative absorbance units per cell and represent the means ± SEM of at least three experiments, 36 samples per experiment.

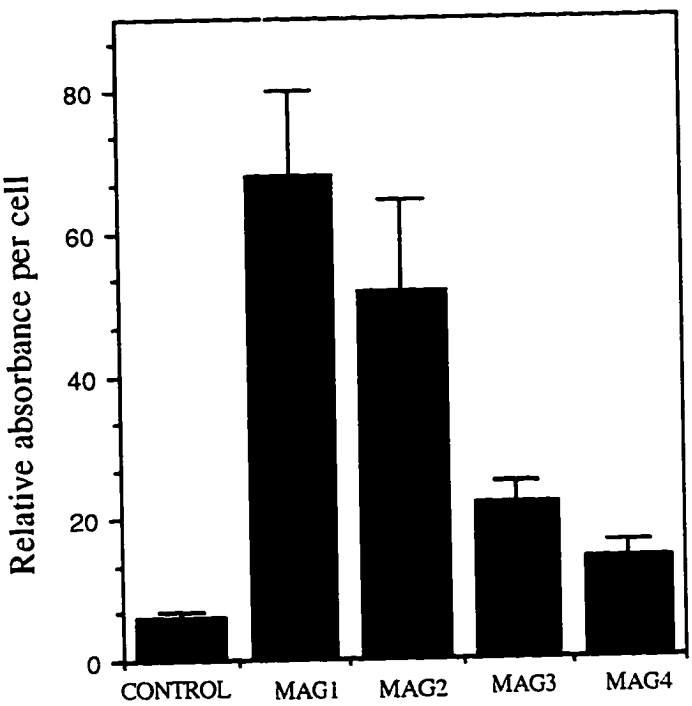


Figure 7: The Effect of MAG on Neurite Outgrowth from Cerebellar Neurons of Different Ages

(a, b, c and d) Dissociated cerebellar neurons from PND1 animals were cultured for 16.5 hr on confluent monolayer of control (a and b) or MAG-expressing (c and d) CHO cells before being fixed and immunostained for GAP43.

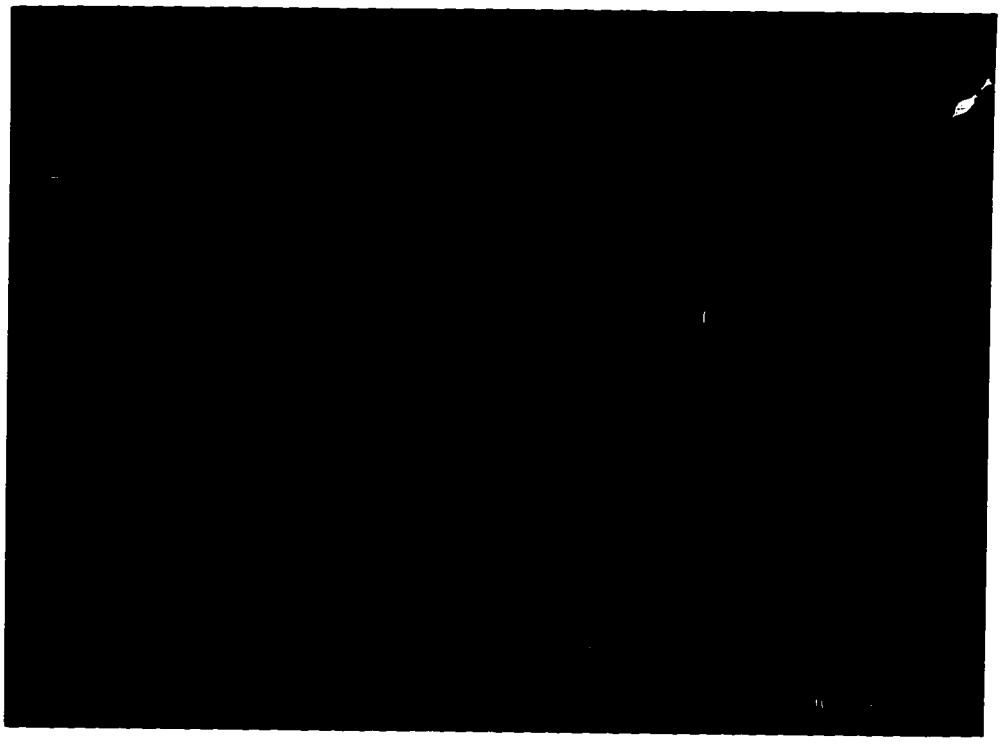


Figure 8: The Effect of Different MAG-Expressing CHO Cell Lines on Neurite Outgrowth from Cerebellar Neurons

(a) Dissociated PND4 cerebellar neurons were cultured on confluent monolayers of control and MAG-expressing CHO cells, i.e., MAG1, MAG2, MAG3 and MAG4 cells for either 16.5 h (open bars) or 24.5 hr (hatched bars). Cocultures were fixed and immunostained . Results show the mean length of the longest neurite per cell (± SEM) for 120-180 individual neurons.

(b) Dissociated PND 1, 4, and 7 cerebellar neurons were cultured for 16.5 hr on confluent monolayers of control (dotted bars) or MAG-expressing (open bars)cells before being fixed and immunostained for GAP43. Afterwards, the neurite length was measured. Results show that mean length of the longest neurite per cell (± SEM) for GAP43. Afterwards, the neurite length was measured. Results show that the mean length of the longest neurite per cell (± SEM) for 120-180 individual neurons.

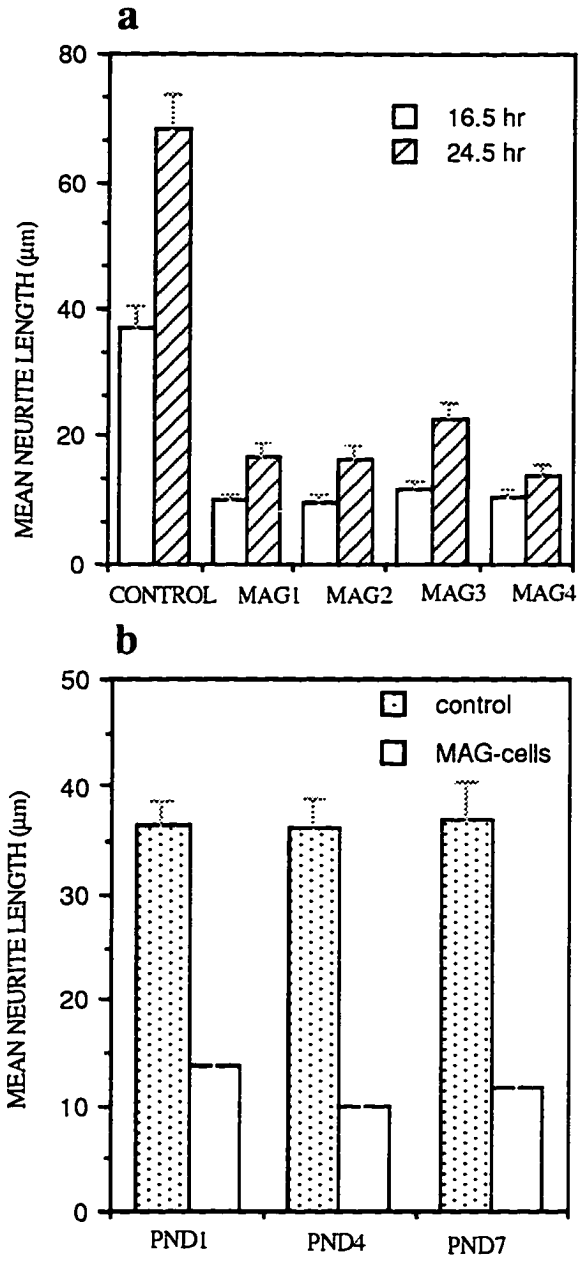


Table 2

Antibody Reversal of MAG Inhibition of Neurite Outgrowth from PND1 Cerebellar Neurons		
	MAG Cells	**Control Cells**
Preimmune	**8.9 ± 0.70 (152)**	**28.3 ± 2.0 (149)**
Anti-MAG Ab	**16.5 ± 1.12 (147)**	**29.1 ± 1.8 (150)**

Figure 9: The Effect of MAG on Neurite Outgrowth from PND1 and Adult DRG Neurons

Dissociated DRG neurons from PND1 or adult rats were cultured for 12 and 17 h, respectively, on confluent monolayers of control CHO (solid bars) or MAG-expressing (stippled bars MAG3;) cells. Results show the mean length of the longest neurite per cell (± SEM) for 100-120 individual neurons.

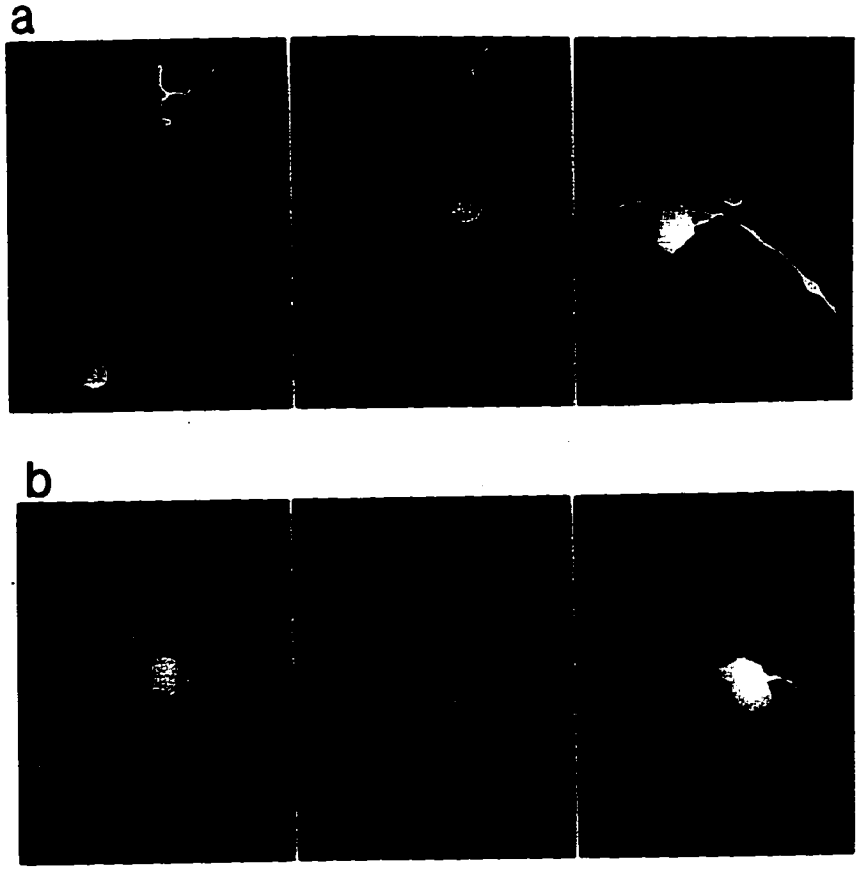


Figure 10: The Effect of MAG on Neurite Outgrowth from Adult DRG Neurons

Dissociated DRG neurons from adult rats were cultured for 17 h on confluent monolayer of control CHO (a) or MAG3 (b) cells before being fixed and immunostained for GAP43

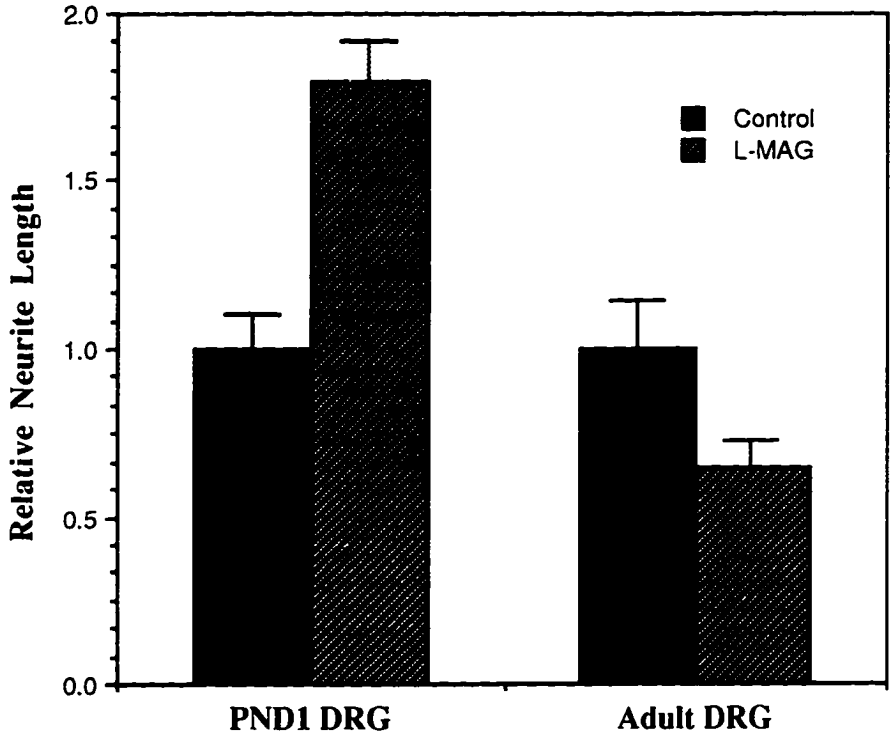
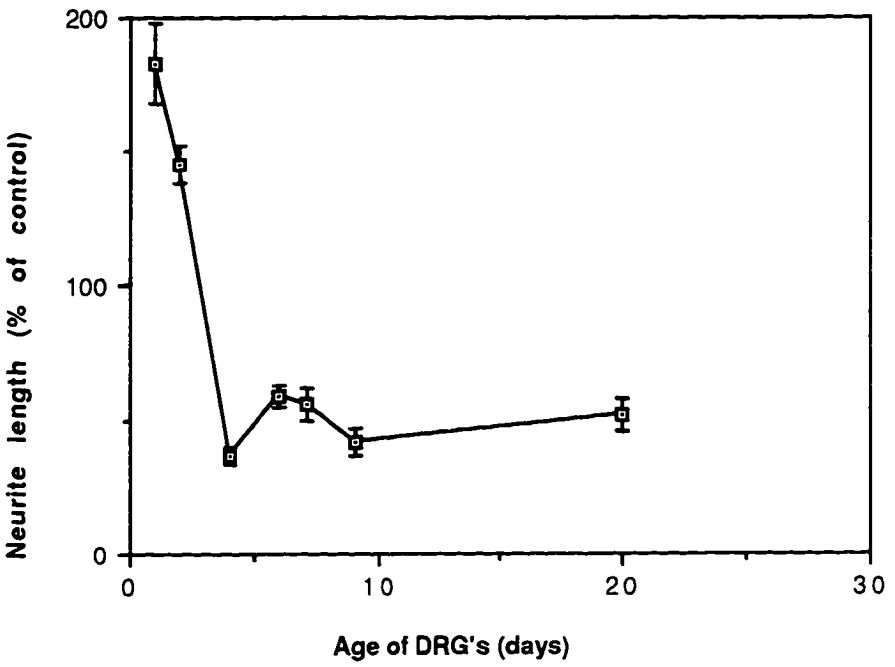


Figure 11: MAG Shows a Developmental Switch from Promotion to Inhibition of Neurite Outgrowth from DRG Neurons

Dissociated PND 1,2,3,4,5,7 and adult DRG neurons were cultured for 12 hr on confluent monolayers of MAG expressing (MAG3) and control CHO cells before being fixed, immunostained for GAP43 and neurite length measured. Results show neurite length on MAG-expressing cells as a percentage of neurite length on control cells (±SEM). For each point 120-180 individual neurons were measured.



