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CHARACTERIZATION OF MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

by

SONG TANG

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1998

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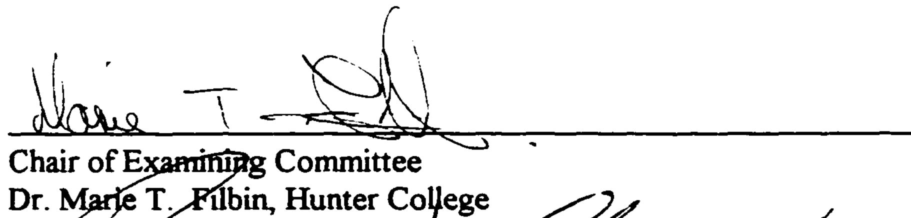

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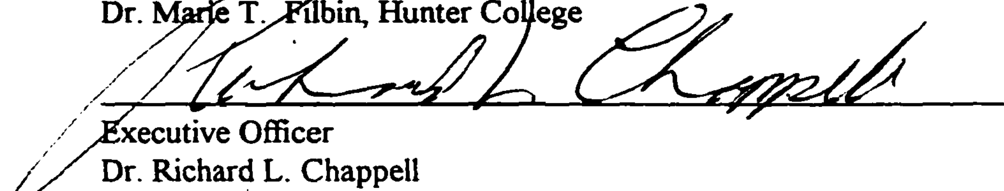

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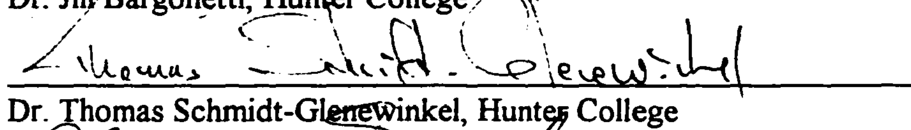

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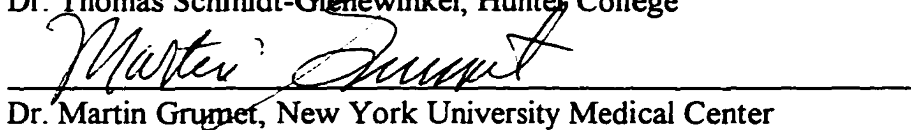

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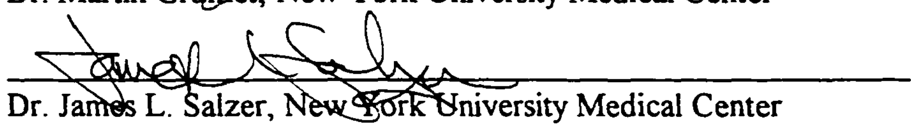

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ABSTRACT

CHARACTERIZATION OF MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

by

SONG TANG

Advisor: Professor Marie T. Filbin

It is believed that inhibitors in myelin are responsible for the lack of axonal regeneration in the mammalian CNS after injury. But the precise nature of the molecules responsible for this effect is not known. Myelin-associated glycoprotein (MAG), a well characterized transmembrane glycoprotein, was shown to inhibit neurite outgrowth from all postnatal cerebellar neurons and adult dorsal root ganglion neurons. However, how MAG brings about this effect is not known. Here, we report that (1) MAG specifically binds to neurons in a sialic acid-dependent manner, (2) the soluble forms of MAG, MAG-Fc and dMAG, inhibit neurite outgrowth, and MAG is a true inhibitor of axonal regeneration, and (3) arginine 118 in the first Ig-like domain of MAG is the sialic acid binding site on MAG. Using a chimeric form of MAG, consisting of the extracellular domain of MAG fused to the Fc portion of human IgG, termed MAG-Fc, we have shown that MAG specifically binds to sialoglycoconjuagtes on neurons, and MAG-Fc specifically inhibits neurite outgrowth in sialic acid-dependent manner. In addition, we have also shown that the soluble MAG found in vivo, termed dMAG, inhibits neurite outgrowth, and the inhibition is MAG-specific and dose-dependent. Furthermore, we have found that mutation at Arg118 in the first domain of MAG abolishes the sialic acid-dependent binding of MAG to neurons and the inhibitory effect of MAG-Fc on neurite outgrowth. However, a truncated MAG-Fc, which also binds to sialic acids on neurons, does not inhibit neurite outgrowth, and the R118-mutated MAG expressed on cell surface still inhibits neurite outgrowth. Therefore, we suggest that MAG has two recognition sites for neurons, the sialic acid binding site at R118 and a distinct inhibition site which is absent from the first three Ig-like domains.

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Chapter I

Introduction

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1.1 Influences on Neurite Outgrowth During Development

An important step in the development of the nervous system is the directed outgrowth of axons, which brings them into contact with their target cells in the peripheral and central nervous systems. Neurite outgrowth is guided by either fixed or diffusible cues during development.

The fixed cues consist of extracellular matrix (ECM) components, such as laminin and fibronectin (for review, see Letourneau et al., 1992), and cell membrane-bound or transmembrane proteins, such as the cell adhesion molecules NCAM and L1 (for review, see Doherty and Walsh, 1992). The fixed cues can have either a positive or negative influence on neurite outgrowth. For example, the cell adhesion molecules NCAM and L1, and ECM components laminin and fibronectin, promote neurite outgrowth (for review, see Letourneau et al., 1992 and Doherty and Walsh, 1992), but the smallest immunoglobulin superfamily molecule, Thy-1, inhibits neurite outgrowth. When a transfected neural cell line expressing Thy-1 on its cell surface (termed Thy-1$^+$ cells) was grown on a monolayer of mature astrocytes, the neurite outgrowth from Thy-1$^+$ cells was inhibited by interaction between Thy-1 and the astrocytes, as this inhibition was reversed when soluble Thy-1 or anti-Thy-1 antibodies were included in the culture (Tiverson et al., 1992).

Similarly, the effect on neurite outgrowth of diffusible cues can also be positive or negative, defined as chemoattraction or chemorepulsion respectively. In addition to this, some molecules can effect neurite outgrowth both in a soluble form or as a membrane-bound molecule. For example, some members of the family of molecules termed the semaphorins inhibit neurite outgrowth from different neuronal populations in a soluble form as well as via cell-cell contact (Kolodkin et al., 1992; Kolodkin et al, 1993; Luo et al., 1993; Luo et al., 1995; Püschel et al., 1995; Messersmith et al., 1995).

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What is of particular interest is that some molecules can have bifunctional effects on neurite outgrowth. For example, in Drosophila, a cell-surface protein called connectin, which is expressed on a subset of embryonic muscles, appears to function as either an attractive or a repulsive molecule for specific neuronal populations during motor neuron growth cone guidance (Nose et al., 1994). Also, Netrin-1, a molecule of the secreted netrin family, attracts commissural axons but repels trochlear motor axons (Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). It is suggested that bifunctionality may be a more efficient way for a molecule to influence neurite outgrowth, specifically and developmentally.

The negative influence on neurite outgrowth during development can be defined as either nonpermissive or inhibitory, although the distinction is not always clear. For example, the ECM molecule tenascin provides an unfavorable substrate for a variety of CNS axons even though it promotes the outgrowth of spinal motor neurons (Faissner and Kruse, 1990; Wehrie and Chiquet, 1990). The semaphorin family molecule, chick collapsin-1, inhibits neurite outgrowth from different neuronal population (Luo et al., 1993). It is important to distinguish between inhibitory molecules and non-permissive substrates. Non-permissive substrates do not support growth cone advance and neurite outgrowth, but inhibitory molecules must, in addition, inhibit neurite outgrowth and induce growth cone collapse (for review, see Luo and Raper, 1994).

It has been shown that inhibitory molecules mediate a negative effect on axonal outgrowth via their ability to collapse or repulse growth cones. For example, chick collapsin-1, a secreted semaphorin molecule, in vitro causes the collapse of growth cones from dorsal root ganglion (DRG) neurons (Luo et al., 1993). The grasshopper semaphorin I, a transmembrane semaphorin in insects, functions in the grasshopper limb bud to steer growth cones as they encounter epithelial cells expressing it (Kolodkin et al, 1992). The collapse of

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growth cones is characterized as induced filopodial retraction and disruption of the lamellipodial structure of the growth cone. The repulsion of growth cones means that the trajectory of a whole growth cone can be altered after a filopodium contacts an inhibitory molecule (Kapfhammer and Raper, 1987; Fan and Raper, 1995). It is postulated that in vivo these inhibitory molecules may function as guideposts for growth cones and hence direct growing axons.

1.2 Major Families of Inhibitory Molecules

To date, four families of molecules have been identified as inhibitors in nervous system development. They are the semaphorin family (for review, see Dodd and Schuchardt, 1995; Kolodkin, 1996), the netrin family (for review, see Culotti and Kolodkin, 1996), the Eph receptor-ligand family (for review, see Brambilla and Klein, 1995; Friedman and O'Leary, 1996; Orike and Pini, 1996; and Drescher et al., 1997), and the Robo family (for review, see Chien, 1998 and Thomas, 1998).

The semaphorin family is found in both vertebrates and invertebrates, and includes both secreted and transmembrane molecules. The semaphorins are characterized by the presence of a conserved domain of 500 amino acids (the semaphorin domain) at their amino termini and several conserved cysteine residues (for review, see Kolodkin, 1996) . It has been shown that the semaphorin family has an evolutionary conserved negative influence on neurite outgrowth in nerve development as different family members have been found in different species (for review, see Kolodkin, 1996). In this family, chick collapsin-1 is the founding member, which shares high homology with grasshopper semaphorin I (formally called fasciclin IV) and human semaphorin III (Kolodkin et al., 1993). It was shown that in vitro chick collapsin-1 causes the collapse of growth cones from DRG neurons (Luo et al., 1993). Similarly, a murine homolog of the human semaphorin III (termed semaphorin D) repelled axons from NGF-dependent

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embryonic DRG sensory neurons in collagen-stabilized cultures (Messersmith et al., 1995). In addition, the grasshopper semaphorin I was implicated in axon guidance in developing limb buds (Kolodkin et al., 1992).

Recently, identification of a binding partner for semaphorin family molecules has been reported. A transmembrane protein, called neuropilin-1, has been shown to be a receptor or a component of a receptor complex for the semaphorin D (murine homolog to chick collapsin-1, human semaphorin III, generally referred to as Sema III), since Sema III binds with high affinity to neuropilin-1. Importantly, antibodies to neuropilin-1 block the ability of the Sema III to repel rat embryonic sensory axons and to induce collapse of their growth cones (He et al., 1997; Kolodkin et al, 1997). In addition, it has been found that another neuropilin protein, neuropilin-2, shows a high degree of sequence and structural similarity to neuropilin-1, and both of them are expressed in overlapping, yet distinct, populations of neurons in the rat embryonic nervous system (Kolodkin et al, 1997; Chen et al., 1997). Furthermore, it has been demonstrated that neuropilin-2 can bind with high affinity to the murine semaphorin E and human semaphorin IV (another secreted semaphorin group structurally related to Sema III), but not to Sema III (Chen et al., 1997). Therefore, it is suggested that neuropilins are a family of candidate receptors (or components of receptors) for members of the semaphorin family that are structurally related to Sema III, and are expressed differentially in the mammalian nervous system during development.

The netrins are a small family of secreted, laminin-related proteins that are conserved from invertebrates to vertebrates and are capable of guiding growth-cone migration (for review, see Culotti and Kolodkin, 1996). It has been found that the netrin family has members in C. elegans (UNC-6; Ishii et al, 1992), in vertebrates (netrin-1 and netrin-2; Serafini et al., 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995), and in Drosophila (netrin-A and netrin-B; Mitchell et al., 1996). In vivo, netrin-1 is expressed in floor plate cells and

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netrin-2 at lower levels in the ventral two-thirds of the chick spinal cord, suggesting that there is a decreasing ventral-to-dorsal gradient of netrin protein that functions to attract commissural axons to the ventral midline of the spinal cord (Kennedy et al., 1994). A netrin gradient may also contribute to repelling some axons, such as trochlear motor axons, away from the ventral midline, since netrin-1 can repel these axons in vitro (Colamarino and Tessier-Lavigne, 1995). In C. elegans, UNC-6, expressed along the ventral midline, is thought to attract ventrally directed axons and to repel dorsally directed axons, since guidance of these axons is impaired in *unc-6* mutants (Hedgecock et al., 1990; Wadsworth et al, 1996).