Conclusion

The inhibition by MAG of neurite outgrowth from cerebellar neurons is 70% and from adult DRG neurons is 40%. The specificity of the inhibitory effect of MAG on the extension of neurites from cerebellar neurons and adult DRG neurons was demonstrated in a number of ways: a) It is possible that the expression of MAG as a foreign protein in CHO cells can directly or indirectly interfere with the function of other resident proteins and as a consequence the property of CHO cells could be changed, affecting neurite outgrowth. To show that this is not the case, we have shown that expression of another myelin protein, Po, on the surface of CHO cells had no effect on the extension of neurites from cerebellar neurons compared to the control CHO cells which are transfected with the plasmid only (results not shown). b) The fact that CHO cells expressing MAG promote outgrowth from newborn DRG neurons clearly shows that they are not compromised by the expression of MAG in their general ability to promote growth. c) The inhibitory effect is not an artifact of a particular clone because four MAG-expressing cells had a similar effect. d) A polyclonal antibody to MAG reversed inhibition by about 50%, whereas pre-immune serum had no effect. This shows that the inhibition of neurite outgrowth is specific. However, 100% reversal, was never achieved. It is possible that the affinity of the receptor for MAG is stronger than the affinity of MAG for this antibody. By densitometeric scanning the concentration of MAG expressed by several cell lines was compared with MAG expression in sciatic nerve (result not shown). It was found that the concentration of MAG (per milligram of protein) expressed by these cell lines spans the approximate concentration of MAG in the sciatic nerve. Therefore, its concentration when expressed by CHO cells is within the physiological range.

It has been suggested that inhibitors function by decreasing adhesion between neurons and their substrate (Caroni and Schwab, 1988a; 1988b). This is unlikely to account for the

inhibition by MAG, because the plating efficiency of all types of neurons on MAG-expressing cells is the same as that on control cells. Furthermore, it has been also shown by others from our laboratory that radiolabelled soluble MAG-Fc (extracellular domain of MAG is fused to the Fc portion of IgG) can bind equally well to cerebellar and DRG neurons and also this binding can be abolished by a monoclonal MAG antibody (Tang et al., 1996, in preparation). This indicates that MAG expressed on CHO cells, is unlikely to decrease the binding of neurons to these cells.

The inhibition of neurite outgrowth from cerebellar neurons did not change in the presence of CHO cells expressing different concentrations of MAG, which indicates that the cell line expressing the least amount of MAG is sufficient to achieve a maximum effect. This observation indicates that MAG is therefore, a very potent inhibitor of neurite outgrowth.

Although both DRG and cerebellar neurons bind equally well to MAG, they show a different response in the growth of their neurites. MAG inhibited axonal outgrowth from all post natal cerebellar neurons tested to date from PND1 to adult. In addition, here we show that as reported before (Johnson et al., 1989), MAG also promotes outgrowth from PND1 DRG neurons by about 80%. In sharp contrast, at PND3 MAG inhibits neurite outgrowth from DRG neurons by about 40%. A similar inhibition was observed for all DRG neurons from animals of PND3 and older. The switch in response from promotion to inhibition of neurite outgrowth from DRG neurons occurs at PND3 and it is not known if there is a physiological significance to this switch. At PND3 DRG axons have reached their targets and myelination is well under way. It is possible that early in development axons are programmed to respond favorably to any early expression of MAG as they must be encouraged to reach their targets. Only late in development would MAG adopt the role of protector, at a time when its inhibitory properties would not affect growing axons. It is not known, how MAG interacts with the neuron to bring about inhibition or promotion of neurite outgrowth. Moreover, how these two apparent opposing effects, promotion

and inhibition, are distinguished at the molecular level is also not known. Recently a number of studies have described molecules that have bifunctional activity, such as netrin-1 (Colamarino and Tessier-Lavigne, 1995) and tenascin (Gotz et al., 1996). Netrin-1 has been shown to be a chemoattractant for commisural axons (Kennedy et al., 1994) but repulses/inhibits the growth of trochlear motor axons (Colamarino and Tessier-Lavigne, 1995). Tenascin (Wehrle and Chiquet, 1990) can promote the growth of spinal motor axons but inhibits the growth of cerebellar neurons (Pesheva, Spiess and Schachner, 1989). Like MAG it is not known how netrin-1 or tenascin exerts these opposing effects. There are two possibilities, either there is a different, separate receptor on the neuron/axon surface for an inhibitory and promoting effect or, that the receptor is the same but the downstream signal is different for different types of neurons and at different times during development.

In summary, MAG inhibits neurite outgrowth from cerebellar neurons of all postnatal ages and adult DRG neurons. MAG promotes neurite outgrowth from newborn DRG and the switch from promotion to inhibition of neurite outgrowth occurs sharply at PND3 for DRG neurons.

Chapter IV

The role of S-MAG as an inhibitor of neurite outgrowth from cerebellar neurons and adult DRG neurons

Introduction

We have shown that MAG is a potent inhibitor of neurite outgrowth from both cerebellar and adult DRG neurons. MAG protein exists in two isoforms, designated as L-MAG (p72) and S-MAG (p67), that are dependent on the differential splicing of the primary mRNA transcript (Lai et al., 1987; Salzer et at., 1987). The S-MAG cDNA contains a 45 bp insert which has an inframe stop codon. Therefore, S-MAG has a unique 10 amino acid sequence at its carboxy end, which is not present in L-MAG. L-MAG also has a unique 54 amino acid region at its carboxy terminus which is not present in S-MAG. Hence, the cytoplasmic domain of S-MAG is shorter than L-MAG by 44 amino acids (Pedraza et al., 1990). Because of the difference in the sequence of the cytoplasmic domains of S-MAG and L-MAG, they interact differently with the non-receptor tyrosine kinase Fyn. It has been shown by co-immunoprecipitation that L-MAG expressed by COS cells is associated with Fyn whereas no association is detected between Fyn and S-MAG expressed by COS cells (Umemori et al., 1994). However, Fyn can be co-immunoprecipitated with both L-MAG and S-MAG from crude brain lysates of rat brain. Therefore, it is suggested that the association of Fyn and L-MAG does not require any other brain-specific components, whereas S-MAG does.

The expression of these two spliced isoforms is developmentally regulated. At the early stages of myelination in the CNS and PNS, L-MAG was shown to be expressed earlier than S-MAG. In the CNS and PNS of adult animals S-MAG is the major form, although L-MAG is also present (Frail, Webster and Braun, 1985). In apparent contradiction, it has been reported by another group that L-MAG is not present in the adult PNS, instead S-MAG is the only form present (Inuzuka et al., 1991).

Experiments have shown that the truncation of the cytoplasmic domain of the transmembrane proteins the integrins (Hayashi et al., 1990), E-cadherin (Nagafuchi and

Takeichi, 1988), and Po (Wong and Filbin, 1994), can affect their extracellular adhesive properties. Although the reason for the different expression pattern of the two spliced isoforms of MAG during development is unknown, it is possible that S-MAG behaves differently from L-MAG regarding inhibition of neurite outgrowth. Experiments have been designed to assess if S-MAG, like L-MAG, affects neurite outgrowth.

Results

1. Expression of S-MAG by CHO cells

S-MAG cDNA was subcloned into the pSJL plasmid (Fig. 2). Transfection of S-MAG-cDNA into CHO cells, G418 selection and gene amplification were as previously described for L-MAG (Chapter III). Two different S-MAG expressing CHO cell lines were used for further studies.

2. Characterization of S-MAG

Before carrying out the neurite outgrowth assay, characterization of the two S-MAG-expressing cells was carried out as follows. Fig. 12 shows a Western blot of the cell lysates of the two S-MAG expressing cell lines. From the Western blot, (Fig. 12) it can be seen that these two cell lines express different amounts of S-MAG and S-MAG2 expresses almost twice as much as S-MAG1. In order to ensure that S-MAG is glycosylated, the cell lysates of S-MAG expressing cells were treated with PNGase F. Fig. 12, lane D shows that after deglycosylation the molecular weight of S-MAG2 is decreased to about 67 kD. From the Western blot it is apparent that the molecular weight of S-MAG-expressing cells is little higher than the molecular weight of MAG in sciatic nerve (compare lanes, C and D with E). Some minor bands are also apparent in CHO cells, which are absent in sciatic nerve and these bands disappear after deglycosylation (compare lanes, D with F). Therefore, there is microheterogeneity in the sugar composition of S-MAG expressed by CHO cells.. A similar effect on S-MAG1 cells was also observed after PNGase F treatment (result not shown).

To confirm that S-MAG is reaching the surface of CHO cells, live S-MAG-expressing cells were immunostained using anti-MAG monoclonal antibody and quantitated by ELISA of live, unpermeabilized cells. Fig. 13 shows surface expression of these two S-MAG expressing

cells. It is suggested from the Western blot that S-MAG2 expresses higher levels of MAG than S-MAG1 and indeed ELISA analysis shows that the amount of MAG reaching the surface of CHO cells in S-MAG2 cells is almost double that of S-MAG1 cells. Surface expression of S-MAG1 and S-MAG2 was also assessed by immunofluorescent staining of live, intact cells using an anti-MAG monoclonal antibody. From the immunofluorescent data, it is obvious that S-MAG is reaching the surface of both two S-MAG expressing cell lines (result not shown). The expression level of S-MAG by S-MAG1 and S-MAG2 is comparable to the expression level of L-MAG4 and L-MAG3 cells (Chapter III) respectively (result not shown).

3. Neurite Outgrowth of Cerebellar Neurons on Different S-MAG-Expressing CHO Cells.

After characterization of S-MAG expression by the two CHO cells, the neurite outgrowth assay with PND1 cerebellar neurons was carried out. Fig. 14, shows that for the two S-MAG-expressing cell lines, the growth of neurites from cerebellar neurons is inhibited by 85.9% compared to control cells. This result demonstrates that the inhibitory effect of S-MAG on neurite outgrowth was not limited to one cell line.

4. The Effect of MAG Antibodies on the Inhibition of Neurite Outgrowth by S-MAG-Expressing Cells

In order to show the specificity of the inhibition by S-MAG on neurite outgrowth, polyclonal MAG antibodies at a concentration of 3 μg/ml were included in the neurite outgrowth assay (Table 3). Inclusion of polyclonal antibody in the cocultures reversed the inhibition by about 66%. Preimmune serum at the same concentration had no effect. Furthermore, this anti-MAG polyclonal antibody had no effect on the length of neurites from neurons grown on control CHO cells (Table 3).

5. Neurite Outgrowth of Cerebellar Neurons of Different Ages on S-MAG-Expressing and Control Cells

To determine whether the ability of S-MAG to inibit neurite outgrowth changes with age, cerebellar neurons from PND1, PND3 and 7 rats were cocultured on S-MAG2 cells. Fig. 15 shows that on S-MAG2 cells, the length of neurites from cerebellar neurons of all ages tested is reduced by 70% when compared to the length of neurites on control transfected cells.

6. Neurite Outgrowth from DRG Neurons of Different Ages on S-MAG-Expressing and Control CHO Cells.

It has been already demonstrated that L-MAG can promote neurite outgrowth from DRG neurons isolated from newborn animals (Johnson et al., 1989). We obtained similar results with DRG neurons from PND1 rat cultured on S-MAG expressing CHO cells; the length of neurites from newborn DRG neurons is about 50% longer on S-MAG2 cells than the length of neurites on control transfected cells. However, growth of neurites from adult DRG neurons was reduced by 40% on S-MAG2 expressing CHO cells (Fig. 16), a response similar to that observed with L-MAG.

Figure 12: Western Blot Analysis and Deglycosylation of S-MAG

Three cell lines, S-MAG1, S-MAG2 and control CHO cells, were subjected to SDS-PAGE in an 8% polyacrylamide gel. The proteins were transferred to PVDF and immunostained with a monoclonal MAG antibody and then with an alkaline phosphatase-conjugated goat antimouse antibody followed by alkaline phosphatase-conjugated, goat anti-mouse (1:1000). The substrate was 5-bromo-4-chloro-3-indoylphosphate, and nitroblue tetrazolium was the choromogen. Molecular weight standards (lane a), 198, 120, 88, and 70 kD. The first lane is the molecular weight marker, the second lane is the control CHO cells. The third and fourth lanes are S-MAG1 and S-MAG2, respectively.

Deglycosylation was performed on S-MAG2 by incubating the lysates with PNGase F for an hour. The lysate were then subjected to Western blotting as described above. The fifth lane is the MAG in sciatic nerve. The last lane represents the deglycosylated S-MAG2 cell at 67 kD.