Recent work in C. elegans provides further insight into how netrins may function during neural development. UNC-40, an integral membrane receptor on pioneering neurons, allows their growth cones to move toward UNC-6 sources. However, when the neurons co-express UNC-5 with UNC-40, they move away from UNC-6 sources. Therefore, UNC-40 is required in neurons for their growth cones migrating toward to UNC-6 cues, and along with UNC-5, for migrating away from same cues (Hedgecock et al., 1990; Chan et al., 1996). More importantly, it has been found that a previously identified vertebrate transmembrane protein of the immunoglobulin superfamily, Deleted in Colorectal Cancer (DCC), is a homolog to UNC-40 in C. elegans and is expressed on the axons and growth cones of spinal commissural neurons. DCC has been shown to be a netrin-1 binding protein that is required to mediate the chemoattractive effect of netrin-1 on commissural axons in vitro (Keino-Masu et al., 1996). Likewise, the Drosophila homolog of DCC, Frazzled, is a transmembrane protein which is expressed on netrin-responsive axons (CNS commissural and peripheral motor axons) and required for their pathfinding and targeting (Kolodziej et al., 1996). Together, all these results indicate that the netrin-dependent axon guidance mediated by DCC is evolutionary conserved, and DCC is a receptor or a component of a receptor for netrin-1.

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In addition to the semaphorins and netrins, it has been shown recently that ligands and their receptors of Eph (Erythropoietin-producing hepatocellular) receptor family are repulsive cues for axonal guidance (for review, see Brambilla and Klein, 1995; Tessier-Lavigne, 1995; Harris and Holt, 1995; Friedman and O'Leary, 1996; Orike and Pini, 1996; and Drescher et al., 1997). The Eph family is the largest subfamily of receptor tyrosine kinases (RTKs), consisting of at least 13 distinct members, many of which are expressed exclusively or predominantly in the nervous system in a variety of vertebrates. The Eph RTKs are typified by their extracellular domains containing an Ig-like domain, a cysteine-rich region, and two fibronectin type III repeats similar to those found in many neural cell adhesion molecules. The ligands for these receptors are cell-surface proteins, anchored either by a GPI-anchor or by a transmembrane domain. Based on their binding interactions, Eph receptors and their ligands can be grouped into two subgroups: one subgroup consists of all the GPI-anchored ligands (such as B61, ELF-1, Lerk3/Ehk1-L, and AL-1/RAGS) and their receptors (such as Eph, Eck/Sek2, Mek4, Sek1/Hek8, Ehk1/Cek7), referred to as the Eck subgroup; the other one comprises all the transmembrane ligands (such as Elk-L/Lerk2, and ELF-2/Lerk5/Htk-L) and their receptors (such as Elk/Cek6, Htk, Sek3/Nuk and Herk2), referred to as the Elk subgroup (for review, see Brambilla and Klein, 1995; Friedman and O'Leary, 1996). Within each subgroup, a ligand can bind and activate many different receptors, but not to the receptors from the other subgroup. In other words, ligand-receptor interaction is promiscuous within each subgroup (Brambilla et al., 1995; Gale et al., 1996).

A major breakthrough in our understanding of the repulsive function of Eph family members occurred when a GPI-anchored ligand called RAGS (for repulsive axon guidance signal) was cloned from chicken, using an in vitro axon guidance assay, termed the stripe assay, to identify its repulsive activity (Drescher et al., 1995). In this assay, axons from retinal ganglion cells are confronted with alternating stripes of anterior and posterior tectal membranes. It was found that RAGS, expressed in a posterior-to-anterior gradient in the optic

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tectum (posterior high, anterior low), repels the axonal outgrowth of retinal ganglion cells in vitro (Drescher et al., 1995). Furthermore, another GPI-anchored ligand, ELF-1, was shown to be expressed in a similar posterior-to-anterior gradient in chicken optic tectum, while its receptor, Mek4 is expressed in a temporal-to-nasal gradient in the retina (temporal high, nasal low; Cheng et al., 1995). Since the most temporal axons innervate the most anterior region of the tectum, the observed gradients of ELF-1 and Mek4 expression are complementary: the retinal ganglion cells with highest Mek4 expression choose the tectal area with lowest ELF-1 expression (Cheng et al., 1995; Nakamoto et al, 1996). More notably, it was recently demonstrated that ELF-1 acts through Mek4 as a repulsive guidance cue for axons from temporal but not nasal retinal ganglion cells in vitro and in vivo (Nakamoto et al, 1996). Therefore, the GPI-anchored Eph ligands and their receptors play a critical role in the developing visual system.

In addition, the transmembrane Eph ligands and their receptors appear to be involved in axon guidance as well. For example, two transmembrane Eph ligands, Lerk2 and Htk-L, inhibit both rat axonal outgrowth from rat motor neurons and neural crest cell migration in the strip assays and induce growth cone collapse of motor neurons (Wang and Anderson, 1997). Moreover, an Eph receptor for transmembrane ligands, Nuk, restricts pathfinding of the cortical axons that form the anterior commissure, since gene disruption of Nuk in mice led to abnormal development and misrouting of the anterior commissure (Henkemeyer et al., 1996). All the results mentioned above suggest that Eph receptors and their ligands play a dynamic role in controlling axon movements in developing nervous systems.

It was found recently that the *robo* gene in Drosophila (called *sax-3* in C. elegans) encodes an axon guidance receptor which defines a novel subfamily of immunoglobulin superfamily proteins highly conserved from C. elegans and Drosophila to mammals (Zallen et al, 1998; Kidd et al., 1998a). Robo is expressed on growth cones and axons of developing

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neurons, and is believed to function as a gatekeeper controlling midline axon crossing. In Drosophila, for those axons that never cross the midline, their growth cones express high levels of Robo from the outset, while for the majority of axons (~90% in Drosophila CNS) that do cross the midline, their growth cones initially express low levels Robo, but upon crossing the midline, the level of Robo is dramatically increased on their growth cones and longitudinal axons (Kidd et al., 1998a). It was also demonstrated that in *robo* mutant Drosophila embryos, many commissural growth cones that normally extend only on their own side, instead projected across the midline, and the axons that normally cross the midline once, instead crossed and recrossed multiple times (Kidd et al., 1998b). Therefore, it is implied that neurons with low Robo concentration can cross the midline of the embryo, but a subsequent increase in the protein level prevents them from crossing back (Kidd et al., 1998a). Notably, the level of Robo expression was found to be influenced by a novel transmembrane protein expressed on midline cells called Comm. In *comm* mutant Drosophila embryos, commissural growth cones initially orient toward the midline but then fail to cross it and instead recoil and extend on their own side. However, *comm* and *robo* double mutant embryos have identical phenotype as *robo* mutant embryos, in which some axons crossed and recrossed the midline freely, suggesting that in the absence of Robo, Comm is no longer required. Furthermore, *comm* mutant embryos have a complementary phenotype to that of *robo* mutants in which too few axons crossed the midline, while overexpression of Comm reduces levels of Robo proteins and generates a *robo* mutant phenotype (Kidd et al., 1998b). Thus, there is an inverse correlation between Comm and Robo expression. Based on all these results, it is proposed that the levels of Comm and Robo are tightly regulated to ensure that only certain growth cones cross the midline and that those growth cones that do cross never do so again (Kidd et al., 1998b).

The high structural and functional conservation in the netrin and Robo families reveals a common underlying system in control of axonal traffic by specialized CNS midline structures in the embryo - the long range chemoattractant effect mediated by netrin-1/DCC and the

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chemorepulsive effect mediated by netrin-1/DCC/UNC-5 bring axons close to or away from the midline; a short-range repellent Robo/Comm controls them crossing the midline (for review, see Thomas, 1998 and Chien, 1998).

The significant progress in studying the guidance molecules in nerve development has produced tools and concepts that start to show their impact on axonal regeneration research. This is particularly true for the rapid growing cellular and molecular understanding of crucial aspect of neurite outgrowth, guidance and target finding.

1.3 Axonal Regeneration of CNS Nerve Fibers

In addition to molecules mediating negative cues for axon growth in development, some axon outgrowth inhibitory activities have been shown to be present in the adult mammalian CNS. The presence of inhibitory molecules is very likely to contribute to the lack of axonal regeneration after brain or spinal cord injury (for review, see Kapfhammer, 1997).

It is well known that after injury axons in the adult mammalian PNS regenerate. Regeneration in the PNS is permitted by removing myelin debris, the production of NGF and appropriate adhesion molecules by Schwann cells, and the down-regulation of expression of all myelin proteins, including myelin-associated glycoprotein (for review, see Aubert et al., 1995). Direct evidence that myelin debris must be cleared before axonal regeneration in the PNS occurs comes from studies with the mutant mice termed C57BL/Ola mice which suffer from delayed lesion-induced PNS myelin degeneration (Brown et al., 1991, 1994). In these mice, after injury, myelin debris is not removed and nerve regeneration occurs very slowly, if at all, and mostly along unmyelinated tracts. Therefore, myelin in the PNS either fails to encourage or actively inhibits nerve regeneration (Brown et al., 1991, 1994). It would appear

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that clearance of myelin debris is a necessary prerequisite for successful regeneration of PNS axons after injury.

Although the axons in the PNS will regenerate after injury, nerve fibers in the CNS show a very poor capacity to regenerate (for review, see Kapfhammer, 1997). The reasons for the poor regeneration within the mature CNS of higher vertebrates have been investigated for several decades. Historically, Ramon y Cajal (1928) argued that lack of trophic support might be the reason for the poor regeneration within the CNS. In the 1950s, the glial scar that forms after injury of CNS tissue was implicated as the major obstacle for nerve regeneration in the CNS (Windle et al., 1952). Importantly, it was recently shown that when dissociated adult rat DRG neurons were injected into adult rat CNS pathway with minimal scarring, the adult CNS white matter can support long-distance regeneration of adult axons in the absence of glial scarring (Davis et al., 1997). In the 1980s, Aguayo and coworkers demonstrated that CNS nerve fibers were indeed able to regenerate, provided they were supplied with a peripheral nerve segment by transplantation (David et al., 1981 and Vidal-Snaz et al., 1987). In addition, Schwann cells alone, when transplanted into the CNS, have been shown recently to provide a microenvironment sufficient to support sprouting and elongation of lesioned CNS nerve fibers (Li et al., 1994a and Paíno et al., 1994). Indeed, application of neurotrophic factors alone may improve the growth capacity of lesioned CNS nerve fibers (Hagg and Varon, 1993). All these findings argue strongly that CNS neurons possess the ability to regenerate, however, a non-permissive environment, including lack of trophic support, glial scar as a physical barrier, and possible growth-inhibitory molecules in the CNS may suppress the ability of CNS neurons to regenerate.

Early experimental evidence for the presence of inhibitory components in the mammalian CNS came from tissue culture experiments carried out by Schwab and coworkers (Schwab and Thoenen, 1985). They studied the difference in neurite outgrowth of neonatal rat

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sympathetic or sensory neurons in co-culture with either sciatic (PNS) or optic (CNS) nerve explants, provided with high concentrations of nerve growth factor (NGF). These neurons grew into sciatic nerve explants, but not into optic nerve explants. These findings were not dependent on living cells within the explant as the results could be reproduced with the tissue explants which were repeatedly frozen and thawed (Schwab and Thoenen, 1985). In addition, Schwab and coworkers showed that differentiated oligodendrocytes were a nonpermissive substrate for neuronal adhesion and neurite outgrowth from dissociated sympathetic or sensory neurons or fetal retinal cells (Schwab and Caroni, 1988). In conclusion, the inhibition of axonal outgrowth in the CNS is likely to be associated with the myelin-forming cells of the CNS, oligodendrocytes.

The neurite growth-inhibitory action of CNS myelin and differentiated oligodendrocytes was further confirmed by a number of studies. Experiments using frozen sections of nervous tissue as a substrate showed that CNS myelin was a very bad substrate for cell attachment, spreading, and neurite outgrowth, in contrast to gray matter areas of the sections (Carbonetto et al, 1987; Savio and Schwab, 1989 and David et al., 1990). Differentiated oligodendrocytes in culture were shown to induce growth cone collapse in a number of studies (Vanselow et al., 1990; and Bandtlow et al., 1990). All these experiments suggested that the presence of myelin-associated inhibitors may contribute to the limited regeneration of adult mammalian CNS following injury.

1.4 Myelin-Associated Neurite Outgrowth Inhibitors

As mentioned above, CNS myelin of higher vertebrates strongly inhibits neurite outgrowth, while gray matter area tested in the same way is a permissive substrate for neurite outgrowth. The CNS myelin was subsequently used for biochemical studies. Myelin proteins were fractionated on SDS-PAGE, and the fractions were tested for their nonpermissiveness

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upon reconstitution into liposomes and adsorption on culture dishes. Two highly inhibitory, minor protein fractions were isolated at 35kD and 250kD, called myelin-associated neurite outgrowth inhibitors of regeneration, termed as NI-35 and NI-250. NI-35 and NI-250 are membrane proteins in CNS myelin, but were not found in PNS myelin (Caroni and Schwab, 1988a). NI-250 appears to be a complex containing NI-35 (Cadelli et al., 1992).

The role of NI-35 and NI-250 was examined by several experimental approaches. First, a monoclonal antibody, called IN-1, was raised against gel-purified NI-250. This monoclonal antibody neutralized the inhibitory activities in tissue culture experiments of NI-250 and NI-35, cultural oligodendrocytes, and of intact CNS myelin (Caroni and Schwab, 1988b). In particular, in the coculture experiments with DRG neurons and oligodendrocytes, the IN-1 antibody successfully prevented the oligodendrocyte-mediated growth cone collapse (Bandtlow et al., 1990). Second, NI-35 incorporated into liposomes caused DRG growth cone collapse (Bandtlow et al., 1993). Third, after application of the IN-1 antibody by implantation of antibody-producing hybridoma cells to lesioned corticospinal tract (CST) fibers, there was elongation over several millimeters, distal to the lesion site. In contrast, no elongation beyond 2mm distal to the lesion site was observed in rats without antibody treatment (Schnell and Schwab, 1990). In addition, regenerated CST fibers were observed at 4.5-6mm distal to the lesion in myelin-free spinal cords where dividing oligodendrocytes were abolished by neonatal x-irradiation, but the CST fibers in normal rats failed to elongate more than 1mm after injury (Savio and Schwab, 1990). Furthermore, recent experiments showed that there was a recovery of specific reflex and locomotor functions after application of IN-1 antibodies to injured spinal cord in adult rats (Bregman et al., 1995). All these experiments indicate that nerve regrowth after injury in the mammalian CNS is greatly enhanced by blocking myelin-associated neurite growth inhibitors.

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Although NI-35 and NI-250 are both recognized by the monoclonal antibody IN-1 and have long been suggested to play a major role in the inhibitory properties of CNS myelin, only limited amount of regeneration was observed (Savio and Schwab, 1989; Schnell and Schwab, 1990), accompanied by partial functional recovery when the IN-1 antibody was applied to a corticospinal cord lesion (Bregman et al., 1995). This suggests that inhibitory molecules, other than the antigens recognized by the IN-1 antibody, may also contribute to the inhibitory effect of CNS myelin. Recently, the myelin-associated glycoprotein (MAG), a member of the immunoglobulin superfamily, has been identified as a potent inhibitor of axonal regeneration (for review, see Filbin, 1996). MAG has been recently reported not only to enhance neurite outgrowth from new-born DRG neurons but also to inhibit neurite outgrowth from adult DRG, all postnatal ages of cerebellar, retinal, spinal, hippocampal and superior cervical ganglion neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996), and a neuroblastoma cell line (NG108-15; McKerracher et al., 1994).

In addition to the myelin-associated inhibitors of axonal regeneration, it has been found that tenascin (Laywell et al., 1992) and chondroitin-sulfate proteoglycans (Bovolenta et al., 1993) accumulate at lesion sites in the CNS. The chondroitin-sulfate proteoglycans purified from injured tissue membrane was capable of preventing neurite outgrowth and causing growth cone collapse in vitro (Bovolenta et al., 1993). Importantly, it was recently demonstrated that the failure of axon regrowth correlated with increased level of proteoglycans within the glial extracellular matrix at the lesion site, whereas successfully regenerating transplants were associated with minimal upregulation of these molecules (Davies et al., 1997).

1.5 Myelin-Associated Glycoprotein: Structure

The myelin-associated glycoprotein (MAG) is a plasma membrane protein of myelin-forming oligodendrocytes in the CNS and Schwann cells in the PNS. MAG was first detected

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by the incorporation of radioactive sugar precursors into glycoproteins that were present in purified CNS myelin fractions (Quarles et al., 1973). The localization of MAG to the periaxonal region in adult myelin suggests that MAG may participate in neuron-glial interactions that lead to myelination (for review, see Quarles et al., 1992).

The independent sequencing of cDNAs for MAG from rat brain in three different laboratories revealed that MAG is a glycoprotein with a single transmembrane domain, a large extracellular region that contains five Ig-like domains and eight potential N-linked glycosylation sites, and one of two possible cytoplasmic domains that contain putative phosphorylation sites (Salzer et al., 1987; Arquint et al., 1987; Lai et al., 1987; for review, see Trapp 1990). The five Ig-like extracellular domains of MAG, are five tandem repeats of internal homology, each approximately 100 amino acids long. The Ig-like domain 2 (D2) to domain 5 (D5) from the N-terminus of MAG contain the cysteine spacing and seven ß-strands typical of C-like domains, but their amino acid sequences are more similar to V-like domains of immunoglobulins. Because of the intermediate nature of these domains, they belong to a subclass of Ig-like domains termed the constant type 2 (C2) (for review, see Salzer et al 1990; Quarles et al., 1992). In contrast, domain 1 (D1) of MAG folds as a V-like domain with nine ß-strands when all other domains fold as C2 domains. The second and third cysteine of D1 form an unusual intrasheet disulfide linkage, and the first cysteine of D1 is at a position to form a interdomain disulfide linkage with cysteine 5 of D2 (Fig. 1.1). The consequence of this conformation is that the tripeptide sequence Arg-Gly-Asp (RGD) is in the interior of D1 with limited accessibility (Pedraza et al., 1990; for review, see Quarles et al., 1992). Even though the tripeptide RGD sequence is an important determinant of the binding site of cell recognition and extracellular matrix molecules that bind to a family of heterodimeric surface receptors, the integrins (Ruoslahti and Pierschbacher, 1987), the steric inaccessibility of the RGD sequence on MAG excludes the possibility that MAG binds to integrins via the RGD sequence (Fig. 1.1).

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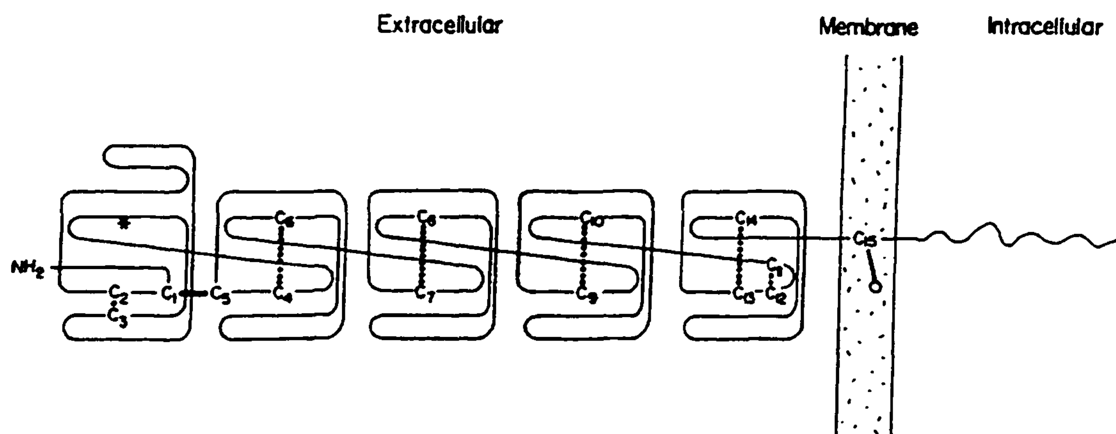

Fig.1.1 Model of the conformation of MAG. The predicted position of the cysteines and their disulfide linkages are indicated including an interdomain disulfide linkage of first and fifth cysteine, and the intrasheet linkage of second and third cysteine. The predicted position of the RGD sequence (*) of MAG within the first domain is also shown (Pedraza et al., 1990).

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Furthermore, structural studies postulated that the first two Ig-like domains form a unit that further folds back over the rest of the molecule (Attia et al., 1993). Consistent with the structural studies, the transmission electron microscopy of a soluble fragment of MAG revealed a rod-like structure for the extracellular domain of MAG with a terminal globular part and two thin parallel arms (Fahrig et al., 1993). The proposed structural model for MAG is that domain 3 and parts of domain 2 and 4 would build up the globular part of MAG, while domain 1 and 5 as well as the parts of the domain 2 and 4 are located in the non-globular arms (Fahrig et al., 1993).

The two developmentally regulated MAG polypeptides large(L)- and small(S)- MAG, which are the result of alternative splicing of the primary MAG transcript, are present in the CNS (Frail and Braun, 1984; Lai et al., 1987; Tropak et al., 1988). The S-MAG and L-MAG isoforms are identical in their extracellular and transmembrane domains but are distinct at their C-terminal ends (Lai et al., 1987; Salzer et al., 1987). L-MAG is the most abundant form at early and active stages of CNS myelination. In the transition from non-compacted to compacted myelin, there is a switch from L-MAG to S-MAG which predominates in the mature CNS (Tropak et al., 1988). In the PNS, S-MAG represents about 95% of the total MAG at all stages of development (Frail et al., 1985; Tropak et al., 1988). The molecular masses of the polypeptides of S-MAG and L-MAG are 67kD and 72kD respectively. However when glycosylated in vivo, these polypeptides co-migrate on SDS-PAGE as a single, broad band of about 100kD. Carbohydrate analysis of rat brain MAG reveals a carbohydrate content of about 30% and a composition characteristic of an N-linked glycoprotein with N-acetylglucosamine, mannose, galactose, fucose, and sialic acid (for review, see Quarles et al., 1992).

More recently, it was noted that the first four Ig-like domains from the N-terminus of MAG share considerable amino acid sequence similarity, 45-50%, with equivalent Ig-like domains of, initially, two other Ig superfamily members CD22 (Stamenkovic and Seed, 1990)

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and sialoadhesin (Crocker et al., 1991). They are both sialic acid binding proteins that bind to their respective receptors with low affinity (Kelm et al., 1994). Therefore, sialoadhesin, CD22 and MAG as well as two other proteins CD33 (Sylvie et al., 1995) and SMP (a protein similar to MAG but found only in avian species; Dulac et al., 1992) form a novel sub-group of Ig-like molecules called the sialoadhesins (for review, see Filbin, 1995). Although sialic acid binding defines a family member, each member binds sialic acid in a specific linkage: CD22 binds to α 2,6-linked sialic acid attached to N-linked glycoconjugates, sialoadhesin recognizes α 2,3-linked sialic acid attached to either N- or O-linked glycoconjugates, MAG binds to α 2,3-linked sialic acid attached to O-linked glycoconjugates (Kelm et al., 1994), and CD33 recognizes the same sialic acid linkage as sialoadhesin, α 2,3-linked sialic acid attached to either N- or O-linked glycoconjugates (Sylvie et al., 1995).

1.6 Myelin-Associated Glycoprotein: Location

MAG is quantitatively a minor constituent of the total protein found in myelin isolated from the CNS (about 1%) and the PNS (about 0.1%). Unlike structural components of compact myelin such as the myelin basic protein and the proteolipid protein, the MAG protein is present in non-compacted myelin membranes. It has been found that MAG locates to the periaxonal myelin membrane (adjacent to the axolemma) of CNS and PNS myelin internodes, as well as to the apposed myelin membranes of the Schmidt-Lanterman incisures, the paranodal loops, and outer mesaxon membranes of PNS myelin (for review, see Salzer et al., 1990; Quarles et al., 1992). The presence of MAG in these locations suggests that MAG may contribute to maintain the architecture of the periaxonal space of myelin.