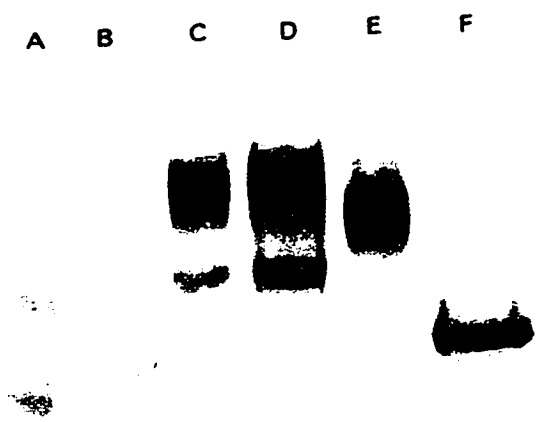


Figure 13: Enzyme-linked Immunosorbent Assay of S-MAG

The relative amounts of surface-bound S-MAG on fixed, unpermeabilized, 3-different cell lines, S-MAG1, S-MAG2 and control CHO cells, were quantified by an ELISA. The relative absorbance and the standard error of the mean (±SEM) for each cell line is shown above. Seventy-two wells were tested for each cell line.

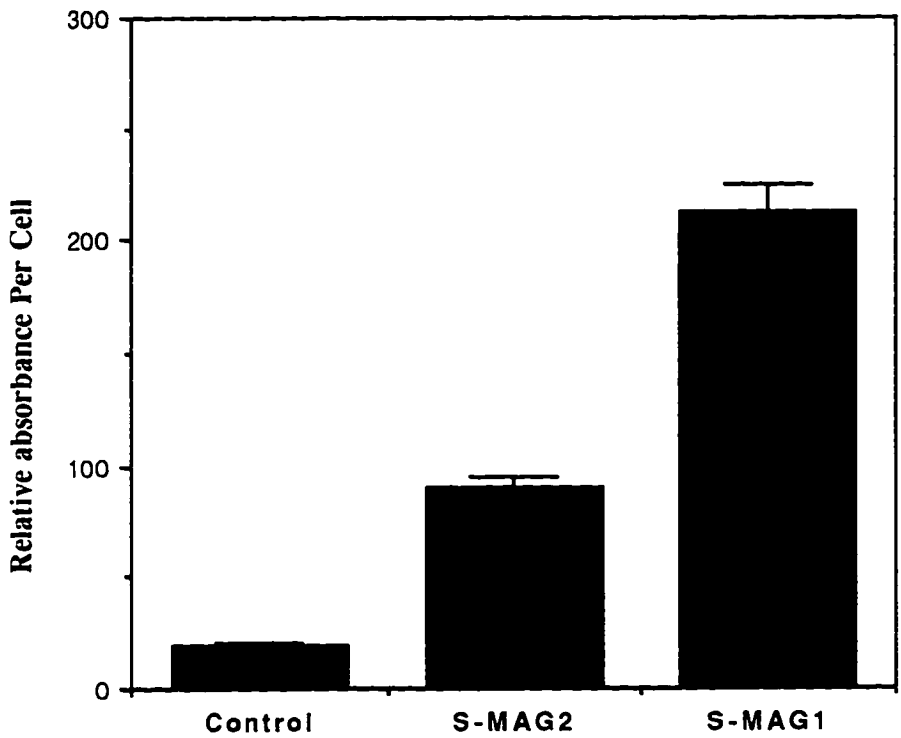


Figure 14: The Effect of Different S-MAG-Expressing CHO Cell Lines on Neurite Outgrowth from Cerebellar Neurons

Dissociated rat cerebellar neurons of PND7 were cultured on 3 different cell lines, S-MAG1, S-MAG2, and control CHO cells, for 16 hours and fixed with a neuron-specific marker, GAP43. The longest neurite among 110 neurites on each cell lines were then measured with an image analyzer. Results shown are the mean length of the longest neurites in μm and their corresponding standard errors of the mean (±SEM)

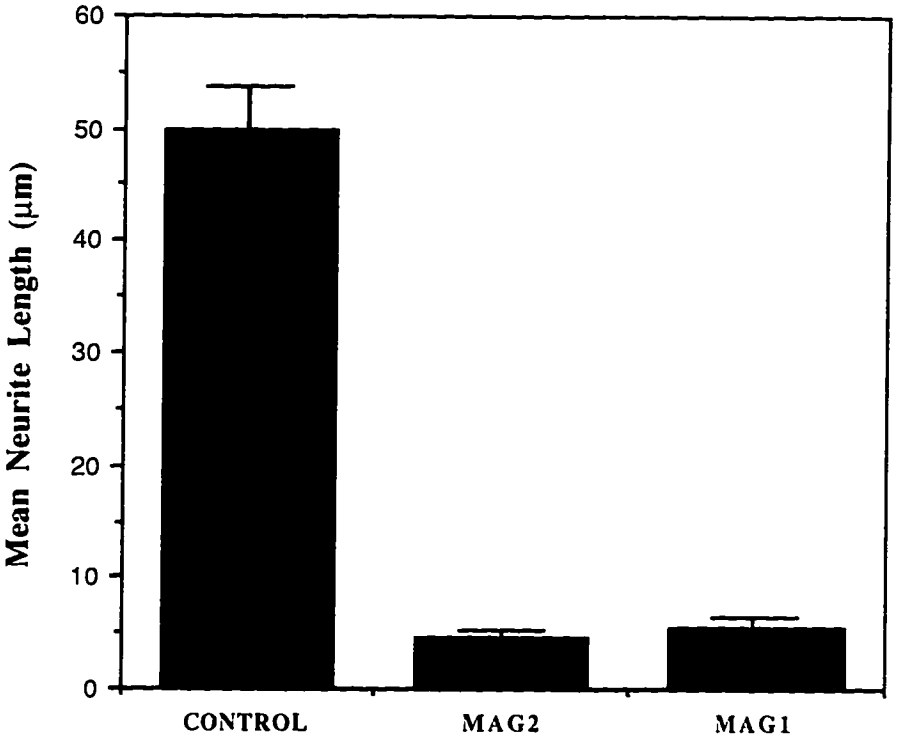


Figure 15: The Effect of S-MAG on Neurite Outgrowth from Cerebellar Neurons of Different Ages

Dissociated rat cerebellar neurons from 3 different ages (PND1, 3 and 7) were cultured on S-MAG (S-MAG2) expressing CHO cells for 16 hours, after which they were immunostained with GAP43, a neuron specific marker. The leading process of 110 neurons on each cell line was then measured with an image analyzer. The results shown are the mean lengths of the longest neurite in μm with their corresponding standard errors of the mean (±SEM)

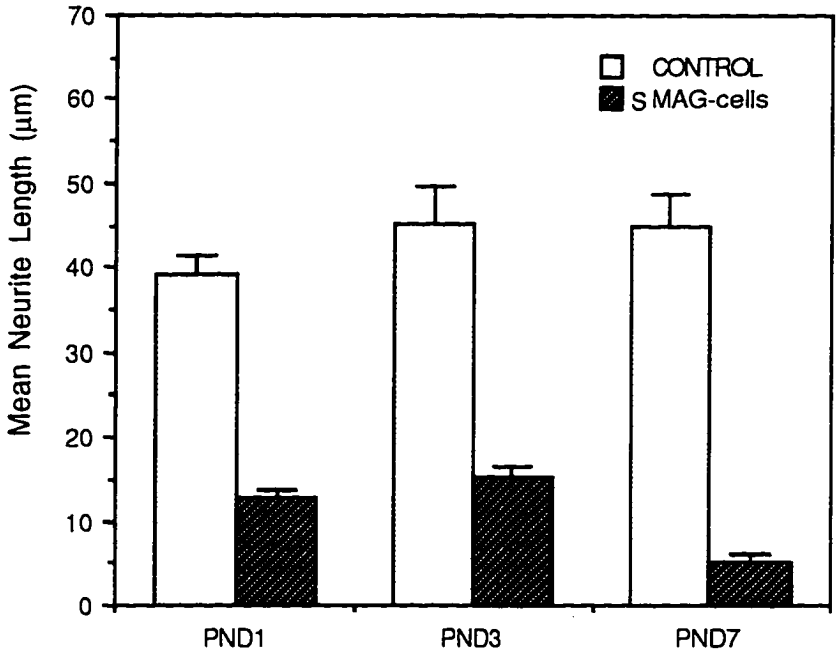


Figure 16: The Effect of MAG on Neurite outgrowth from PND1 and Adult DRG Neurons

Dissociated DRG neurons from PND1 and adult rats were cultured on S-MAG expressing CHO cells (S-MAG2). PND1 DRG neurons were cultured for 24 h while adult DRG neurons were cultured for 10 h. They were stained with GAP43, a neuron specific marker. The length of longest process of 110 neurites on each cell line are in μm and their corresponding standard errors of the mean (±SEM).

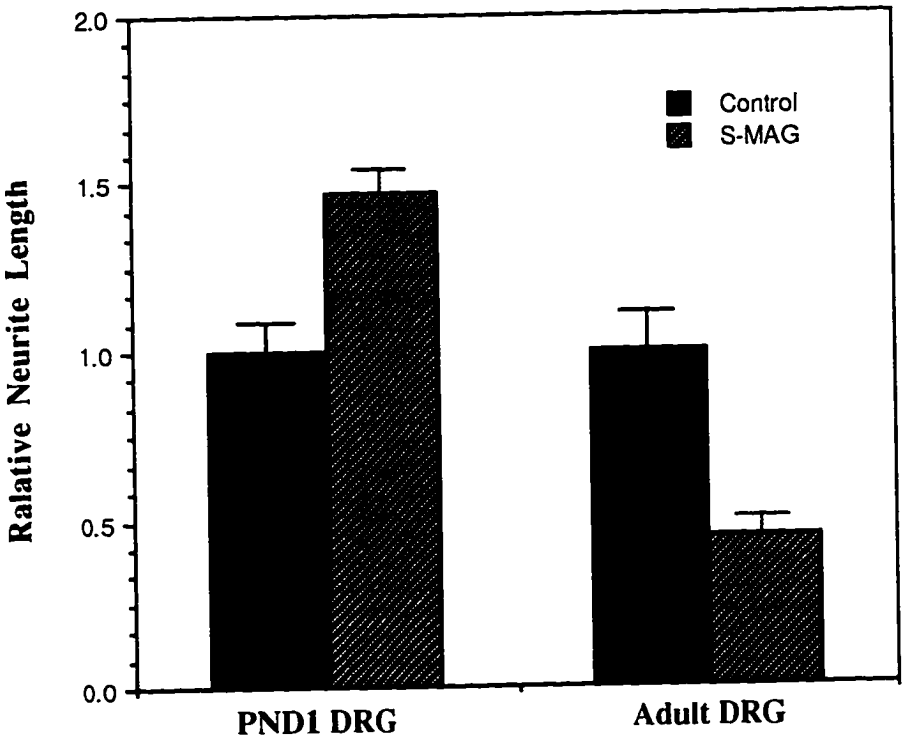


Table 3

Antibody Reversal of S-MAG Inhibition of Neurite Outgrowth		
	S-MAG cells	Control CHO cells
Preimmune	7.9± 0.96	68.0 ± 5.89
MAG antibody	23.8 ± 3.32	65.7 ± 6.45

Conclusion

The growth of neurites from cerebellar neurons is inhibited by 70 to 90% on S-MAG expressing cells (Fig. 15). Similarly, the growth of neurites from adult DRG neurons is inhibited by S-MAG by 40% (Fig. 16). In contrast, S-MAG promotes neurite outgrowth by 50% from newborn DRG neurons (Fig. 16).

The specificity was demonstrated by the following observations. a) CHO cells expressing another adhesion molecule Po, have no effect on the length of neurites extended from cerebellar neurons (results not shown). b) We have shown that the inhibition of neurite outgrowth by S-MAG can be reversed with a polyclonal MAG antibody. c) To show that inhibition of neurite outgrowth by MAG on CHO cells is not a clonal artifact, a second S-MAG expressing cell line was also shown to inhibit neurite outgrowth.

Although S-MAG has a shorter cytoplasmic domain than L-MAG by 44 amino acids and also has a unique ten amino acid sequence, it behaves like L-MAG in the inhibition of neurite extension. Experiments suggest that integrins (Hayashi et al., 1990), E-cadherin (Nagafuchi and Takeichi, 1988), and Po (Wong and Filbin, 1994), bind to the cytoskeletal proteins because these proteins are partially insoluble in the nonionic detergent NP40. Truncation of the cytoplasmic domains of these proteins disrupts the interaction of cytoskeletal proteins with their cytoplasmic domains. It is suggested that the function of the extracellular domain of these proteins is affected by the loss of cytoplasmic domain:cytoskeleton interaction. For example, in the case of integrins, truncation of the cytoplasmic domain can disrupt its binding to the extracellular matrix protein laminin or fibronectin (Hayashi et al., 1990). Therefore, the integrity of the cytoplasmic domain is required for the extracellular domain to function correctly. It is known that MAG binds to some component on the surface of neurons, and the inhibition/promotion effect of MAG depends on this specific binding. Therefore, the extracellular domain of MAG must have the

correct conformation to bind to the neuronal surface. However, until now there is no evidence supporting that cytoplasmic domain of MAG binds to cytoskeletal proteins. Therefore from our result it is not surprising that S-MAG is as potent as L-MAG promoting or inhibiting neurite outgrowth from different populations of neurons.

In conclusion, we have shown that like L-MAG, S-MAG can also inhibit neurite outgrowth from developing cerebellar neurons. In addition, S-MAG promotes neurite outgrowth from newborn DRG neurons. In contrast, it inhibits neurite outgrowth from adult DRG neurons.