As mentioned above, MAG is a 100kD integral membrane glycoprotein found in both CNS and PNS myelin. However, in the course of isolating MAG from human white matter for chemical characterization, it was observed that half the amount of MAG is converted to its

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soluble form after 30 min incubation at neutral pH (Sato et al., 1982). The soluble form of MAG contains of the entire extracellular domain with a molecular weight of 90kD, and is termed dMAG (Sato et al., 1982, Stebbins et al., 1997). Further studies indicated that intact MAG is converted to dMAG by a calcium-activated, myelin-associated, neutral protease in the CNS (Sato et al., 1982 and Sato et al., 1984a). In addition, the release of dMAG also occurs in vivo, since examination of human cerebrospinal fluid (CSF) revealed the presence of dMAG, but no intact MAG, in CSF from both normal subjects and patients with demyelinating diseases (Yanagisawa et al., 1985). The presence of dMAG in the CSF of healthy humans suggests that the proteolysis of MAG may be a naturally occurring process in the CNS. Notably, homogenates of white matter from multiple sclerosis (MS) brains show high levels of dMAG, and MAG is lost preferentially at the edges of some MS plaques (Sato et al., 1984b; for review, see Quarles et al., 1992). Thus the high susceptibility of human MAG to conversion to dMAG could be related to human neurological diseases.

1.7 Myelin-Associated Glycoprotein: Function in Myelination

As MAG is one of the earliest myelin proteins to be transported to the tips of the many processes on oligodendrocytes where glial:neuronal contacts will be made during development, it is suggested that MAG plays an early role in the process of myelination (Dubois-Dalcq et al., 1986). More evidence of MAG's role in myelin formation was provided by tissue culture experiments. In myelinating co-cultures of DRG neurons and Schwann cells, MAG was up-regulated on myelinating Schwann cells relative to MAG expression on non-myelinating Schwann cells (Owens and Bunge, 1989). Furthermore, Overexpression of L-MAG in Schwann cells led to a more rapid ensheathment of axons (Owens et al., 1990). In contrast, Schwann cells infected with a recombinant retrovirus expressing MAG antisense RNA failed to form myelin (Owens and Bunge, 1991). Therefore, based on this evidence, it is suggested that MAG most likely plays a critical role in the formation of myelin.

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In apparent contradiction to the studies in vitro, it was shown that mice with a homozygous null mutation for MAG (-/-), termed MAG knockout mice, had essentially normal myelin structure in the PNS (Montag et al., 1994; Li et al., 1994b). A possible explanation for the apparent contradiction on the requirement for MAG in myelinating co-cultures but not in intact animals is that another molecule must compensate for the absence of MAG in MAG knockout mice, and this compensation can be effected only in the whole animals, not in isolated cells in culture. Indeed, in the PNS of MAG knockout mice, NCAM was up-regulated and located to the periaxonal regions normally occupied by MAG (Montag et al., 1994). However, it was recently found that PNS myelin was formed normally in double mutants deficient in both MAG and NCAM (Carenini et al., 1997). It is suggested that, in the double mutant, the putative compensator NCAM itself is replaced by other molecules. In other words, a high degree of flexibility in molecular compensation may be present in Schwann cells during myelin formation (see discussion in Carenini et al., 1997).

Although myelin formation in PNS was not affected in MAG knockout mice and double mutants deficient in both MAG and NCAM, the organization of the periaxonal region in the CNS myelin of MAG knockout mice was impaired (Montag et al., 1994; Li et al., 1994b). Compared to normal mice, significant more CNS nerve fibers of MAG knockout mice lost their cytoplasmic collars, had swollen collars or redundant myelin (Li et al., 1994b), and the onset of CNS myelination was delayed (Montag et al., 1994). Interestingly, similar abnormalities in periaxonal region were reported for the PNS of the quaking mouse, which shows an altered expression of myelin protein genes including MAG (Trapp et al., 1984). Thus, MAG may not be essential for myelin formation, but is necessary for formation and maintenance of the cytoplasmic collar and periaxonal space of myelinated fibers.

More importantly, Fruttiger et al. (1995a) reported that MAG knockout mice older than 8 months displayed significant myelin abnormalities and axonal degeneration in the PNS. In

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addition, it was recently found that, in adult MAG knockout mice, there was a significant increased number of unmyelinated axons of optic nerves compared to age-matched wild-type mice (Bartsch et al., 1997; Li et al., 1998), and the absence of MAG caused reduced axonal calibers for optic and sciatic nerves (Bartsch et al., 1997; Yin et al., 1998). Therefore, MAG appears to be redundant for the formation of myelin sheaths, but is essential for the long-term maintenance of the integrity of both myelin and axons.

Recently, Carenini et al. (1997) showed that PNS myelin and axon degeneration occurred significantly earlier in the double mutants deficient in both MAG and NCAM than in MAG knockout mice. It is suggested that in MAG knockout mice, although N-CAM does not compensate for MAG in myelin formation, it partially substitutes MAG in the maintenance of axon-myelin integrity (Carenini, et al., 1997).

In addition, another kind of MAG mutant mice, termed L-MAG mutant mice, was recently generated (Fujita, et al., 1998). L-MAG mutant mice express a truncated from of L-MAG and the wild-type S-MAG. The CNS myelin in L-MAG mutant mice displays most of the pathological abnormalities reported for the MAG knockout mice, however, in contrast to the MAG knockout mice, PNS axons and myelin of adult L-MAG mutant animals do not degenerate. Thus, these results suggest that S-MAG expression is sufficient to maintain PNS integrity (Fujita, et al., 1998).

1.8 Myelin-Associated Glycoprotein: Function in Axonal Regeneration

Besides its role in myelination, it has been recently shown that MAG is a bifunctional molecule that influences neurite outgrowth temporally and spatially, to either promote or inhibit depending on the type and age of neuron (for review, see Filbin, 1996). For example, fibroblast cells transfected to express MAG enhanced DRG neurite outgrowth from 1-day-old

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(PND1) mice (Johnson et al., 1989). Recently, it was demonstrated that recombinant MAG expressed by transfected Chinese hamster ovary (CHO) cells not only promoted neurite outgrowth from cultured neonatal DRG neurons, but also inhibited neurite outgrowth from adult DRG neurons (Mukhopadhyay et al., 1994).

Importantly, MAG was reported to inhibit neurite outgrowth from adult DRG, cerebellar, retinal, spinal, hippocampal and superior cervical ganglion neurons at all postnatal ages (Mukhopadhyay et al., 1994; DeBellard et al., 1996). Furthermore, it was shown that fractionation of solublized bovine CNS myelin by DEAE chromatography yielded at least two peaks of inhibitory activity for the extension of axons from neuroblastoma cells, NG108-15. The first major peak of inhibitory activity corresponded to the elution profile of MAG, and immunodepletion of MAG from the column eluant removed the inhibitory effect of the first peak. Immunodepletion of MAG from total extracts of CNS myelin restored neurite outgrowth up to 63% of control level, neurite outgrowth on polylysine (McKerracher et al., 1994). Importantly, about 60% of axonal growth cones of postnatal day 1 hippocampal neurons collapsed when they encountered polystyrene beads coated with recombinant MAG (rMAG). In contrast, only 7% of the growth cones encountering beads coated with denatured rMAG collapsed (Li et al., 1996). All these results suggest strongly that MAG is a potent inhibitor for axonal regeneration in CNS myelin.

Although the studies in culture indicate that MAG is a potent inhibitor for axonal regeneration, the role of MAG on axonal regeneration in vivo has yet to be resolved. Neurite outgrowth after injury in the MAG knockout mice was compared to that in normal mice, but conflicting results have been reported by different research groups (Bartsch et al., 1995; Ng et al., 1996; and Li et al., 1996). One group found that after complete transection of the corticospinal tract, there was an increase in the number of axons regenerated and an improvement in the length of regenerating axons in MAG knockout mice (Li et al., 1996). For

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example, some axons in MAG knockout mice extended even up to 13.2mm past the lesion site. In contrast, no axons grew more than 2mm distal to the injury site in normal mice (Li et al., 1996). However, another group reported that regeneration of lesioned optic and corticospinal fibers in MAG knockout mice was not significantly different from that in control mice (Bartsch et al., 1995).

In addition, the neurite outgrowth on purified CNS myelin from MAG knockout and normal mice has been compared by two research groups (Ng et al., 1996; and Li et al., 1996). Although Ng et al. (1996) showed that CNS myelin from MAG knockout mice exerted an inhibitory effect for neurite outgrowth from NG108-15 cells similar to that found for CNS myelin from normal mice, Li et al. (1996) observed an improvement in average neurite length from NG108-15 cells grown on CNS myelin from MAG knockout mice. However, the difference in neurite length from NG108-15 cells on CNS myelin from normal and MAG knockout mice is statistically insignificant because of tremendous variation from one experiment to another (Li et al., 1996). This high variability in results highlights the problems in using purified myelin as a substrate. As we mentioned previously, in the course of myelin purification, it was observed that half the amount of MAG is converted to its soluble form, dMAG, after 30 min incubation (Sato et al., 1982). Thus, for purified myelin used as a substrate in neurite outgrowth assays, if myelin is freshly prepared, the MAG content is high in myelin, in contrast, if the myelin is stored and washed before use, the MAG may be lost as dMAG is washed away.

Furthermore, Li et al. (1996) found that although the difference in neurite outgrowth on myelin purified from MAG knockout and normal mice is insignificant, differences were observed in the fractionated myelin from MAG knockout mice. The major inhibitory peak that is associated with MAG in normal mice was significantly reduced in MAG knockout mice.

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Therefore, it is suggested that the inhibitory effect on neurite regrowth in MAG knockout mice may due to the presence of other non-MAG inhibitors (Li et al., 1996).

However, it was recently found that disruption of the gene for MAG improved axonal regeneration in the PNS of C57BL/Ola mice (Schäfer et al., 1996). As mentioned above, in C57BL/Ola mice, axonal regeneration was poor along persisting PNS myelin sheaths, vigorous axonal regrowth could only be detected along Schwann cells in which myelin was degraded (Fruttiger et al., 1995b). But in the MAG-deficient C57BL/Ola double mutant mice, significantly more axons grew in association with persisting PNS myelin sheaths than in C57BL/Ola mice. This argued strongly in favor of MAG being a true inhibitory molecule which has to be removed or down-regulated to facilitate axonal regrowth from lesioned PNS nerves (Schäfer et al., 1996).

1.9 The Goals of My Work

Although it has been shown that MAG expressed on CHO cell's surface inhibits axonal regeneration from adult DRG neurons and the cerebellar neurons at all ages, the mechanism for this inhibition is unknown. In addition, it has been reported that MAG is subjected to specific proteolytic cleavage which breaks it down to a smaller derivative (dMAG). dMAG is soluble and quite stable and is present in the human cerebrospinal fluid of normal human subjects and patients with demyelinating diseases (Sato et al., 1982; Yanagisawa et al., 1985). However, the possible effect of dMAG on neurite regrowth has not been studied. To begin to address these questions, I will investigate the interaction between MAG and neurons, find out the possible effect of soluble MAG on neurite outgrowth, and finally map the possible epitopes on MAG which are responsible for MAG-neuronal interactions:

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1. A chimeric, soluble MAG was made by fusing the extracellular region of MAG to the Fc region of human Fc, termed MAG-Fc. The MAG-Fc will be used in binding assays to investigate the interaction between MAG and neurons (Chapter III).

2. The inhibitory effect of MAG-Fc on neurite outgrowth from cerebellar neurons will be studied by an in vitro neurite outgrowth assay (Chapter IV).

3. Soluble MAG found in vivo (dMAG) will be tested for its inhibitory effect in our in vitro neurite outgrowth assay. In addition, we will assess if dMAG is released after spinal cord injury (Chapter V).

4. By site-directed mutagenesis, the sialic acid binding site on MAG will be identified and the effect of mutating this site on the function of MAG will be investigated (Chapter VI).

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Chapter II

Methods and Materials

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2.1 Isolation of Neurons

To isolate cerebellar, spinal, retinal, and hippocampal neurons, the cerebella, spinal cords, retina and hippocampus were removed from two to five rat pups up to postnatal day (PND) 7 (DeBellard et al., 1996). Like tissue was combined and placed in 10ml of 0.025% trypsin in PBS, gently triturated, and incubated for 10min at 37°C. For dorsal root ganglion (DRG) and superior cervical ganglion (SCG) neurons, ganglia were removed from rat pups up to PND7 and incubated in 5ml of L15 media containing 0.025% trypsin and 0.3% collagenase type I (Worthington) for 30min at 37°C. The ganglia were triturated with a fine polished Pasteur pipette. Trypsinization was stopped by adding 10ml DMEM containing 10% FCS, and the cells were centrifuged at 1000rpm at 4°C for 5min. For the neurons being used in binding assays, the isolated neurons were washed twice in PBS, and then resuspended to a single-cell suspension. For the cerebellar and DRG neurons being used in neurite outgrowth assay, the isolated neurons were washed once in DMEM, and then resuspended to a single-cell suspension in Sato medium (Doherty et al., 1990), without FCS for neurite outgrowth on cell monolayers, or with 2% FCS for neurite outgrowth on immobilized Fc-chimeras and other growth-promoting substrates. Cells were counted by a Coulter counter (Coulter Electronics).

2.2 Fixation and Desialylation of Cerebellar and DRG Neurons

Glutaraldehyde fixation allows in advance preparation of neuronal cells and harsh treatment condition that may not be tolerated by viable cells. In fixation, 1-5x10^7 fresh neuronal cells in PBS were incubated in 0.25% glutaraldehyde solution (Sigma) for 10min at room temperature and then washed 3 times with PBS/1%BSA buffer. For desialylation, 1-5x10^7 isolated neurons in PBS (either fresh or fixed, used for binding assays) or in DMEM (used for neurite outgrowth assays) were treated with 100mU/ml Vibrio cholerae sialidase, termed VCS (Behringwerke/Calbiochem) for 3hrs at 37°C in a final volume of 0.5ml, and then washed 3 times with PBS/0.25%BSA buffer, or DMEM. The desialylated neurons will be resuspended in PBS for binding assays or in Sato medium for neurite outgrowth assays.

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2.3 Preparation of Fc-Chimeras

The pIG plasmid (Simmons, 1993) containing the cDNA for Fc-chimeras was constructed by fusing the extracellular domain of MAG, MUC18, Sialoadhesin or L1 to the Fc region of human IgG (Kelm et al., 1994; Doherty et al., 1995). To obtain Fc-chimeras of the mutated MAG proteins, a fragment containing the mutated nucleotides was cut from R118-mutated MAG cDNA in pBluescript and subcloned into the pIG1 plasmid which already harbored the extracellular domain of MAG (Tang et al.,. 1997b). The plasmids for Fc-chimeras were transiently transfected into COS-1 cells with DEAE-dextran (Kelm et al., 1994). 70%-80% confluent COS-1 cells were covered by the transfection solution with the following concentrations: 100ng Fc-chimera plasmids per 1.0×10^5 cells, 250μg/ml DEAE-dextran, and 100μM chloroquine diphosphate in 10mM HEPES-buffered DMEM. The COS-1 cells were incubated in transfection solution at 37°C until the cells began to look vacuolated. The transfected COS-1 cells were osmotically shocked by PBS/10%DMSO for 2min, washed twice with HEPES-buffered DMEM, incubated in DMEM with 1% IgG-depleted FCS for 8-10 days.

The chimeric proteins were purified by affinity chromatography using protein A Sepharose following transient expression of recombinant plasmid DNA in COS-1 cells. The media of transfected COS-1 cells were incubated with 5ml protein A Sepharose slurry overnight at 4°C. The bound chimeric proteins were eluted from protein A Sepharose by 5ml 100mM glycine solution at pH 3.0, then neutralized with 0.5ml 1M Tris buffer at pH 8.0. The eluted proteins were concentrated to about 1-2mg/ml by Centricon-30 microcentrators (Amicon) via repeating centrifugation, sterilized by filtration with sterile Ultrafree-MC filter units (Millipore). Protein concentration was estimated using a Bio-Rad kit.

2.4 Preparation of Myelin- and Gray Matter-Conditioned Media

Myelin from adult rats was purified as described previously (Norton and Poduslo, 1973). After osmotic shocking with sterile water, the myelin membranes were centrifuged at

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40,000g for 1hr at 4°C, and resuspended in Sato medium at the final concentration of 15-20mg/ml. Gray-matter (GM) from two week old rat pups was homogenized in sterile water, centrifuged and resuspended in Sato medium to a final concentration of 15-20mg/ml. The myelin and GM membrane preparations were aliquoted and incubated at room temperature for 1, 4, 7 and 18hrs. After incubation, the aliquots were spun at 100,000g for 1hr at 4°C. The supernatant from each aliquot was collected, termed as myelin-conditioned medium or GM-conditioned medium respectively. The protein concentrations of myelin-conditioned and GM-conditioned media were measured by using a Bio-Rad kit. The samples of myelin- and GM-conditioned media were loaded onto SDS-PAGE gel, transferred to Western blotting membrane, immunostained for MAG with chemiluminescence detection system (KPL) as previously described (Mukhopadhay et al., 1994). An aliquot of the myelin-conditioned medium was subjected to immunodepletion of MAG.

2.5 Immunodepletion of dMAG from Myelin-Conditioned Medium

For each milliliter of myelin-conditioned medium, 100μl of the 513 MAG monoclonal antibody (Boehringer-Mannheim) was added and incubated overnight with rocking at 4°C. After incubation, 50μl of 10% wt/vol Protein A-Sepharose and 50μl Protein G-Agarose slurry (Pharmacia) were added and incubated 3hrs at room temperature. Then the medium was centrifuged to remove the Protein A-Sepharose and Protein G-Agarose beads. The collected supernatant was dMAG-depleted medium, which was used in the neurite outgrowth assays as described below. The beads were washed, the bound protein was released and denatured by boiling in 2% SDS sample buffer. The dMAG-depleted medium and protein released from the beads were subjected to SDS-PAGE gel and Western blotting described as above.

2.6 Release of dMAG from Injured Spinal Cords

The spinal cords, each about 40mm long, from six one-month old rats, were removed and placed in Sato media. Three spinal cords were uncrushed and used as the control. The

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other three spinal cords were damaged by crushing at a number of points along its length. Both crushed and uncrushed spinal cords were incubated overnight at room temperature in Sato media. After incubation, crushed and uncrushed spinal cords were homogenized and spun down at 20,000g for 1hr at 4°C. The supernatants were collected, concentrated in Centricon-30 microcentrators (Amicon), filtered with Ultrafree-MC filter units (Millipore). Protein concentration was estimated using a Bio-Rad kit. The supernatants were subjected to SDS-PAGE and Western blotting for detecting the presence of dMAG described as above.

2.7 An ELISA Assay for dMAG

A 96-well ELISA plate (Dynatech) was coated with the 513 MAG monoclonal antibody at 10μg/ml for 2hrs at room temperature. After washing with PBS/0.25%BSA, the plate was added with MAG-Fc and MUC18-Fc in PBS with 5% skim milk at various concentrations, and myelin- and spinal cord-conditioned media at different dilutions. The plate was then incubated overnight at 4°C. After incubation, the plate was washed with PBS/0.25% BSA, blocked for 30min with 5% skim milk in PBS and fixed with 4% paraformaldehyde for 30min at room temperature. After washing, the plate was incubated for 2hrs at room temperature with a polyclonal MAG antibody developed from rabbit (a gift provided by Dr. J. L. Salzer) at the dilution of 1:10,000 in PBS/0.25%BSA, blocked for 30min with 5% skim milk in PBS and further incubated with peroxidase-conjugated anti-rabbit IgG at a dilution of 1:15,000. Color was developed with 4% wt/vol O-phenylenediamine and 0.2% H_2O_2 in citrate buffer, and measured in an ELISA reader at the wavelengths of 490nm and 630nm. The absorbance values from MAG-Fc at different concentrations were used as the standard to estimate the concentrations of dMAG in myelin-conditioned and spinal cord-conditioned media.

2.8 Isolation of dMAG from Myelin-Conditioned Medium by Gel Filtration

A HR 16/50 Superose 12 FPLC column was washed with PBS and calibrated with Molecular Weight Marker Standards (BioRad). The myelin-conditioned medium was dialyzed

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overnight with PBS, concentrated in Centricon-30 microcentrators (Amicon), and filtered with Ultrafree-MC filter units (Millipore). Protein concentration was estimated using a Bio-Rad kit. Then the myelin-conditioned medium was loaded onto the Superose 12 column and eluted with PBS. The proteins in the myelin-conditioned medium were separated based on their molecular weights. The eluted fractions were collected, concentrated in microcentrators, and subjected to SDS-PAGE and Western blotting for detecting the presence of dMAG described as above.

2.9 Soluble Binding Assay with Fc-Chimeras

The purified MAG-Fc molecules were iodinated with Iodogen (PIERCE). I^{125}-MAG-Fc at 0.4μg/ml was incubated for 1hr at 4°C with various concentrations of anti-Fc (Sigma), 1μg/ml-40μg/ml, on a 96-well microtiter plate (Dynatech), then 1.5×10^5 either fresh or glutaraldehyde-fixed cerebellar or DRG neurons were added. After 1hr incubation at 4°C, the neuronal cells were washed with PBS/1%BSA by repeating centrifugation and suspension. The bound radioactivity was measured by scintillation counter. Where indicated, the 513 MAG monoclonal antibody (Boehringer-Mannheim) was preincubated with MAG-Fc aggregates for one hour at a concentration of 1μg/ml or neurons were desialylated before being used.

In addition, it was shown that MAG-Fc bound to cerebellar neurons in suspension without forming immune complexes (Tang et al., 1997a). 25μg/ml MAG-Fc or the control Fc-chimera, MUC18-Fc was incubated with isolated cerebellar neurons, 6.0×10^5 in PBS for 1hr at 4°C. After incubation, the cells were washed with PBS/1%BSA. Then the cells were incubated with Cy3-conjugated anti-Fc (Sigma) at 25μg/ml for 1hr at 4°C, washed with PBS with 1%BSA, and viewed under a fluorescent microscope and photographed.

2.10 Solid-phase Binding Assay with Immobilized Fc-Chimeras

In order to maximize the chances of correct orientation of Fc-chimera, 15μg/ml anti-Fc was first coated onto an Immulon-3 96–well ELISA plate (Dynatech) for 2hrs at 37°C in 0.1M bicarbonate buffer. MAG-Fc and the control Fc-chimera, MUC18-Fc, with increasing

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concentrations, 0.01μg/ml-50μg/ml, were absorbed for 2hrs at 37°C to the coated plate. Prior to the binding assay, isolated neurons were vitally labeled with fluorescent dye, 10μM calcein AM (Molecular Probe) for 15min at 37°C, then washed and resuspended in PBS. Where indicated, the 513 MAG monoclonal antibody (Boehringer-Mannheim) was incubated with MAG-Fc for 2hrs at a concentration of 10μg/ml or neurons were desialylated before being used. Then the fluorescent-stained neurons ($1\text{-}3 \times 10^5$/well) were added into each well and incubated for 1hr at room temperature. The plate was washed 2 to 4 times with PBS/0.25%BSA applied to each well under gravity. Bound cells were measured by FluorImager (Molecular Dynamics). In addition, other soluble Fc-chimeras such as sialoadhesin-Fc, R118-mutated MAG-Fc or MAG(d1-3)-Fc were tested for binding to cerebellar neurons in the same way at the concentration 10μg/ml.

2.11 Binding of MAG-Fc to Cell Monolayers and Immobilized Proteins

As a positive control, anti-Fc was coated as a substrate at 15μg/ml on an Immulon-3 96–well ELISA plate (Dynatech) by incubating for 2hrs at 37°C in 0.1M bicarbonate buffer. After washing, MAG-Fc was added at various concentrations, from 0.5-25μg/ml, and incubated for another 2hrs at 37°C. Unbound MAG-Fc was washed away with PBS/0.25%BSA, bound MAG-Fc was quantitated in an ELISA. Similarly, binding of MAG-Fc to cell monolayers and immobilized proteins was determined by adding MAG-Fc at 25μg/ml, which is the maximum concentration of anti-Fc binding and is the concentration used in our neurite outgrowth assays, to confluent cell monolayers or immobilized proteins at the concentrations used in the neurite outgrowth assays. The ELISA plate was incubated for 2hrs at 37°C, following by washing with HEPES-buffered DMEM or PBS/0.25% BSA. The bound MAG-Fc was quantitated in an ELISA. For the ELISA used here, the plate was blocked with 3% normal goat serum for 1hr at 37°C, then incubated with the 513 MAG monoclonal antibody (Boehringer-Mannheim) at a concentration of 1μg/ml for 2hrs at 37°C. After washing, the plate was further incubated with peroxidase-conjugated anti-mouse IgG at a

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dilution of 1:1500. Color was developed with 4% wt/vol O-phenylenediamine and 0.02% H_2O_2 in citrate buffer and read in an ELISA reader at the wavelengths of 490nm and 630nm.