Chapter V

Inhibitory and promoting role of MAG after disrupting its sialic acid binding site

Introduction

Like other members of the Sialoadhesin family, MAG is a sialic acid binding protein. It also shares 45-50% amino acid sequence similarity in its four N-terminal Ig-like domains with the four N-terminal Ig-like domains of both CD22 and sialoadhesin (Kelm et al., 1994). Previously, it was shown that binding of MAG to the neuronal surface is sialic acid-dependent (Kelm et al., 1994). This conclusion derives from studies in which MAG-Fc does not bind to desialylated neurons. Furthermore, results show that inhibition or promotion of neurite outgrowth by MAG can be reduced or abolished completely by desialylation of the neurons prior to the assay (DeBellard et al., 1996a). Therefore, it is likely that the inhibitory and promoting effects of MAG depend directly or indirectly on the interaction of MAG with a sialylated component on the surface of neurons. MAG recognizes a specific linkage of sialic acid, which is NeuAcα2-3 Gal from the terminal sugar (Table 1). A small sialic acid-bearing analogue, 2,3-dideoxy sialic acid (DD-NANA) and the trisaccharide sugar sialo 2,3 α lactose, were shown to reverse the effect of inhibition and promotion of neurite outgrowth by MAG in a dose dependent manner (DeBellard et al., 1996a).

As a first step to map more precisely the sialic acid binding site on sialoadhesin and CD22, Crocker and his colleagues made different truncated chimeric constructs of sialoadhesin and CD22 (Nath et al., 1995). They attached various truncated domains of sialoadhesin or CD22 with Fc fragments of human IgG and allowed these chimeric molecules to bind to untreated and desialylated erythrocytes. They found that the N-terminal (membrane-distal V-set) domains 1 of sialoadhesin and domain 1 and 2 of CD22 were necessary and sufficient for sialic acid-dependent binding to erythrocytes. To identify which specific amino acids within domain 1 of sialoadhesin and CD22 were responsible for sialic acid dependent binding, site directed mutagenesis was carried out. For sialoadhesin, initially it was found that six amino acids are important for sialic

acid binding (Vinson et al., 1996). By drastic changes (changing acidic amino acids to basic amino acids or visa versa) to all 6 of these amino acids, sialic acid binding site was lost. However, it was shown that Arg97 is the key amino acid among these 6 important amino acids, for sialic acid binding. The reasons are as follows: a) Instead of a drastic mutation, these 6 important amino acids were individually mutated to alanine. It was found that only Arg97→Ala mutation caused abolition of the sialic acid-dependent binding. b) Mutation of Arg97→Lys, a conservative substitution which retains the positive charge after mutation, abolishes sialic acid dependent binding. In addition, this Arg is conserved at Arg130 in CD22 and mutation of Arg130 of CD22 to either alanine and glutamic (drastic mutation) acid also abolished sialic acid-dependent binding (Van der Merwe et al., 1996). All these mutated sialoadhesin or CD22 were still recognized by conformationally dependent monoclonal antibodies. Therefore, it is unlikely that the mutations, cause a misfolding of the proteins. Therefore, Arg97 in sialoadhesin or Arg130 in CD22 is a key residue in mediating sialic acid dependent binding because without changes to its structure, mutation of Arg abolishes binding of sialoadhesin or CD22 to the erythrocytes.

To identify the sialic acid binding site of MAG, Arg118 was chosen as a candidate to mutate because this arginine is conserved in sialoadhesin and CD22 (Fig. 17). Here, Arg118 of L-MAG will be mutated either to Ala or Asp, changing the positive charge to neutral or negative respectively. After characterization of the expression of mutated L-MAG in CHO cells as described for unmutated L-MAG, neurite outgrowth assays will be conducted to see whether or not the inhibitory or promotive properties of L-MAG are altered by these mutations. In addition, we will also test if mutated MAG-Fcs can still bind to the neurons.

Results

1. Transfection of Mutated MAG-cDNA into CHO Cells

The Arg 118 of L-MAG in pBluescript vector was mutated to either Ala or Asp by site directed mutagenesis, using the kit provided by Stratagene (General method section). After mutagenesis, the mutations for Arg118→Ala (R118A) and Arg118→Asp (R118D) were confirmed by DNA sequencing (Fig. 17 and 18). Fig. 18a shows that after mutation the wild type coding sequence of Arg, "CGA" is changed to "GCC" which codes for the amino acid alanine. Fig. 18b shows that wild type coding sequence of Arg, "CGA" is changed to "GAC" a sequence coding for aspartic acid. After subcloning these two mutated MAG cDNAs (R118A-MAG and R118D-MAG) into a suitable plasmid, namely pSJL (Fig. 2), the transfection of these two mutated MAG cDNAs into CHO cells, later G418 selection and gene amplication were carried out similarly as described for unmutated MAG. Two different cell lines were chosen for each mutation.

2. Characterization of R118A-MAG and R118D-MAG Expressed by CHO cells.

Mutated MAG expressed by a single clone for each mutation was determined by Western blot. Fig. 19 shows a Western blot, immunostained for MAG, of cell lysates from three different cell lines, one wild type and two mutated MAG-expressing cell lines (termed L-MAG, R118A-MAG and R118D-MAG) and control CHO cells also grown in MTX but not expressing MAG. MAG expressed by all these cell lines (Fig. 19, b-d) is of the same molecular weight as MAG from sciatic nerve (Fig. 19, lane e), i.e., approximately 100 kD. This suggests that wild type and mutated proteins are glycosylated to the same extent as they are *in vivo* (Quarles et al., 1984). From Fig. 19, it is also apparent that the amount of MAG expressed by each cell line is comparable to MAG in sciatic nerve (per milligram of protein). To confirm that the mutated

forms of MAG are glycosylated, cell lysates were treated with the enzyme PNGase F. Fig. 20a and 20b show that the molecular weight of R118A-MAG and R118D-MAG are decreased to about 70 kD after treatment with PNGase F. To test the effect of mutated MAG on neurite outgrowth, it has to be first established that the mutated MAG is reaching the surface. Surface expression of R118A-MAG and R118D-MAG was quantitated relative to the surface expression of wild type L-MAG. As shown in the ELISA analysis in Fig. 21, R118D-MAG and R118A-MAG express atleast the same amount of MAG on the surface as cells expressing wild type. Surface immunostaining as shown in Fig. 22a and 22b, using a MAG monoclonal antibody, shows that mutated R118A-MAG and R118D-MAG are readily detected at the surface of CHO cells. There was no staining of the control transfected cells (results are not shown).

3. Neurite Outgrowth of Cerebellar Neurons on Mutated MAG-Expressing CHO Cells

After characterization of R118A-MAG and R118D-MAG expressing CHO cells, rat cerebellar neurons isolated at PND 7 were cultured on transfected CHO cells and the neurite outgrowth assay were carried out. After 16-18 h the cocultures were fixed and average length of GAP43 positive neurites was determined. It was found that the growth of cerebellar neurons was inhibited when cultured on R118A-MAG and R118D-MAG expressing CHO cells (Fig. 23). Both of these mutated forms of MAG and wild type MAG inhibited neurite outgrowth from PND7 cerebellar neurons by about 70%.

4. Neurite Outgrowth from PND1 DRG Neurons on Mutated MAG-Expressing CHO Cells

Neurite outgrowth from DRG neurons on cells expressing mutated MAG was compared to cells expressing wild type MAG and control cells. Neurite outgrowth was promoted on the mutated R118A-MAG and R118D-MAG-expressing cells to the same extent as on cell

expressing wild type MAG (Fig. 24). Neurites were about twice as long when grown on cells expressing wild type L-MAG, R118A-MAG or R118D-MAG compared to control transfected cells.

5. The Effect of Mutated MAG on Neurite Outgrowth from Desialylated Neurons

Previously it has been shown that the inhibition and promotion of MAG on neurite outgrowth are dependent directly, or indirectly, on a sialo-glycoprotein on the surface of neurons (DeBellard et al., 1996a). This conclusion derives from the fact that when sialic acid was removed from cerebellar neurons by neuraminidase treatment prior to the neurite outgrowth assay, the length of neurites from cerebellar neurons were twice as long as untreated neurons on MAG-expressing cells. Newborn DRG neurons were also treated with neuraminidase to remove sialic acid prior to the neurite outgrowth assay. The length of neurites from neuraminidase-treated newborn DRG neurons on MAG-expressing cells was reduced to the length of neurites on control cells.

To test the effect of desialylation on inhibition and promotion by mutated MAG a coculture experiment was set up in which cerebellar neurons from PND7 and newborn DRG neurons were desialylated prior to the neurite outgrowth assay and grown on R118A-MAG and R118D-MAG and L-MAG expressing CHO cells. Plating efficiency of desialylated neurons onto either mutated or wild type MAG-expressing or control cells was unaffected by neuraminidase treatment (result not shown). The length of neurites from PND7 cerebellar neurons, treated with neuraminidase was significantly longer than untreated neurons on L-MAG, R118A-MAG or R118D-MAG-expressing CHO cells (Fig. 25a). The length of neurites from cerebellar neurons on the control transfected cells was unaffected by neuraminidase treatment (Fig. 25a).

The neurites from untreated PND1 DRG neurons grown on L-MAG, R118A-MAG and R118D-MAG-expressing cells are about twice as long as those from neurons grown on control cells. Desialylation of the neurons with neuraminidase reduced the neurite outgrowth from both wild type and mutant MAG expressing CHO cells to that measured on control cells. It is surprising that even though sialic acid binding site in MAG was apparently destroyed, still sialic acid on neurons had some role in the affect of MAG on neurite outgrowth (Fig. 25a and 25b). Furthermore, it is shown by others from our laboratories that Arg118 in MAG is also crucial to its sialic acid dependent binding to the neurons because mutation of this amino acid, to either Ala or to Asp, abolishes binding completely (Tang et al., 1996a; 1996b).

Figure 17: Alignment of Sialoadhesins (MAG, Sn, CD22, and CD33) with CD8∝, a Member of the Ig Superfamily of Known Structure.

The predicted protein sequence of the V set Ig domain of mouse MAG was aligned with the V-set domains of mouse CD22 (Torres et al., 1992), sialoadhesin(Vinson et al., 1996), human CD33 (Simmons et al., 1988) and human CD8∝ (Littman et al., 1985) Numbering of amino acids corresponds to the mature protein sequence of MAG (Salzer et al., 1987). The β-strand assignments (solid bars) were based on the structure of CD8∝. Dotted lines instead of bars are shown where there are no grounds for making precise assignments to β-strands. It should be noted that for the assignment of residues that are identical between members of the sialoadhesin family but are not characteristic of other V-set domains within the Ig superfamily. The other species homologues are taken into account, namely rat and human MAG (Salzer et al., 1987) human CD22 (Wilson et al., 1991) and mouse CD33 (Tchilian et al., 1994). (Modified from, Vinson et al., J.Biol Chem, 1996)

```
1                                                  50
MAG  .HWGAWMPST ISAFEGTCVS IPCRFDFPDE .LR.PAV.VH GVWYFNSPYP
Sn   .TWGVSSPKN VQGLSGSCLL IPCIFSYPAD .VPVSNG.IT AIWYYD..YS
CD33 ..FWLQVQES VTVQEGLCVL VPCTFFHPIP YYDKNSP.VH GYWFREGAII
CD22 .DWTVDHPQT LFAWEGACIR IPCKYKTPLP KARLDNILLF QNYEFDKATK
CD8  SQFRVSPLDR TWNL.GETVE LKCQ.....  .VLLSNPTSG CSWLFQPRGA
       a_____ b_____ c_____
51                                                 100
MAG  KNYPPVVFKS RTQVVHESF.  .....QGRSR LLGDLGLRNC TLLLSTLSPE
Sn   GKRQVVIHSG DPKLVDKRF.  .....RGRAE LMGNMDHKVC NLLLKDLKPE
CD33 SGDSPVATNK LDQEVQEET.  .....QGRFR LLGDPSRNNC SLSIVDARRR
CD22 KFTGTVLYNA TKTEKDPESE LYLSKQGRVT FLGNR.IDNC TLKIHPIRAN
CD8  AASPTFLLYL SQNKPKAAEG L.....DTQR FSGKRLGDTF VLTLSDFRRE
                                   d_____ e_____
101     f                          128
MAG  LGGKYYFRGD LGGYNQYTFS EHSVLD..
Sn   DSGTYNFRFE ISDSNRWLDV KGTTVTVT
CD33 DNGSYFFRME RTSTKYSYKS PQLSVHVT
CD22 DSGNLGLRMT .AGTERWMEP IHLNVSE.
CD8  NEGYYFCSAL SNSIMYFSHF VPVFLPA.
       f_____ g_____
```

Figure 18a: Sequencing Data for R118A-MAG

Autoradiogram of sequencing gel shows mutation of the amino acid position 118 in MAG from nucleotide coding for arginine "CGA" to nucleotide coding for alanine"GCC".

Figure 18b: Sequencing Data for R118D-MAG

Autoradiogram of sequencing gel shows mutation of the amino acid position 118 in MAG from nucleotide coding for arginine "CGA" to nucleotide coding for aspartic acid "GAC".

Figure 19: Western Blot Analysis of L-MAG, R118A-MAG and R118D-MAG

Four cell lines, control CHO cells, L-MAG, R118A-MAG and, R118D-MAG were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in an 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and immunostained with a monoclonal MAG antibody and then with an alkaline phosphatase-conjugated goat antimouse antibody followed by alkaline phosphatase-conjugated, goat anti-mouse (1:1000). The substrate was 5-bromo-4-chloro-3-indoylphosphate, and nitroblue tetrazolium was the choromogen. Molecular weight standards (lane a), 198, 120, 88, and 70 kD. The first lane is the control CHO cells. The second, third and fourth lanes are L-MAG, R118A-MAG and R118D-MAG, respectively. The last lane is sciatic nerve.

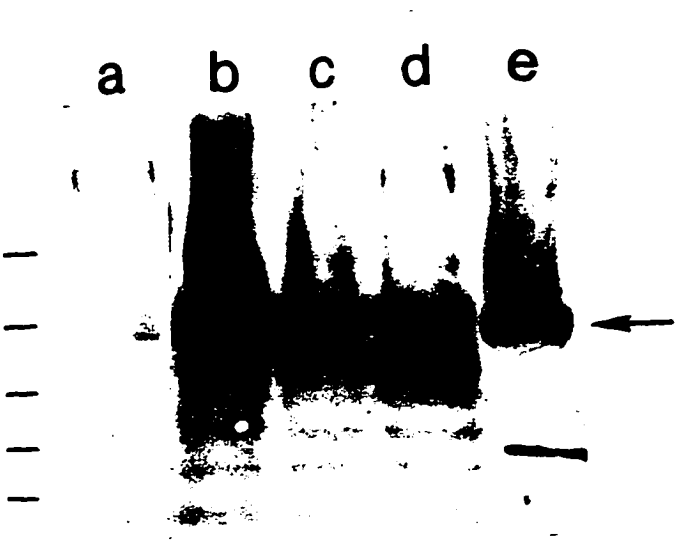


Figure 20: Deglycosylation of R118A-MAG and R118D-MAG

(a) Deglycosylation was performed on R118A-MAG by incubating the lysates with PNGase for overnight. The lysate were then subjected to Western blotting as described above (Fig. 19). The first lane is the glycosylated R118A-MAG cells. The second lane represents the deglycosylated L-R118A-MAG cell at 72 kD.