2.12 Binding of Human Erythrocytes to CHO Cells and Schwann Cells

A 96-well flat-bottomed tissue culture ELISA plate (Falcon) was coated with poly-L-lysine at 16.6μg/ml by incubating 1hr at room temperature. After washing, the plate was further incubated with fibronectin at 10μg/ml for 1hr at 37°C. Confluent cell monolayers of CHO or Schwann cells expressing wild-type or R118-mutated MAG, or control cells were established by incubating 2.0×10^4 cells/well overnight at 37°C. The cell monolayers were treated for 1hr with 200mU/well VCS at 37°C in DMEM. At the same time, an aliquot of human erythrocytes can be desialylated by incubating the cells with 100mU/ml VCS at 37°C in PBS for 1 hr. Finally, after washing both desialylated or untreated human erythrocytes and the cell monolayers with DMEM, 2.0×10^4 human erythrocytes/well were added onto the cell monolayers and incubated for 1 hr at room temperature. Unbound human erythrocytes were washed away by DMEM and the bound cells were examined under a phase-contrast microscope and photographed.

2.13 Neurite Outgrowth on Immobilized Substrates

This assay was developed based on MAG-Fc soluble binding assay and solid-phase binding assay, and used to show that MAG-Fc inhibits neurite outgrowth from cerebellar neurons grown on different outgrowth-promoting substrates (Tang et al, 1997a, b). An 8-chamber slide (Lab-Tek) was first coated with 16.6μg/ml poly-L-lysine (Sigma) in sterile water for 1hr at room temperature. After washing twice with DMEM, the poly-L-lysine coated wells were either ready to use for the neurite outgrowth assay or incubated with 10μg/ml fibronectin or 5μg/ml laminin for 2hrs at 37°C. Excess fibronectin and laminin were washed away by DMEM, and the fibronectin or laminin coated wells were ready to use. For coating wells with L1-Fc, the poly-L-lysine coated wells were washed twice with 0.1M bicarbonate buffer, then

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incubated with anti-Fc 15μg/ml in 0.1M bicarbonate buffer for 2hrs at 37°C. After washing three times with DMEM, L1-Fc was immobilized onto anti-Fc by incubating 30μg/ml L1-Fc in the wells for two more hours at 37°C. Unbound L1-Fc was washed off with DMEM.

For neurite outgrowth assays with Fc-chimeras, the cerebellar single-cell suspension in Sato medium with 2% FCS was mixed with MAG-Fc or MUC18-Fc at the various concentrations, from 1μg/ml to 30μg/ml. Similarly, other soluble Fc-chimeras such as sialoadhesin-Fc, R118-mutated MAG-Fc and MAG(d1-3)-Fc were added into cerebellar neuron suspension in the same way at the final concentration 25μg/ml. For the neurite outgrowth assay with myelin-conditioned media, the isolated cerebellar neurons were suspended in myelin-conditioned media or GM-conditioned media as described above with 2% FCS. Where indicated, the 513 MAG monoclonal antibody was preincubated with MAG-Fc for 1hr at a concentration of 20μg/ml. When desialylated cerebellar neurons were used, the neurons were desialylated as described above, and sialidase was included in the culture at a concentration of 25μU/ml. Finally, 4.0-6.0x10^4 isolated cerebellar neurons were added into each well, and incubated for 16-18 hours at 37°C.

The neurites and neurons were fixed for 30min with 4% paraformaldehyde, and permeabilized with ice cold methanol for 2min. The cells were then blocked for 30min with DMEM containing 10% FCS and incubated for 4hrs at room temperature with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins). The cells were washed 3 times with PBS containing 0.5mg/ml BSA and incubated for 30min with a biotinylated donkey anti-rabbit Ig (1:300, Amersham) , then washed 3 times and incubated for 45min with streptavidin-conjugated Texas Red (1:300, Amersham). After 3 more washes, the neurite outgrowth slides were mounted in Permfluor (Baxer) and viewed with a Zeiss fluorescent microscope. The length of the longest neurite for each GAP-43 positive neuron was measured by using an Oncor image analysis system.

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2.14 Neurite Outgrowth on Cell Monolayers

An 8-chamber slide was coated with poly-L-lysine described as above. The coated slide was incubated with fibronectin at 10μg/ml for 2 hours at 37°C. Confluent cell monolayers were established by plating $4.0\text{-}6.0 \times 10^4$ cells overnight at 37°C. The cells used for the monolayers were 3T3 cells or transfected 3T3 cells expressing NCAM, L1 or N-cadherin, Schwann cells or transfected Schwann cells expressing wild-type or R118-mutated MAG. Then a coculture was established by adding 6.0×10^3 cerebellar neurons onto the cell monolayers in Sato medium without FCS. Where indicated, a soluble chimeric protein, MAG-Fc, MUC18-Fc, sialoadhesin-Fc, MAG(d1-3)-Fc or R118-mutated MAG-Fc was added into a coculture at the final concentration 25μg/ml on the cell monolayers of untransfected 3T3 cells or Schwann cells. Finally, the coculture was incubated for 16hrs at 37°C. After incubation, the neurons were fixed and stained with GAP43 described as above.

2.15 Expression of Mutated MAG by Transfected Schwann Cells

A spontaneously transformed Schwann cell line that does not express MAG (kindly provided by Dr. J. L. Salzer) was used for the transfection. The pSJL plasmids containing the cDNAs for L-MAG, R118D- and R118A-mutated forms of MAG, and the control, MAG in 3'-5' orientation were transfected into this Schwann cell line by using Lipofectin Reagent (GibcoBRL). The transfected cells were selected in 200μg/ml G418. Colonies were combined and the cells were stained for MAG by incubating with the 513 MAG monoclonal antibody at 1μg/ml for 1hr at room temperature. After washing with PBS, the cells were incubated with the second antibody, phycoprobe-conjugated anti-mouse IgG at the dilution of 1:100 for 30min at 37°C, then washed and resuspended in DMEM. The cells expressing high level of MAG were selected by FACS, and further expanded (Tang et al.,. 1997b). The expression of MAG by the transfected Schwann cells were characterized by Western blotting and ELISA as described previously (Mukhopadhay et al., 1994; Tang et al.,. 1997a).

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2.16 Mutation of R118 in MAG

The Chameleon double-stranded site-directed mutagenesis kit (Stratagene) was used to mutate the nucleotide sequence coding for R118 in L-MAG to either an alanine or an aspartate as described by the manufacturer. L-MAG in the antisense orientation in pBluescript (Stratagene) was used for the initial annealing step. The mutations were confirmed by dideoxy sequencing. The R118D- and R118A-mutated forms of MAG were then sub-cloned into the pSJL plasmid for expression in CHO and Schwann cells. To obtain Fc-chimeras of the mutated MAG proteins, a XhoI restriction fragment which cuts MAG cDNA at nucleotides 182 and at 1627, and which contains the mutated nucleotides, was cut and purified from the R118-mutated MAG cDNAs in pBS and subcloned into the pIG1 vector already harboring the extracellular domain of MAG.

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Chapter III

Characterization of Binding of MAG to Neurons

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3.1 Introduction

As mentioned before, it has been shown that MAG plays a role in the formation and maintenance of the integrity of both myelin and axon, and is a potent inhibitor of axonal regeneration. The functions proposed for MAG suggest that it is an adhesive cell surface constituent of myelinating glial cells. To investigate whether MAG is involved in cell adhesion between glial cells and neurons, adhesion of a single cell suspension of cerebellar neurons to monolayers of oligodendrocytes was measured in the absence and presence of Fab fragments of a monoclonal MAG antibody. The result showed that Fab fragments of the monoclonal antibody against MAG blocked adhesion between oligodendrocytes and neurons (Poltorak et al., 1987). To investigate further whether MAG itself is the binding ligand, MAG-containing liposomes were added onto monolayers of cerebellar, spinal cord and dorsal root ganglion neurons. Under these conditions, binding of MAG-containing liposomes to neuronal cell bodies and neurites was observed. This binding was inhibited by Fab fragments of mono- and ploycIonal MAG antibodies which recognize the extracellular portion of MAG (Poltorak et al., 1987; Johnson et al., 1989; Sadoul et al., 1990). These experiments demonstrated that MAG itself is a binding ligand, mediating adhesive interactions between neurons and oligodendrocytes.

MAG does not appear to be involved in homophilic binding since no binding of MAG-containing liposomes to MAG-expressing cells could be measured (Sadoul et al., 1990), and soluble MAG did not bind to MAG plated as a substrate (Fahrig et al., 1987). The fact that MAG-containing liposomes bind to neurons that are MAG-negative shows that MAG must be involved in a heterophilic interaction. However, the heterophilic interaction is not mediated by L1 or N-CAM which are expressed on the surface of neurons, as antibodies to L1 and N-CAM did not influence the binding of MAG-containing liposomes to neurons (Sadoul et al., 1990), and no binding was observed for soluble MAG to L1 or N-CAM (Fahrig et al., 1987).

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In addition, it was also found that during extracellular matrix formation, MAG associated with extracellular matrix constituents such as collagen type I (Fahrig et al., 1987; Probstmeier et al., 1992; Bachmann et al., 1995). However, binding of soluble or liposome-incorporated MAG to laminin or fibronectin was never detected (Fahrig et al., 1987; Sadoul et al., 1990).

It is clear from these studies that MAG is an adhesive, cell surface constituent of myelinating glial cells, but the mechanism for MAG mediated glial:axonal interaction has not been determined. As Arg-Gly-Asp (RGD)-containing peptides did not inhibit binding of MAG-containing liposomes to neurons (Sadoul et al., 1990), the previous assessment that the steric inaccessibility of the RGD sequence on MAG excludes the possibility that MAG binds to integrins via the RGD sequence, is strengthened. In addition, although MAG has the L2/HNK-1 carbohydrate epitope (McGarry et al., 1983; Noronha et al., 1986; Pedraza et al., 1995) and this epitope appears to be involved in adhesion on other molecules (Künemund et al., 1988), the L2/HNK-1 carbohydrate epitope on MAG does not mediate the binding of MAG to neurons since antibodies to the L2/HNK-1 epitope did not influence the binding of MAG-containing liposomes to neurons (Sadoul et al., 1990). Furthermore, only about 30% of all MAG molecules isolated from adult mouse brain carry the L2/HNK-1 epitope (Poltorak et al., 1987), and there is substantial species variability for MAG with regard to the expression of this epitope (O'Shannessy et al., 1985). It is implied that the L2/HNK-1 carbohydrate epitope does not mediate the adhesion of MAG in glial:axonal interaction.

Recently, some light was shed on the possible mechanism of MAG mediated glial:axonal interaction, because MAG was shown to belong to a subgroup of the Ig superfamily, termed the sialoadhesins (Kelm et al., 1994). All members of the sialoadhesins have significant amino acid sequence similarity among their first four Ig-like domains, and all bind to sialic acid. The first two family members described were sialoadhesin (Crocker et al.,

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1991) and CD22 (Stamenkovic and Seed, 1990). Sialoadhesin is an adhesion molecule restricted to macrophages (van den Berg et al., 1992). When expressed on macrophages or following purification, sialoadhesin was shown to bind glycoproteins and glycolipids in a sialic acid-dependent manner (Crocker et al., 1991; van den Berg et al., 1992). CD22 is a B-cell specific adhesion molecule (Stamenkovic and Seed, 1990). When one isoform of CD22, CD22β was transfected into COS cells, it is able to mediate sialic acid-dependent adhesion of lymphocytes (Stamenkovic et al., 1991). As the first four Ig-like domains from the N-terminus of MAG shares 45-50% amino acid sequence similarity with equivalent Ig-like domains of sialoadhesin and CD22, it raises an important question of whether MAG is also a sialic acid-binding protein. Using a soluble binding assay and a solid-phase binding assay, we have demonstrated that MAG is indeed a sialic acid-dependent adhesion molecule (Kelm et al., 1994; Tang et al., 1995; DeBellard et al.; 1996).

3.2 Results

3.2.1 Purification of MAG-Fc Chimera

To characterize the specific binding of MAG to neurons, a soluble chimeric form of MAG, termed MAG-Fc, was used. As shown in Fig. 3.1, the recombinant MAG-Fc chimera was made by fusing the extracellular domain (ECD) of MAG to the Fc region of human IgG (Hinge-CH2-CH3) (Kelm et al., 1994).