(b) Deglycosylation was performed on R118D-MAG, by incubating the lysates with PNGase for overnight. The lysate were then subjected to Western blotting as described above. The first lane is glycosylated R118D-MAG, cells. The second lane represents the deglycosylated R118D-MAG, cell at 72 kD.

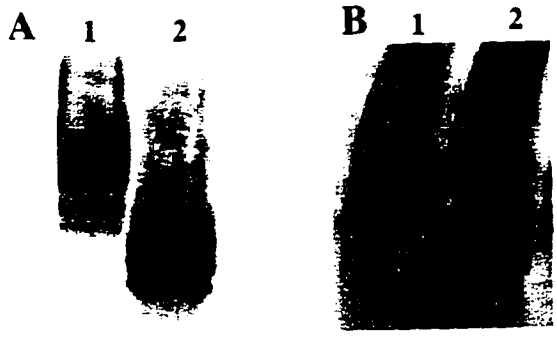


Figure 21: Enzyme-linked Immunosorbant Assay of L-MAG, R118A-MAG and R118D-MAG,

The relative amounts of surface-bound on fixed, unpermeabilized, 4-different cell lines, L-MAG, R118A-MAG, R118D-MAG, and control were quantified by an ELISA. The relative absorbance and the standard error of the mean (±SME) for each cell line is shown above. Seventy-two wells were tested for each cell line.

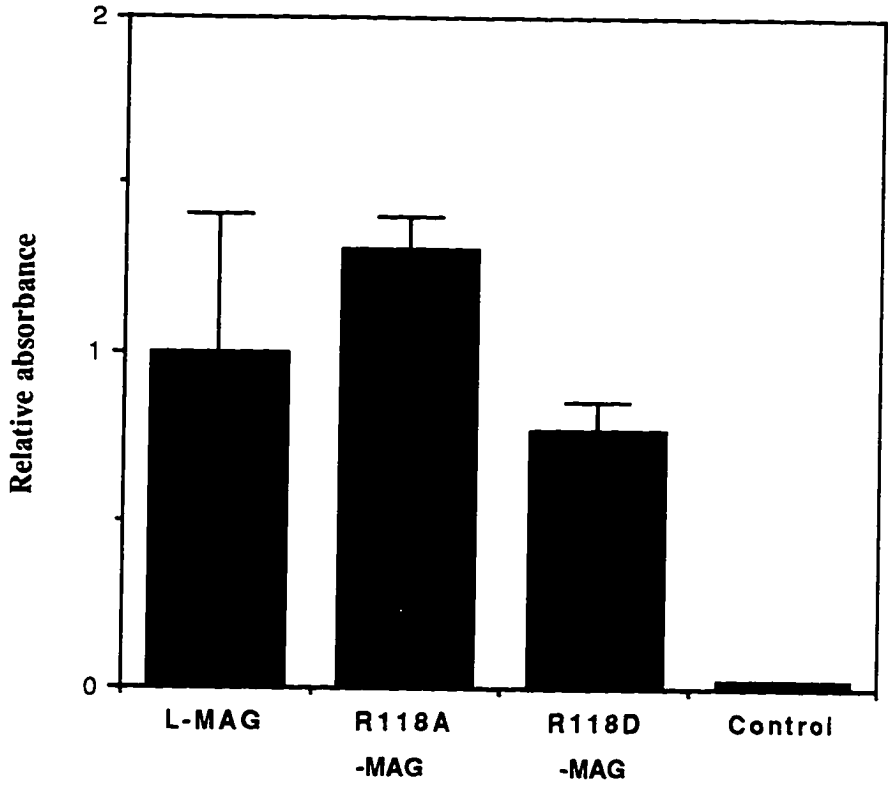
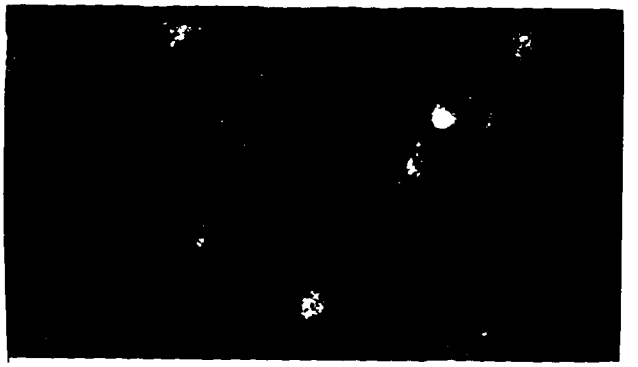


Figure 22: Surface Detection of Mutated MAG on Transfected CHO Cells by Immunofluroscent Staining

Live CHO cells, either (a) R118A-MAG or (b) R118D-MAG, cells were incubated with a monoclonal antibody to chick MAG (5 μg/ml), then fixed with 4% paraformaldehyde and incubated with phycoprobe-conjugated, goat anti-mouse IgG (1:50)

a



b

Figure 23: The Effect of Mutated R118A-MAG and R118D-MAG on Neurite Outgrowth from Cerebellar Neurons of PND7

(a and b) Dissociated cerebellar neurons from PND2 animals were cultured for 16 hr on confluent monolayer of control (a), L-MAG (b), R118A-MAG (c), R118D-MAG (d) CHO cells before being fixed and immunostained for GAP43. Afterwards, the neurite length was measured. Results show that mean length of the longest neurite per cell (± SME) for GAP43. Afterwards, the neurite length was measured. Results show that the mean length of the longest neurite per cell (± SME) for 100-180 individual neurons.

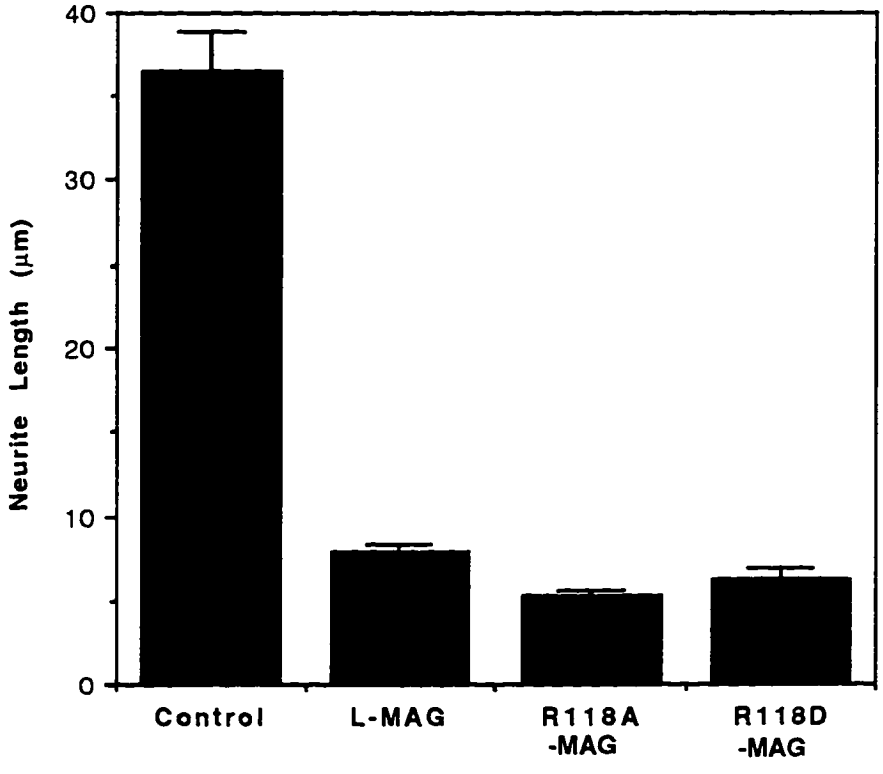


Figure 24: The Effect of Wild type and Mutant MAG-Expressing CHO Cell Lines on Neurite Outgrowth from Newborn DRG

Dissociated PND1 DRG neurons were cultured on confluent monolayers of control (a) L-MAG (b), R118A-MAG (c) and R118D-MAG, CHO cells before being fixed and immunostained for GAP43. Results show the relative neurite length of the longest neurite per cell (± SME) for 100-180 individual neurons.

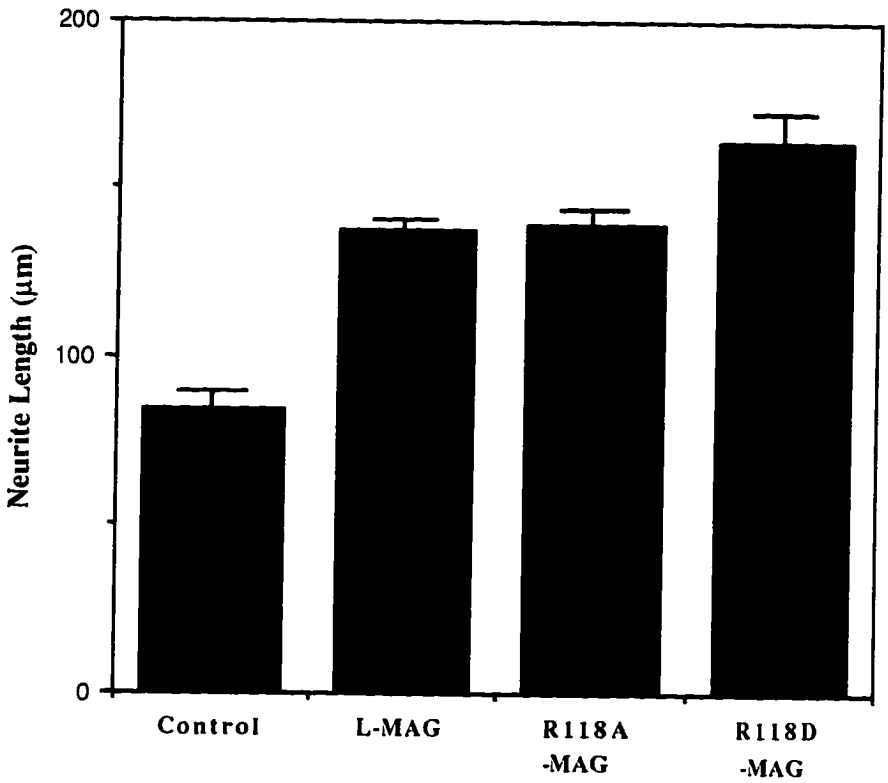
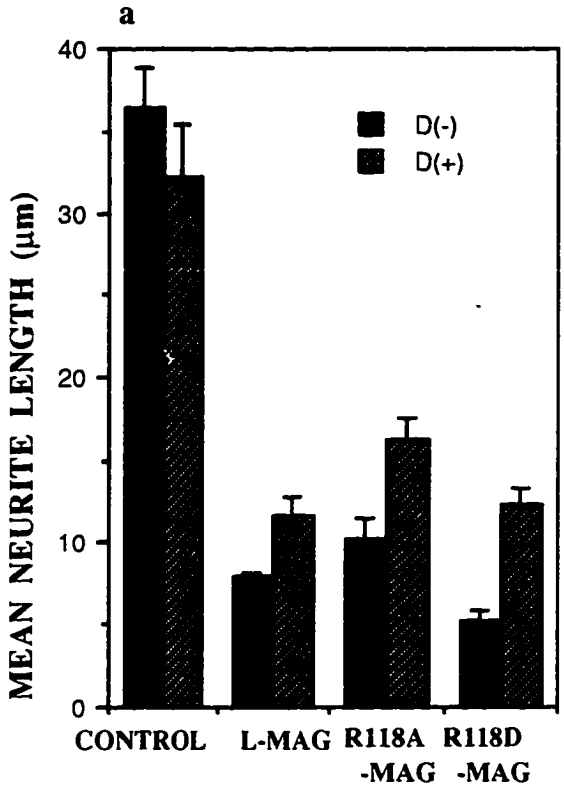


Figure 25: The Effect of MAG on Neurite Outgrowth from Desialylated PND7 Cerebellar and Newborn DRG Neurons

(a) The effect of MAG on neurite outgrowth from desialylated PND7 cerebellar neurons. Neurons were isolated from either PND7 or PND1 animals. One half of the population was treated with the neuraminidase (D+) and other was treated in the same way but without the enzyme (D-), before being cultured for 16 h on confluent monolayers of L-MAG expressing, R118A-MAG, R118D-MAG, and control CHO cells before being fixed and immunostained for GAP43.

(b) The effect of MAG on neurite outgrowth from desialylated PND1 DRG neurons were co-cultured for 12 h. Neuraminidase treatment, fixing and staining of DRG neurites were same as in (a).