The MAG-Fc construct, contained in the pIG1 plasmid, was transiently expressed in COS-1 cells after DEAE-dextran transfection (Simmons 1993). On translation, the recombinant plasmid produced a chimeric protein, MAG-Fc, with the MAG extracellular domain fused to the N-terminus of human IgG Fc (Fig. 3.2). Similar to immunoglobulins,

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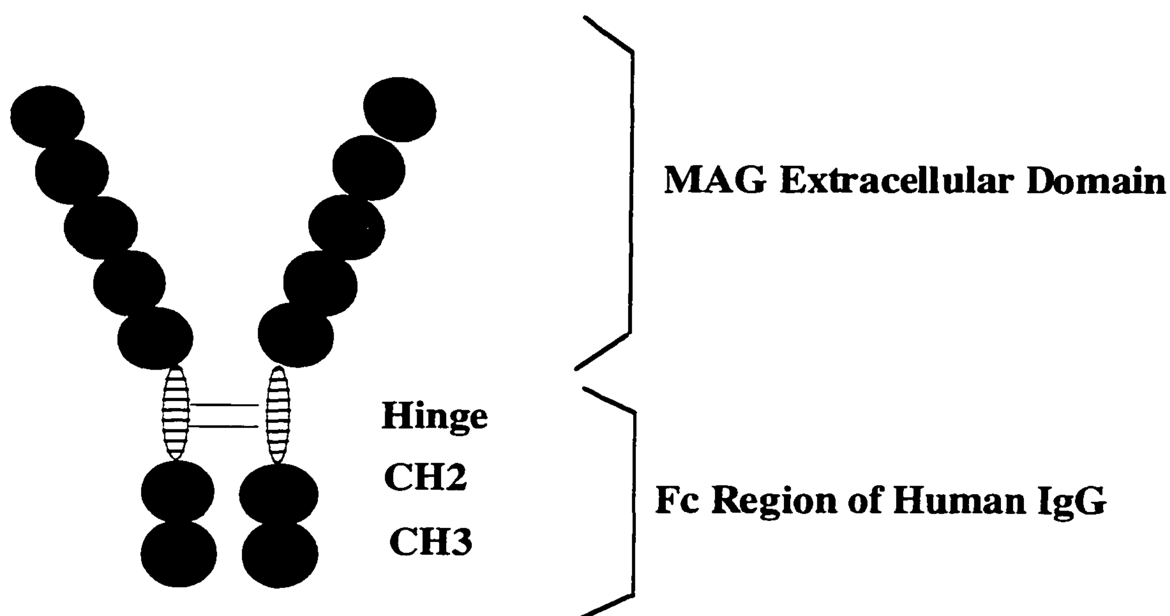

Fig. 3.1 Schematic diagram showing MAG-Fc. MAG-Fc consists of the extracellular domain of MAG fused to the Fc region of human IgG (Hinge-CH2-CH3). Two disulfide bonds are present within the hinge of the Fc-region, resulting in covalent dimerization of MAG-Fc protein.

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two disulfide bonds are present within the hinge of the Fc region, resulting in covalent dimerization of MAG-Fc protein (Fig. 3.1).

The MAG-Fc protein was purified by affinity chromatography (Simmons, 1993). Protein A sepharose, which binds to the Fc domain of IgG, was incubated with media from transfected cells. Bound MAG-Fc was eluted from the protein A sepharose column with a low pH glycine solution and then concentrated by Centricon-30 microcentrators (Fig. 3.2). The yield of MAG-Fc is routinely about 4mg/liter. The purified MAG-Fc molecules were still in a native form as they were recognized by the conformation-dependent MAG monoclonal antibody, mAb513, in an ELISA assay. mAb513 binds to a conformational epitope in the Ig-like extracellular domain 1 to domain 3, comprising the N-terminus of MAG (Fahrig et al., 1993 and Meyer-Franke et al., 1995).

The presence of an Fc tag on MAG (as well as other sialoadhesin family members we used) has a number of advantages: (1) MAG-Fc makes it possible to study MAG in its soluble form. For example, anti-human IgG (Fc specific, termed anti-Fc) was used to complex MAG-Fc in solution to allow high avidity binding to neurons (Kelm et al., 1994). (2) MAG-Fc allows a wide range of assays to be performed. For example, the complexed MAG-Fc can be used to detect its receptor by immunoprecipitation. In addition, ELISA plates coated with anti-Fc antibody to high density can be used to trap MAG-Fc in the correct orientation (DeBellard et al., 1996). (3) MAG-Fc with mutation in the MAG extracellular domain is very useful in identifying the binding site on MAG (Tang et al., 1997b). (4) Fc-proteins, in general, are a powerful tool not only in identification of their binding components, but also in their purification through affinity chromatography (Crocker and Kelm, 1997).

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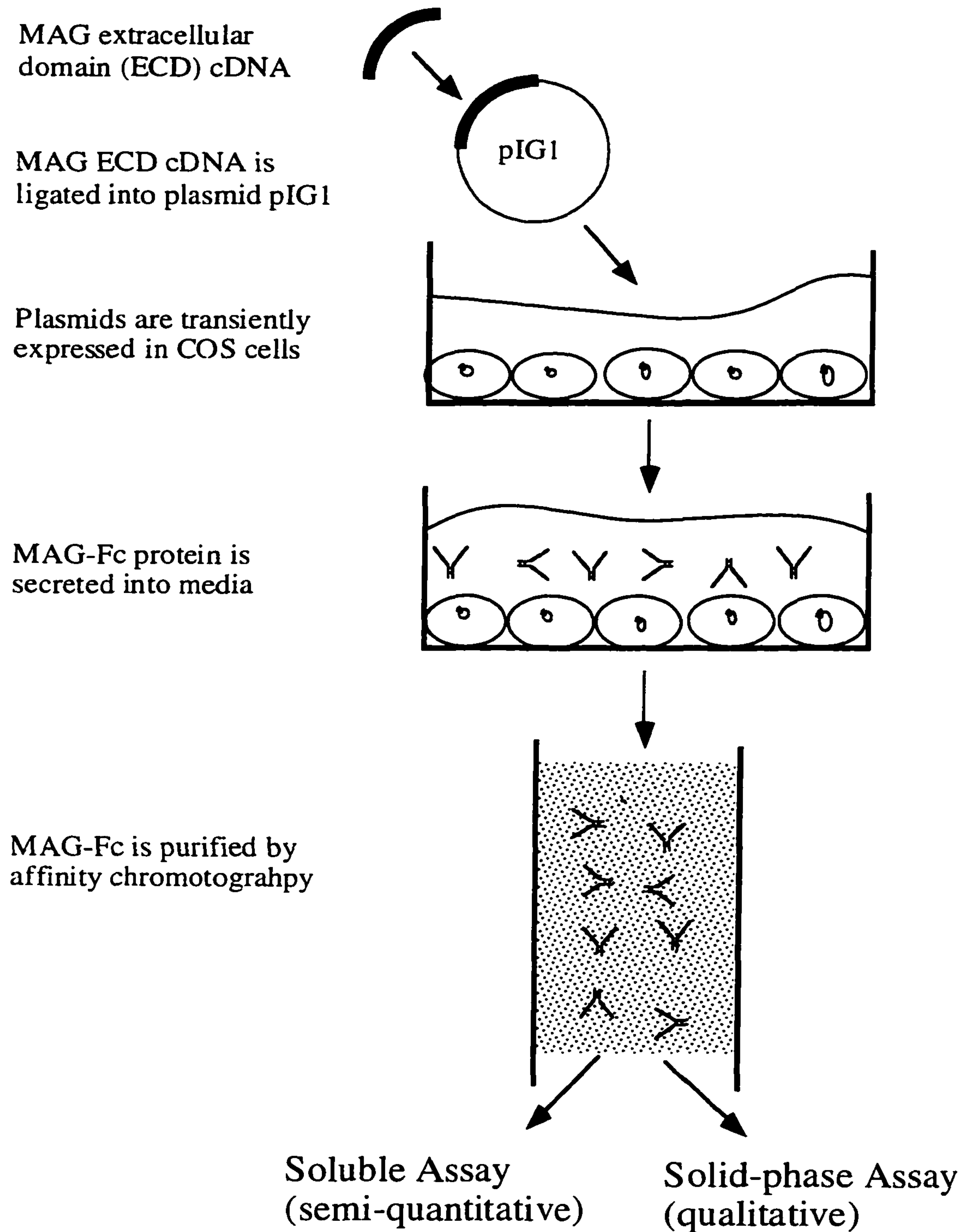

Fig. 3.2 Schematic diagram showing transfection of MAG/pIG1 construct and purification of MAG-Fc.

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3.2.2 Binding of MAG to Cerebellar and DRG Neurons in a Sialic Acid-Dependent Manner (MAG-Fc Soluble Binding Assay)

An important feature of protein-carbohydrate interactions is the rather low affinity of carbohydrate ligands at their individual binding sites of proteins. Consequently, multivalent interactions are often essential to obtain stable, high-avidity binding between proteins and their carbohydrate ligands (Crocker and Kelm, 1997).

To increase the valency of the dimeric MAG-Fc, MAG-Fc molecules, iodinated with Iodogen, were incubated with anti-Fc to form soluble immune complexes. These complexes allow multimeric interactions of MAG-Fc with the putative neuronal cell surface receptor, which is more likely to mimic the interaction in vivo. The iodinated MAG-Fc complexes were incubated with isolated cerebellar or dorsal root ganglion (DRG) neurons. Bound MAG-Fc complexes were separated from free complexes by washing, and quantitated by counting for radioactivity (Fig. 3.3). MUC18-Fc was used as a control. MUC18 is a marker for tumor progression in human melanoma and like MAG, a five-domain Ig superfamily member (Lehmann et al., 1989; Hampel et al., 1997).

In order to determine the ratio for optimal complex formation, it is important to titrate anti-Fc antibody with a fixed amount of iodinated MAG-Fc. The results show that there was a peak of binding that corresponds to the ratio for optimal complex formation, when the concentrations of anti-Fc antibody and MAG-Fc were in a ratio of 10:1 (Fig. 3.4). An explanation to this ratio is that, if the concentration for anti-Fc is too low, the complexes may not reach their optimal size, consequently, the binding will not be optimized; in contrast, if the concentration of anti-Fc is too high, the complexes would fail to form and the binding would

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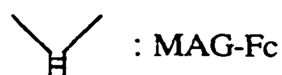
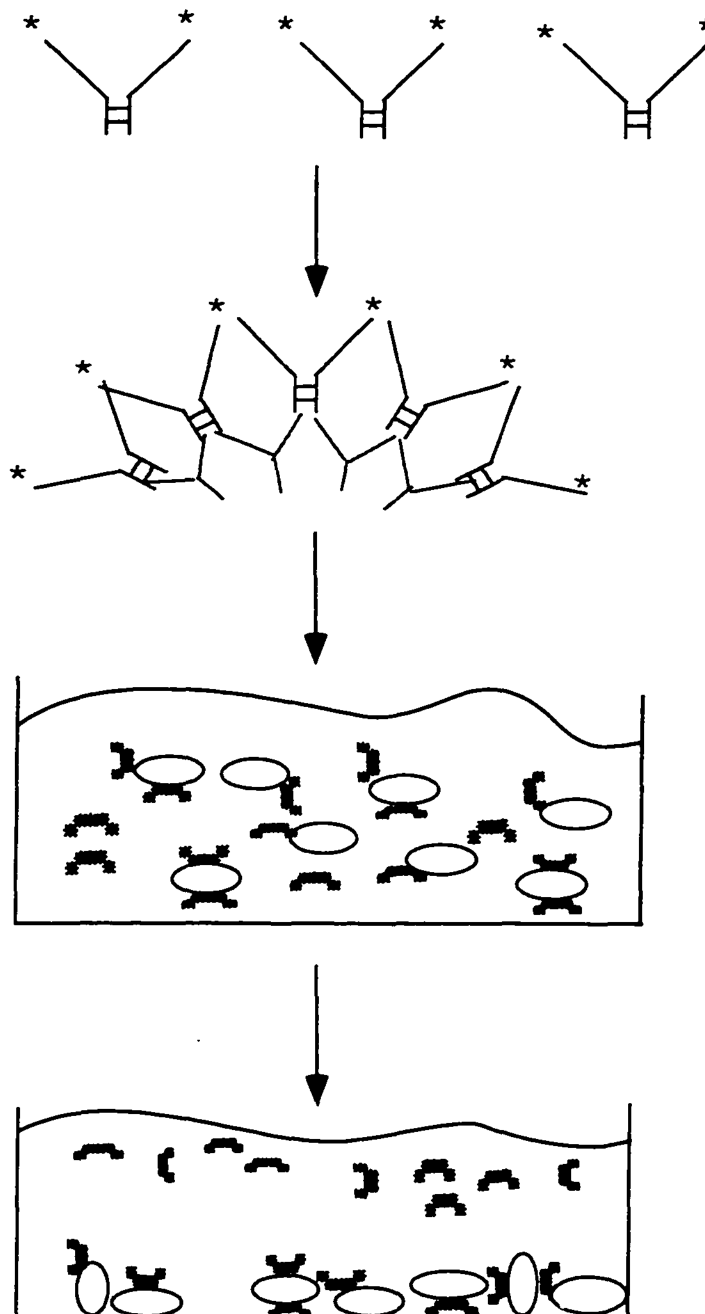
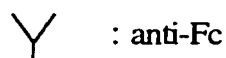
MAG-Fc is radio-iodinated (*)

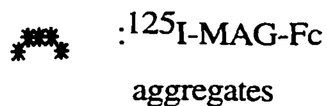




^{125}I-MAG-Fc is aggregated by incubation with anti-Fc



^{125}I-MAG-Fc aggregates are incubated with neurons in suspension



Bound aggregates are separated from free aggregates by centrifugation

Bound aggregates are quantified by counting for radioactivity

Fig. 3.3 Schematic diagram illustrating the soluble binding assay with MAG-Fc. The dimeric MAG-Fc are rendered multivalent by complexing with anti-Fc in solution.