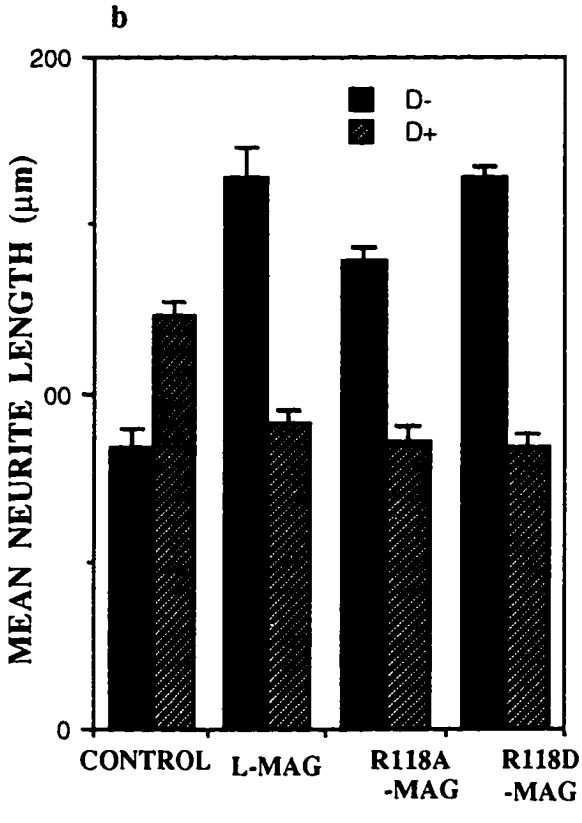
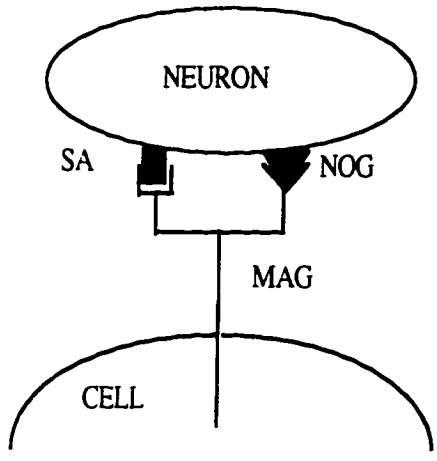


Figure 26: The Sialic Acid Binding Site on MAG (R118) is Critical for Inhibition of Axonal Regeneration by Soluble MAG-Fc but not MAG Expressed by CHO Cells.

There are two recognition epitopes on MAG, a sialic acid binding epitope (SA, square symbol) and a neurite outgrowth inhibition epitope (NOG, triangle symbol). When MAG is expressed by CHO cells, both epitope engage the neuron and neurite outgrowth is inhibited (Panel A). When MAG mutated at its sialic acid binding site is expressed in CHO cells, another sialic acid binding protein on the CHO cell surface engages the neuron along with the neurite outgrowth epitope and neurite outgrowth is still inhibited (Panel B). When soluble MAG-Fc is added to neurons, both the sialic acid and inhibition epitope engage the neuron and neurite outgrowth is inhibited (Panel C). However, when MAG-Fc mutated at the sialic acid binding site is added to neurons, it cannot bind to neurons and consequently the inhibition epitope cannot engage and there is no inhibition of neurite outgrowth.



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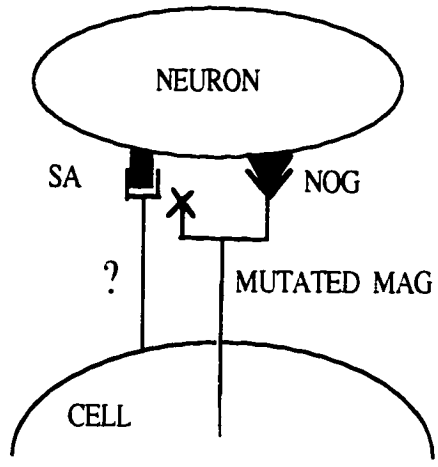

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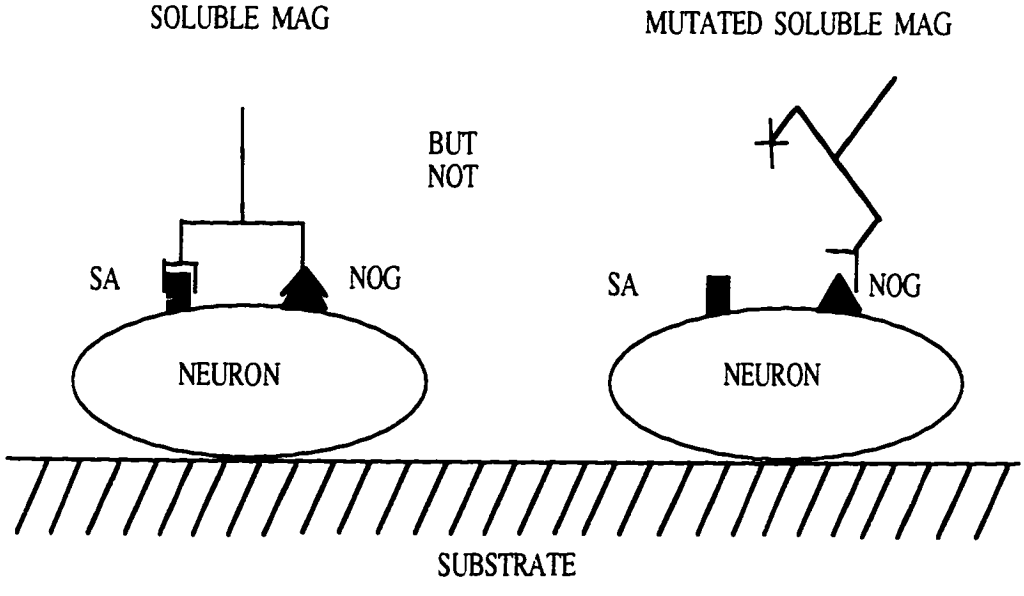


INHIBITION

NO INHIBITION

Conclusion

It was shown by others from our laboratory that mutated soluble MAG -Fc i.e., R118A-MAG-Fc and R118D-MAG-Fc can not bind to the neurons. In addition, they have also shown that wild type soluble L-MAG-Fc can inhibit neurite outgrowth from cerebellar neurons however, soluble R118A-MAG-Fc and R118D-MAG-Fc cannot inhibit neurite outgrowth from cerebellar neurons (Tang et al., 1996).

When arginine118 of MAG in domain 1 was changed to either alanine or aspartic acid, mutated MAG expressed in the CHO cells still inhibited neurite outgrowth from cerebellar neurons by 70% and promoted neurite outgrowth from newborn DRG neurons by about 50 to 70% (Fig. 23, and 24).

From ELISA analysis (For MAG-Fc, see Tang et al., 1996) and surface immunostaining, we have shown that both of these forms of mutated MAG either MAG-Fc or protein expressed by CHO are recognized by a monoclonal antibody (mAb513). As this monoclonal antibody is recognizes only native MAG, the lack of binding of mutated MAG-Fc to the surface of neurons, or inhibition exerted by mutant proteins by CHO cells is not a consequence of misfolding of the protein.

Desialylation of DRG neurons reverses the effect of MAG, up to 100% for promotion, however, desialylation of cerebellar neurons reverses inhibition of neurite outgrowth by about 30% to 50%. The possible explanation for this difference could be as follows. a) Cerebellar neurons may replace sialic acid more rapidly and efficiently during the course of the culture period than do DRG neurons. b) CHO cells bind to cerebellar neurons through other sialic acid-independent component as well and not bind to DRG neurons.

Now the question to be addressed is how Arg118 binds to sialic acid ? Why it is more important than other amino acids? From the crystal structure of polyoma virus VP1, it has been

shown that guanidium group of arginine which is important for sialic acid dependent binding, which forms a salt bridge with the carboxylate of sialic acid (Stehle et al., 1994).

As R118A-MAG and R118D-MAG are still capable of inhibiting neurite outgrowth when expressed by CHO cells but are unable to bind to the neurons, we propose that the sialic acid binding site at Arg118 is distinct from the inhibition site of MAG. It is possible that two sites exist on MAG, one site can interact with the sialic acid component on the neuronal surface and the other site affects inhibition of neurite outgrowth. From our proposed model Fig. 26, it is possible that neurons can still bind to other sialic acid binding proteins expressed by CHO cells, when sialic acid binding domain in MAG is destroyed by mutation. Therefore, these mutated forms of MAG are still active for inhibition and promotion of neurite outgrowth. In contrast, isolated MAG-Fc binds to the neurons only by its sialic acid binding site. So, if sialic acid binding site is mutated, MAG can no longer bind to the surface of neuron and as a consequence, it can not inhibit or promote neurite outgrowth. As mentioned above, it was confirmed by others from our laboratory that these mutated MAG-Fcs are no longer capable of inhibiting neurite outgrowth (Tang et al., 1996). From this model we also can explain desialylation events. When desialylated neurons when grown on wild type MAG, the length of neurites were increased by about 50 to 100% (DeBellard et al., 1996). Taking this in consideration, we are expect that there should not be any change in neurite length from desialylated neurons when cocultured with mutated R118A-MAG or R118D-MAG-expressed CHO cells. However, we do find an increase in the length of neurites from desialylated neurons cocultured on mutated MAG. Hence, from our model Fig. 26, we can explain this situation in which other molecules from CHO cells bind to the sialylated component of the neuronal surface and compensating the function of MAG. Therefore, both wild and mutant type of MAG are affecting neurite outgrowth in a similar way from desialylated neurons.

All these results indicate that arginine118 is necessary for binding of MAG to the neuronal surface but insufficient for the inhibition and promotion of neurite outgrowth by MAG.

Chapter VI

PNS myelin is inhibitory for neurite outgrowth

Introduction

It is well established that CNS myelin is inhibitory for axonal regeneration both *in vivo* (Schnell and Schwab,1990; Schnell et al., 1993; for review see Johnson 1993) and *in vitro* (Carbonetto et al., 1987; Crutcher 1989; Savio and Schwab, 1989; Schwab and Caroni, 1988). We have shown that, depending on the age of neurons, MAG, can either promote or inhibit axonal regeneration: MAG promotes neurite outgrowth from newborn DRG neurons but inhibits neurite extension from adult DRG and cerebellar neurons of all post-natal ages (Mukhopadhyay et al., 1994). Moreover, it was also established that MAG contributes significantly to the inhibitory properties of CNS myelin *in vitro* as immunodepletion of MAG from this membrane results in a 60% loss of its inhibitory properties (McKerracher et al., 1994). However, a number of studies have shown that PNS myelin is permissive for neurite outgrowth *in vitro* (Carbonetto et al., 1987; Caroni and Schwab, 1988a; Savio and Schwab, 1989) and axonal regeneration *in vivo* (Aguayo et al., 1978) yet MAG, although at a concentration 10-fold lower than in the CNS, is also present in PNS myelin (for review see Trapp 1990). The apparent differences in permissiveness of CNS and PNS myelin cannot be accounted for by a dose effect as expression of MAG by a transfected fibroblast cell line, at a level equivalent to that found in the PNS, is sufficient to potently inhibit neurite outgrowth from certain neurons (Mukhopadhyay et al., 1994). However, before describing the permissiveness of PNS myelin a number of important issues should be considered. First, PNS myelin is cleared before regeneration takes place *in vitro*. Hence regenerating axons never encounter myelin. It has been shown that in a strain of mutant mice, C57BL/Ola, in which Wallerian degeneration takes place very slowly, axonal regeneration is limited (Brown et al., 1992). Second, laminin is a constituent of the Schwann cell basal lamina, hence during isolation of PNS myelin laminin can contaminate the preparation. It was shown by David and co-workers in *in vitro* studies that the effects of PNS myelin are

influenced by the content of laminin in the myelin preparation. They found that preparations enriched in laminin are permissive for neurite outgrowth, whereas preparations with little laminin are inhibitory (David et al., 1995). Third, previously, permissiveness of either PNS myelin or PNS tissue was demonstrated using neurons whose axonal growth is promoted by MAG (Johnson et al., 1989; Salzer et al.,1990) namely newborn DRG and embryonic neurons (Caroni and Schwab, 1988b; Carbonetto et al., 1987; Schwab and Thoenen, 1985).

Hence we predict that if neurons known to be inhibited by MAG are grown on PNS myelin, neurite outgrowth will be inhibited. Here we test this hypothesis by comparing the ability of newborn DRG neurons and cerebellar neurons to extend neurites on purified myelin. We demonstrate, as has been reported previously, that newborn DRG neurons extend long neurites on PNS myelin (Carbonetto et al., 1987; Sandrock and Matthew, 1987; Caroni and Schwab, 1988a; Savio and Schwab, 1989). In contrast, cerebellar neurons are inhibited from extending neurites on PNS and CNS myelin.

Figure 27: Neurite Outgrowth from Newborn DRG and Grown on 6 μg/well of CNS and PNS Myelin.

Newborn DRG or cerebellar neurons were cultured on 6 μg/well PNS (a)and CNS (b) myelin for 16 h before being fixed and stained for GAP43 as described in the methods.

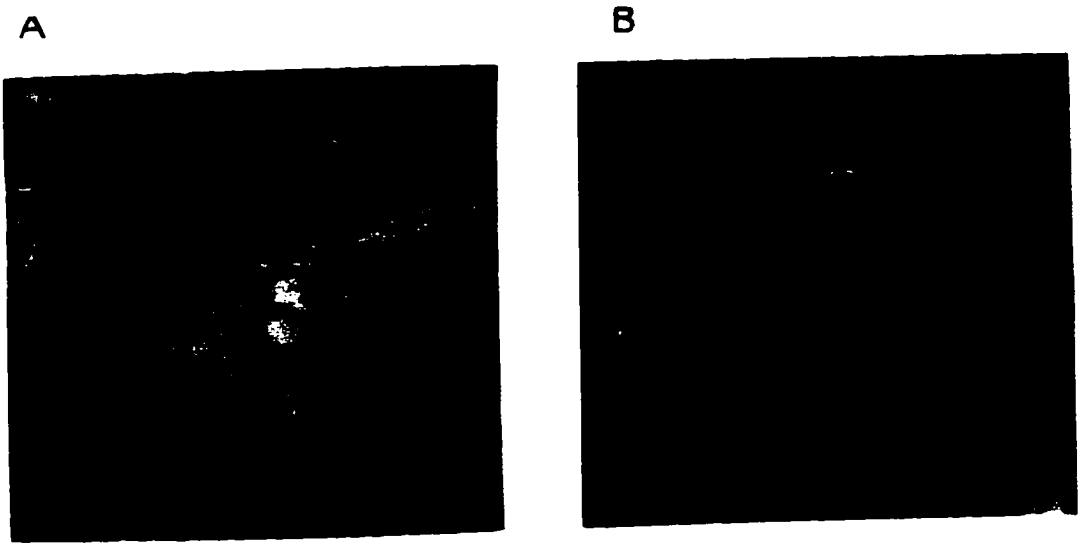


Figure 28: Neurite Outgrowth from Cerebellar Neurons on Various Concentrations of CNS and PNS Myelin

Cerebellar neurons from newborn animals were cultured for 16 h on various concentrations of freshly prepared PNS and CNS myelin as indicated, before being fixed , stained for GAP43 and neurites measured. results show the mean of the longest neurites per cell (+/- SEM) for 120-180 individual neurons.

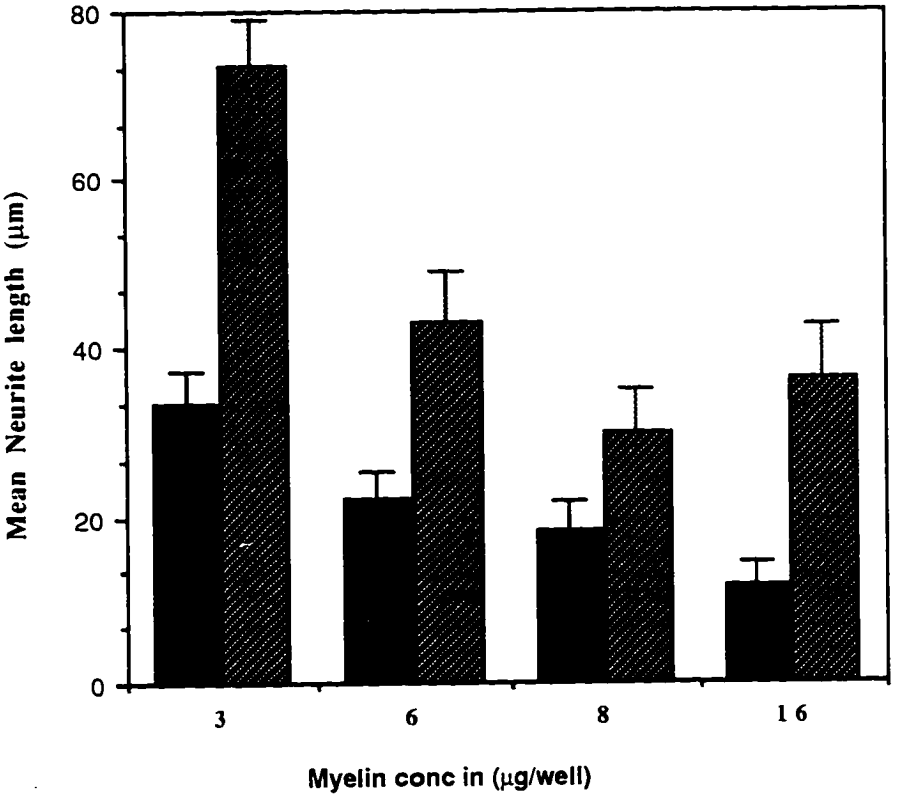
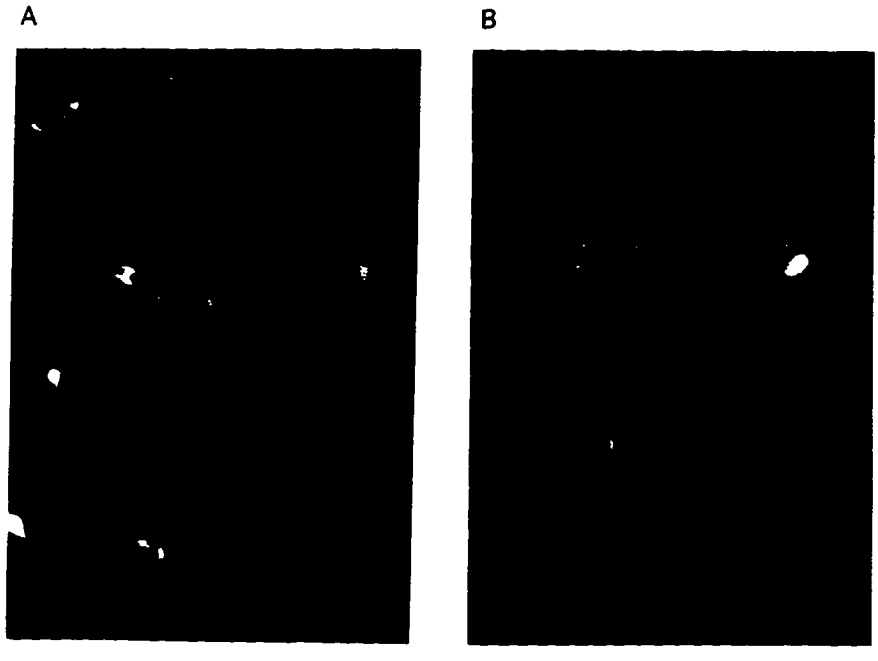


Figure 29: Neurite Outgrowth from PND4 Cerebellar Neurons and Grown on 6 μg/well of CNS and PNS Myelin.

PND4 cerebellar neurons were cultured on 6 μg/well PNS (a)and CNS (b) myelin for 16 h before being fixed and stained for GAP43 as described in the methods.



Conclusion

It is concluded from these studies that PNS myelin is not universally permissive for neurite outgrowth, rather, the type of neuron and membrane purification must be considered before this conclusion can be drawn. These results are consistent both with the presence of MAG in PNS myelin and with our previous observation that depending on the type and age of neuron, MAG can either promote or inhibit axonal regeneration (Chapter III). As mentioned before, David and his co-workers have shown that PNS myelin enriched in laminin is not inhibitory for neurite outgrowth (David et al, 1995). Laminin was barely detectable by Western blotting in our PNS myelin preparations (results not shown). This result is in agreement with the potent inhibition we observe.

Although both PNS and CNS myelin contain MAG, additional CNS myelin-specific inhibitory factors have been identified, namely the factors recognized by the monoclonal antibody IN-1 (Caroni and Schwab, 1988a and 1988b). This finding is consistent with the observation that, unlike PNS myelin and MAG, CNS myelin is inhibitory for neurite outgrowth from newborn DRG neurons.

Unlike newborn DRG neurons, neurite outgrowth from adult DRG neurons is also inhibited by PNS myelin (results not shown). However, MAG is unlikely to affect the regeneration of the mature PNS myelin after injury as regeneration takes place only after myelin debris has been removed by Wallerian degeneration. Therefore, it can be argued that not the absence of MAG but Wallerian degeneration, changing the environment allows regeneration of injured axons. Recently, Martini and his co-workers (Schafer et al., 1996) reported improved regeneration in C57BL/Ola mice that were MAG-deficient. These mice were created by crossing C57BL/Ola with MAG-/- mice. They showed that in the MAG-/-:C57BL/Ola mice after peripheral nerve leison there were twice as many regenerating axons in contact with myelinated

fibers than in the MAG-/-:C57BL/Ola mice. In addition, although an up-regulation of the axonal growth-promoting molecule, N-CAM was previously reported in MAG-/- mice (Montag et al., 1994), it was shown that in the C57BL/Ola and the MAG-/-:C57BL/Ola mice N-CAM was upregulated to the same extent in each after injury (Schafer et al., 1996). It was concluded that an absence of MAG and not an increase in N-CAM accounted for improved regeneration in the MAG-/-:C57BL/Ola mice.

Chapter VII

General discussion

1. MAG as an Inhibitor of Neurite Outgrowth *in vitro*

MAG has a different effect on different ages of DRG neurons. Here, we show that MAG promotes neurite outgrowth from DRG neurons up to PND2. In contrast, MAG inhibits neurite outgrowth from PND3 through to adult DRG neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996a). The post-natal switch in response from promotion to inhibition occurs sharply at PND3. It is not known if MAG interacts with the same molecule on neurons at various stages during development to affect regeneration. It is possible MAG interacts with the same neuronal receptor but that the downstream signals are different. Alternatively, perhaps during development DRG neurons express different types of receptors.

Recently, Shewan and his colleagues have shown that mCD24, (murine CD24), a GPI linked protein, can inhibit neurite outgrowth from DRG or RGC (retinal ganglion cells) and like MAG, inhibition of neurite outgrowth by mCD24 also depends on the age of the neurons (Shewan et al., 1996). CD24, belongs to the immunoglobulin superfamily and is expressed transiently by the hematopoietic lineage (Hardy et al., 1991), neurons (Rougon et al., 1991) and some epithelial cells (Shirasawa et al., 1993). The mCD24 protein expressed by C6 glioma cells can inhibit neurite outgrowth from neonatal RGC, neonatal DRG and adult DRG neurons by 75%, 62% and 57% respectively compared to the neurite length on control untransfected C6 glioma cells. In contrast, neurite outgrowth from embryonic RGC and DRG neurons is not inhibited by mCD24. However, it is known that mCD24 is expressed by embryonic and postnatal RGC and DRG neurons. Therefore, the authors speculated that mCD24 can interact in a heterophilic manner with a developmentally regulated molecule expressed by only postnatal DRG and RGC neurons, but not by embryonic neurons. Heterophilic interaction of mCD24 was demonstrated by studies in which neurite outgrowth from mCD24-negative DRG or RGC neurons isolated from mCD24 knock-out mice were shown to be inhibited by mCD24 expressed

by C6 glioma cells. However, the neurite outgrowth from mCD24-negative DRG or RGC neurons was not inhibited on control untransfected C6 glioma cells (Shewan et al., 1996). Therefore, mCD24 has an effect similar to MAG, as a switch in response from no effect to inhibition is observed for early postnatal neurons.

In addition to our studies, a number of laboratories have demonstrated a role of MAG as an inhibitor of neurite outgrowth (McKerracher et al, 1994; David et al., 1995; Li et al 1996; Schafer et al., 1996). McKerracher and her group demonstrated the inhibitory role of MAG by immunodepleting MAG from total extracts of CNS myelin. They found that immunodepletion of MAG from total extracts of CNS myelin restored neurite growth up to 63% of control levels. Recently David and his colleagues have shown that rMAG (recombinant MAG, i.e., extracellular domain of MAG expressed by insect cells and purified) inhibits neurite outgrowth from rat hippocampal and neonatal cerebellar neurons by about 80%, compared to the length of neurites on laminin (Li et al., 1996). They have also shown that 60% of axonal growth cones of PND1 hippocampal neurons collapse when they encounter polystyrene beads coated with recombinant MAG, while denatured MAG in a similar experiment resulted in only 8% collapse. Previously, Schachner and her colleagues reported that detergent (CHAPS) solubilized myelin proteins isolated from MAG-/- mice contained similarly potent growth cone-collapsing activities for adult DRG neurites as proteins from MAG+/+ mice (Bartsch et al., 1995). Schachner and her group also used immunopurified MAG to test its growth collapsing activity and they concluded that MAG does not have growth cone collapsing activity on adult DRG neurites. The possible explanation for the growth cone collapse of hippocampal neurons by MAG observed by David's group and not by Sachachner's group could be a difference in how MAG is presented to the neurons. When MAG is coated onto beads, it is presented to the growth cone in a multimeric form. In contrast, the MAG purified from myelin and added in solution is monomeric.

Previously it was shown by our laboratory that in order to demonstrate binding of MAG to neurons, the avidity of the interaction must be increased by presenting MAG in a multimeric form (Kelm et al., 1994). This implies that a multimeric interaction between MAG and neurons, which would induce clustering of both MAG and its putative receptor on the opposing membrane, is required to initiate a response of either inhibition or promotion of neurite outgrowth (Filbin 1995).

Apart from PND3 through adult DRG, we have also shown that MAG inhibits neurite out growth from cerebellar neurons of all postnatal ages (Mukhopadhyay et al., 1994). However, the ability of MAG to inhibit neurite outgrowth is not limited to its expression by CHO cells. It was shown by others from our laboratory that Schwann cells normally permissive for neurite outgrowth, are inhibitory when they are induced to express MAG after transfection (Shen et al., 1996). Furthermore, the inhibition of neurite outgrowth from a variety of different neurons, namely, retinal, superior cervical ganglion, spinal and hippocampal was also reported by DeBellard from our laboratory (DeBellard et al., 1996a). She also reported that the length of neurites from cerebellar neurons were about double that on MAG-/-myelin than the neurite length on myelin from MAG+/+ mice. Recently, David and his colleagues had also reported that the length of neurites from neuroblastoma (NG108-15) cells were 40% longer on myelin from MAG-/- mice than on MAG+/+ myelin, but they reported that this difference was statistically insignificant because of tremendous variation from one experiment to another (Li et al., 1996). This high variability in results could be a reflection of problems associated with purified myelin as a substrate. Initially we experienced wide differences in neurite outgrowth on individual myelin preparations, varying all the way from no growth to occasional extensive growth from cerebellar neurons on different concentrations of either CNS or PNS myelin. We therefore changed the dilution protocol by serially diluting purified myelin instead of diluting each time

from the stock myelin. Hence by using only fresh myelin preparations, dilution protocol and also modifying the slide coating procedure (see method section), we minimized experimental variation.

Furthermore, Li et al used another strategy to show that MAG in CNS myelin is an inhibitory molecule for neurite outgrowth (Li et al., 1996). When myelin from MAG+/+ mice fractionated by DEAE anion exchange chromatography according to molecular weight, was used as a substrate a number of inhibitory fractions were identified and one of them contained MAG. However, as MAG is absent from MAG-/- mice, its inhibitory effect is also significantly reduced in that corresponding fraction by about to 52% compared to that of MAG+/+ mice (Li et al., 1996). These results support the previous data obtained by DeBellard from our laboratory, which is discussed above. In apparent contradiction of these findings, Schachner and her colleagues reported that there were no differences in the length of the neurites from cerebellar and adult DRG neurons on the either myelin or optic nerve cryosection of both wild type and MAG-/-mice as substrates (Bartsch et al., 1995). However, they do find a 20% improvement in neurite length on MAG-/- myelin for cell line NG108. Therefore, in conclusion, combining all data from different groups, we propose that MAG plays a significant role in CNS myelin as an inhibitor of neurite outgrowth.

In 1992 Bedi et al reported that adult DRG neurons only extend neurites on tissue sections of peripheral nerve which was previously lesioned and allowed to undergo Wallerian degeneration *in vivo* (Bedi et al., 1992). In these sections there is very little if any myelin and, consequently, no MAG. The same adult DRG neurons fail to grow on intact peripheral nerve tissue which was not previously leisoned and therefore it has intact myelin which contains an abundance of MAG. In contrast, embryonic neurons or newborn DRG neuron will extend their neurites regardless of the stage of degeneration. Hence, from our results, both the promotion of

neurite outgrowth from newborn DRG and inhibition of neurite outgrowth from adult DRG neurons by MAG could account for the previous findings of Bedi in 1992.

Previously PNS myelin was reported to be permissive for the growth of axons both *in vivo* and *in vitro* (Carbonetto et al., 1987; Caroni and Schwab, 1988a; Savio and Schwab, 1989; and Aguayo et al., 1978). As MAG is present in PNS myelin, how can we reconcile the permissiveness of PNS myelin with axonal regeneration? First, it is known that during regeneration in the PNS, axons are unlikely to encounter MAG because myelin debris is cleared before regeneration takes place (Griffin and Hoffman, 1993). However, Wallerian degeneration is very slow in the CNS, which can explain how CNS myelin can affect axonal regeneration. Second, from *in vitro* experiments, so far only embryonic RGC and newborn DRG neurons were tested for their growth on PNS myelin (Caroni and Schwab, 1988b) or PNS tissue section (Carbonetto et al., 1987; Schwab and Thoenen, 1985) Therefore, it is possible that embryonic neurons may not carry receptors for inhibitor molecules. Third, permissiveness of PNS myelin may depend on the amount of laminin co-purified during the isolation procedure (David et al., 1995). Our PNS myelin preparation which has barely detectable amounts of laminin, promotes neurite outgrowth from PND1 DRG neurons. In contrast, it inhibits neurite outgrowth from cerebellar neurons, consistent with the effects of MAG on these neurons.

2. MAG as an Inhibitor of Axonal Regeneration *in vivo*

Recently, David and his co-worker demonstrated the inhibitory role of MAG in the CNS by an *in vivo* experiment (Li et al., 1996). They studied axonal regeneration in MAG-/- mice after thoracic lesions of the CST. They studied the total number of regenerating axons and the longest axon per animal. They found that the number of the anterogradely labeled axons that extended to 13.2 mm from the lesion site was greater in the MAG-/- mice than in wild type mice.

In MAG-/- animals, axons never extended more than 6.7 mm from the lesion site. Although there is some enhancement of axonal regeneration growth, there is still poor growth after spinal cord injury in MAG-/- mice. This may be due to the presence of other non-MAG inhibitors as mentioned before (Caroni and Schwab, 1988a and 1988b). Schachner and her group also studied axonal regeneration in the CNS of MAG-/- mice. They compared the longest regenerating axon per animal from both crushed optic nerve and leisoned CST of MAG+/+ and MAG-/- mice (Bartsch et al., 1995). They found that the extent of axonal regrowth in both MAG+/+ and MAG-/- mice was similar in both and was poor. However, application of the IN-1 antibody (Caroni and Schwab, 1988a and 1988b) improved axonal regeneration in MAG+/+ and MAG-/- mice. This discrepancy in findings on regeneration in the MAG-/- mice between Schachner's group and David's group remains to be resolved, however, there are two possible explanations. First it is possible that Schachner's group recorded no difference in regeneration in MAG+/+ and MAG-/- mice because they examined only one regenerating axon per animal and for RGC they only assessed a total of 5 animals. However, David's group compared regeneration from 15 animals from MAG-/- mice with 10 animals from MAG+/+ mice. Second, the line of MAG-/- mice in each study was created independently by two groups (Montag et al., 1994; Li et al., 1994). The two lines of mice may not be identical and may not respond in the same way after injury.

Other studies show that MAG can inhibit axonal regeneration *in vivo*. It has been shown that in C57BL/Ola mutant mice, in which myelin remains intact and is not rapidly removed after injury, regeneration is very slow and occurs mostly along unmyelinated fiber tracts (Brown et al., 1992). Recently Martini and his colleagues made double mutant mice generated by crossing C57BL/Ola mice with MAG-deficient mice. They reported that in crush nerves of C75BL/Ola mice expressing MAG, only 15.7% of myelin sheaths were associated with regenerating axons

whereas, this number was about double in MAG-/-:C75BL/Ola mice. This report demonstrates that a more favorable environment for the regeneration of crushed nerve in the presence of myelin debris is provided by an absence of MAG (Schafer et al., 1996). Although N-CAM upregulation has been reported in MAG-/- mice, after injury N-CAM expression is upregulated in both MAG+/+:C57BL/Ola and MAG-/-:C57BL/Ola. Therefore, the absence of MAG in the myelin of MAG-/-:C57BL/Ola mice compared to the same extent to MAG+/C57BL/Ola mice is likely to account for the improved regeneration after injury, rather than an upregulation of N-CAM.

3. Mapping the Sialic Acid Binding Domain of MAG.

Reports from our laboratory have shown that MAG binds to a sialic acid-bearing component on both cerebellar and DRG neurons at all postnatal ages, regardless of whether neurite outgrowth is promoted or inhibited (DeBellard et al., 1996a). In this assay a single-cell suspension of neurons (either cerebellar or DRG neurons) was allowed to bind to various concentrations of a MAG-Fc was immobilized on a 96-well microtiter plate. As a control chimera, another five-domain Ig-family member, MUC18, was used. As previously described that binding of neurons to the MAG-Fc was abolished when a monoclonal antibody to MAG, the 513 antibody, was included in the binding assay (Kelm et al., 1994). Furthermore, it was also shown that both inhibition and promotion of neurite outgrowth by MAG can be reduced, or abolished completely, either by including small sialic acid-bearing sugars in the culture or by desialylation of the neurons prior to the neurite outgrowth assay (DeBellard et al., 1996a). It was also shown that binding of MAG to the surface of neurons is trypsin-sensitive. Therefore, the inhibition and promotion effects of MAG are dependent, either directly or indirectly, on the interaction of MAG with a sialoglycoprotein on the neuronal surface.

Sialoadhesins are a distinct subgroup of the immunoglobulin superfamily, comprising to date, sialoadhesin, CD22, MAG, CD33 and SMP (Kelm et al., 1994). All members of this family of molecules are capable of mediating sialic acid-dependent binding to cells with distinct specificity (Table 1). By analyzing sequences it has been shown that the four N-terminal Ig-like domains of MAG share 45 to 50% sequence similarity with the four N-terminal Ig-like domains of sialoadhesin and CD22 (Kelm et al, 1994). In addition, by truncating different domains, it has been shown that the sialic acid binding site is located within the NH_2-terminal (membrane-distal) V-set domain of sialoadhesin (Vinson et al., 1996) or CD22 (Van der Merwe et al., 1996). In this V-set domain, Arg118 (R118) in MAG is conserved in the same location in all members of this family of molecules. This V-set domain contains two β sheets GFCC'C'' sheet and the ABED sheet (Williams and Barclay, 1988). It has been shown that sialic acid binding can be abolished by drastic mutations of five amino acids from sialoadhesins and nine amino acids from CD22. Drastic mutation means a change from an acidic amino acids to a basic amino acid or visa versa. However, unlike four other amino acids of sialoadhesin, when Arg 97, was mutated to lysine, a conservative mutation which retains the positive charge, sialic acid binding was lost. Therefore, this arginine appears to be a key residue for sialic acid-dependent binding of sialoadhesin and CD22.

Based on this mutation analysis and considering the alignment studies of CD22, sialoadhesin, and MAG with CD8α whose crystal structure is known (Leathy et al., 1992), it has been speculated that the sialic acid binding site with the conserved arginine surrounded by five amino acids in sialoadhesin and nine amino acids in CD22 is in the F strand of the GFCC'C'' β sheet (Vinson et al., 1996; Van der Merwe et al, 1996). Because of these findings, we mutated Arg118 of MAG to either alanine or aspartic acid, changing the positive charge to either neutral or negative respectively. As was predicted, like sialoadhesin or CD22, mutation

of Arg118 in MAG to either alanine or aspartic acid, abolished completely its sialic acid-dependent binding to cerebellar or DRG neurons completely (Tang et al., 1996). However, surprisingly, whenever these mutated MAG's are expressed on the surface of CHO cells they still inhibit neurite outgrowth from cerebellar neurons and adult DRG. In addition, these mutated MAGs on the surface of CHO cells are also capable of promoting neurite outgrowth from newborn DRG neurons. So, after disrupting the sialic acid binding domain in MAG, although, mutated MAG can no longer bind to the neurons, it can still inhibit or promote neurite outgrowth from different neuronal populations to the same extent as does wild type MAG.

Furthermore, it has been shown by others from our laboratory that a soluble form of MAG, MAG-Fc can also inhibit neurite outgrowth from cerebellar neurons (Tang et al., 1996). However, R118A or R118D mutated MAG-Fc did not inhibit neurite outgrowth from cerebellar neurons. The neurite length was the are same as with the control Fc-chimera, MUC18-Fc. Therefore, consistent with its inability to bind to neurons mutated MAG-Fc cannot inhibit neurite outgrowth from cerebellar neurons. Taking all these results into consideration, we have proposed a model which is described in Fig. 26. This model requires that the sialic acid binding domain in MAG is distinct from the inhibition/promotion domain in MAG. It was also shown that R118 is necessary for sialic acid-dependent binding of MAG to the neurons. However, after expressing MAG on the surface of CHO cells it is obvious that R118 is not required for the inhibition or promotion of neurite outgrowth from the neurons by MAG. This implies that binding of neurons to R118 in MAG is necessary but it is not the sole component for the inhibition/promotion exhibited by MAG on the extension of neurites from cerebellar or DRG neurons. When MAG is expressed on the surface of CHO cells, the neurons can bind to the CHO cells by some other surface molecule and the neurite outgrowth epitope of MAG can engage and can inhibit axonal growth. In contrast, adding soluble MAG-Fc, neurons only can

see chimeric MAG and if the important binding site R118 in MAG is mutated, neurons can no longer bind to MAG. As a consequence, the inhibition site cannot engage and MAG can not exhibit its inhibitory function. The question is how to explain the results of the desialylation experiments with the wild type and mutant MAG based on our model? From previous experiments it is known that neurite outgrowth depends directly or indirectly on the binding of MAG to the neurons via sialic acid (DeBellard et al., 1996). Because after desialylation of neurons there are 50 to 100% increase in the length of neurites. Taking this in consideration we destroy this sialic acid binding site R118 in MAG by mutation which are discussed above. We are expecting no change in the neurite length from desialylated neurons cocultured on this mutated MAG compared to the untreated neurons. However, using this mutated MAG we do find an increase in the length of neurites from desialylated neurons, an effect similar to wild type MAG (Fig. 25a and 25b). Hence even though we destroy sialic acid binding site in MAG still sialic acids from neurons are occupied by some other molecules expressed by CHO cells (Fig. 26).

In summary, from our results it is possible to speculate that the inhibition domain in MAG is distinct from its sialic acid binding domain.

4. What is the Physiological Relevance of the Inhibition Property of MAG?

Schwab and his colleagues have suggested that myelin stabilizes axonal sprouting and fiber number (Schwegler, Schwab and Kapfhammer, 1995; Colello et al., 1994). This conclusion stems from studies in which myelination was prevented in the spinal cord by killing dividing oligodendrocytes of new born rats by irradiation. As a consequence, the collateral sprouting of primary afferents was increased. In addition, it is also known that the total number of rat optic nerve becomes stabilized at the end of the first week of birth (Colello et al., 1994).. At the end

of the first week myelination has also started. So there is an overlap during the end of the first postnatal week between stabilization of the number of axons and the onset of myelination. Now the question is, does myelin have any role in stabilizing the fiber number? In the absence of myelin the fiber number is high and this number is reduced and stabilized after the onset of myelination. To test this hypothesis Schwab and his colleagues prevented myelin formation of optic nerves in rats of age PND15 by X-ray irradiation, and found that the total fiber number in a myelin free optic nerve was 10-30% higher than that of a myelinated nerve (Colello et al., 1994). The location of MAG in the periaxonal membrane of the CNS, (which is at the axon-myelin interface) places it in an ideal position to influence sprouting. In addition, another role for MAG is suggested in which it stabilizes the axon: myelin interface. MAG could actively prevent growing axons from entering and disrupting the sites of axon:myelin interaction, because myelin inhibitors of neurite extension assign territories of different fiber tracts from each other in the CNS.

Furthermore, since it is known that FGF (fibroblast growth factor) can increase cell survival (Sievers et al., 1988), Schwab and his colleaque also wanted to test whether they could overcome the inhibitory effect of myelin inhibitors by treatment with FGF (Collelo and Schwab, 1994). They treated both X-ray irradiated (myelin-free) and unirradiated (myelinated) animals with FGF. Treatment of FGF in the myelin-free animal resulted in an increase in the total number of fibers by 40%. In contrast, there was no increase in fiber number for myelinated, FGF-treated animals. This result indicates that in the absence of myelin, FGF can increase fiber number. Therefore, the promoting effects of FGF can not override the inhibitory functions of CNS myelin. Therefore, as well as oligodendrocyte membrane proteins NI35/250 (Caroni and Schwab, 1988a and 1988b), MAG is another candidate which can serve as an inhibitor of the sprouting of neurites. The inhibitory effects of MAG on axonal regeneration we observe is likely

to be an unwanted carryover of this "protector" function. The same unwanted function could come into play and could be detrimental after injury to the CNS.

It is unlikely that MAG has a role in the axonal pathfinding during development. Unlike other axonal pathfinding molecules, N-CAM (Doherty et al., 1990), L1 (Williams et al., 1992), the Netrins, (Serafini et al., 1994; Kennedy et al., 1994; Colamarino et al., 1995), and semaphorin/collapsin (Luo et al., 1993; Puschel et al., 1995; Messersmith et al., 1995), MAG is not expressed during embryonic development. MAG, like other myelin-specific proteins, is believed to be expressed only after the axon has reached its target and myelination has begun, which is relatively late in development (Quarles 1984; Maritini et al., 1986; and Bartsch et al., 1989). These observations would suggest that MAG cannot play a role in axonal guidance during development. However, recent findings have shown that a number of myelin-specific proteins are expressed in migrating, immature oligodendrocytes (Yu et al., 1994; Timsit et al., 1995). This contradicts the previous belief that they are only expressed late in development. Similarly, MAG may be expressed at low levels during the early stages of development. This could allow MAG to guide the axon but still remains to be determined.

Chapter VIII

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