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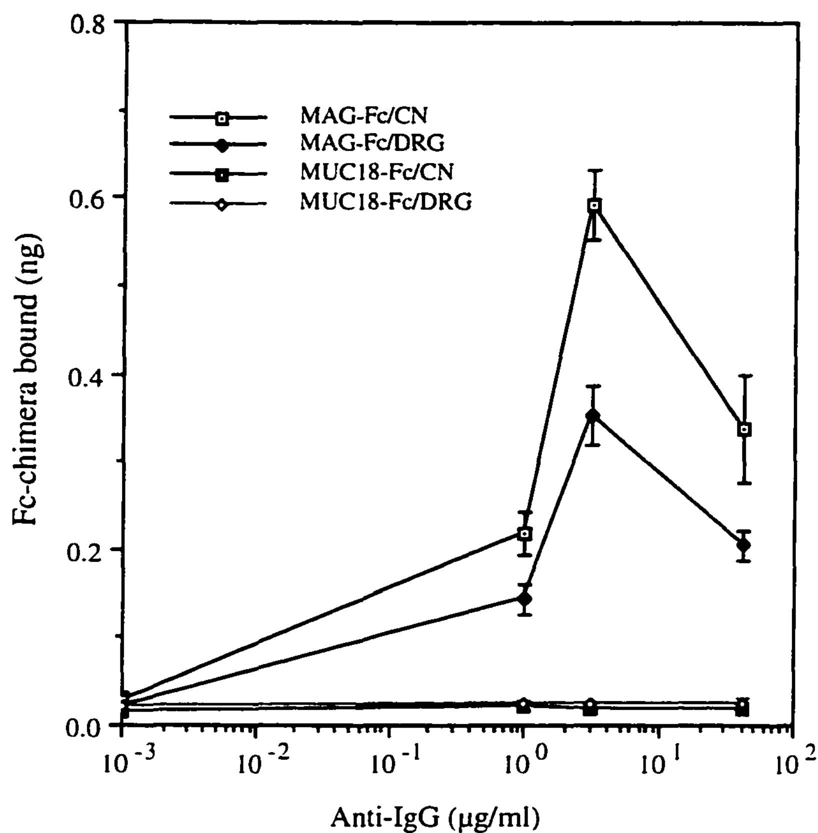

Fig. 3.4 Titration of ^{125}I-MAG-Fc/anti-Fc ratio for specific binding of MAG-Fc to cerebellar and DRG neurons. Iodinated Fc-chimeric proteins, 0.4µg/ml, were incubated for 1hr at 4°C with anti-Fc, 1.0µg/ml to 40µg/ml on a 96-well microtiter plate. 1.5×10^5 cerebellar or DRG neurons were added to each well and incubated for 1hr at 4°C. After washing, the bound radioactivity was measured. Results represent the mean ± SE.

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again be less than optimal. Therefore, as shown in Fig. 3.4, the binding was not simply dose-dependent on MAG-Fc or anti-Fc. Using a ratio of anti-Fc:MAG-Fc of 10:1, we found that iodinated MAG-Fc bound to both cerebellar and DRG neurons while the control, MUC18-Fc, did not bind to either neuronal population (Fig. 3.4).

Under the conditions of optimal complex formation, when MAG-Fc complexes were preincubated with the 513 MAG monoclonal antibody, the binding of complexed MAG-Fc to both cerebellar and DRG neurons was reduced to background (Fig. 3.5a). There was no effect on binding of MAG-Fc to the neurons if a murine IgG was included (data not shown). This further indicates that MAG-Fc binds to neurons specifically. Importantly, if cerebellar or DRG neurons were pretreated with the desialylating enzyme, Vibrio cholerae sialidase (VCS), binding of MAG-Fc to neurons was reduced by 60%-90% (Fig. 3.5b). Vibrio cholerae sialidase specifically hydrolyses terminal sialic acid residues from glycoconjugates. In addition, MAG-Fc bound equally well to glutaraldehyde-fixed neurons and this binding was also abolished by desialylation of neurons (Fig. 3.5b). Therefore, it is suggested that the terminal sialic acid is sufficient to mediate the binding of MAG to neurons.

These results shown above demonstrate specific, sialic-acid dependent binding of MAG to neurons (Kelm et al., 1994; Tang et al., 1995).

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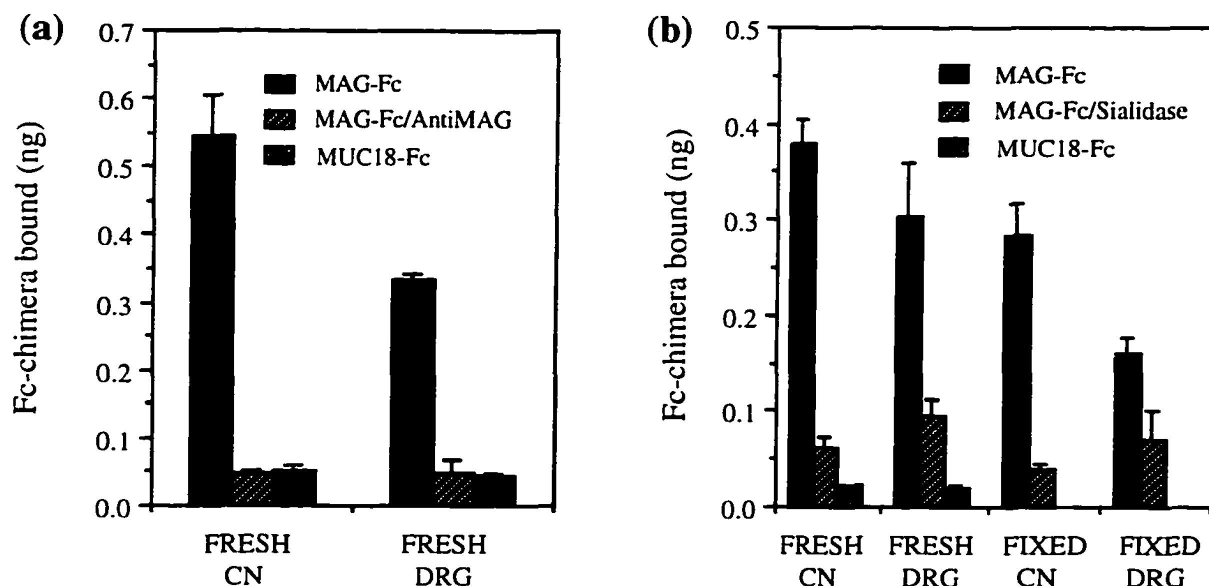

Fig. 3.5 (a) Blocking ^{125}I-MAG-Fc binding to neurons by MAG antibody treatment. Iodinated Fc-chimeric proteins, 0.4μg/ml, were incubated with anti-Fc, 4μg/ml for 1hr at 4°C. The 513 MAG monoclonal antibody, 1μg/ml, was then incubated with the aggregated chimeric proteins for 1hr at 4°C before adding cerebellar and DRG neurons. (b) Abolishing ^{125}I-MAG-Fc binding by desialylation of neurons. The cerebellar and DRG neurons were fixed by incubating 2-5x10^7 neurons with 0.25% glutaraldehyde for 10min at room temperature. For desialylation, 2-5x10^7 either fresh or fixed neurons were treated with 100mU *Vibrio cholerae* sialidase for 3hrs at 37°c prior to the soluble binding assay. 1.5x10^5 cerebellar or DRG neurons were added into each well. After incubation and washing, the bound radioactivity was measured. Results represent the mean ± SE.

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3.2.3 Binding MAG to a Variety of Postnatal Neurons (MAG-Fc Solid-Phase Binding Assay)

We have already shown that MAG binds specifically to both cerebellar and DRG neurons in a sialic acid-dependent manner. To assess the sialic acid-dependent binding of MAG to other neurons such as hippocampal (HN), spinal (SN), superior cervical ganglion (SCG) and retinal (RN) neurons, we used a solid-phase, non-radioisotope binding assay (Crocker and Kelm, 1997). In this assay, MAG-Fc molecules were immobilized in the correct orientation on a 96-well plate by precoating the plate with anti-Fc antibody. Isolated neurons were vitally labeled with the fluorescent dye calcein AM and incubated with the immobilized MAG-Fc. Unbound neurons were washed off and bound neurons were counted in a FluorImager (Fig. 3.6).

At first, we showed that in the solid-phase binding assay, cerebellar neurons bound specifically to immobilized MAG-Fc, because when the MAG-Fc concentration was increased, the number of bound neurons increased until saturation was reached at a concentration of 5μg/ml. There was only background binding of neurons to the control Fc-protein, MUC18-Fc at all concentrations tested (Fig. 3.7). A further demonstration of specificity of MAG-Fc binding was that the binding was abolished when the 513 MAG monoclonal antibody was added to the binding assay (Fig. 3.7) and when the cerebellar neurons were desialylated prior to binding (results not shown). These results demonstrate that the solid-phase binding assay, like the soluble assay, is suitable to assess the specific, sialic acid-dependent binding of MAG to neurons.

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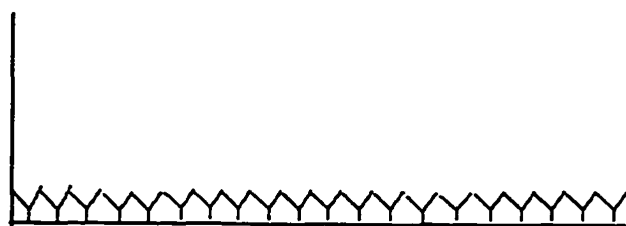
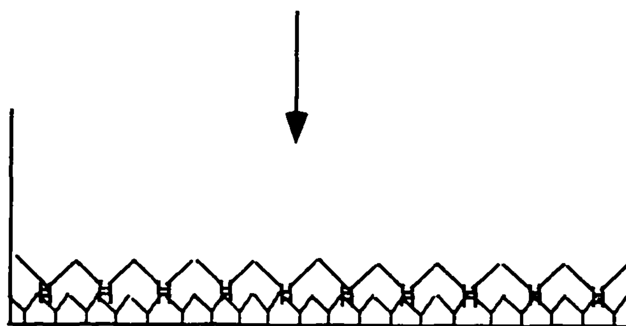
An ELISA plate is coated with anti-Fc

: anti-Fc



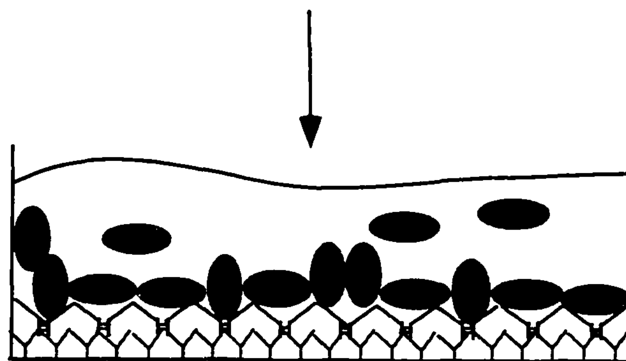
MAG-Fc is immoblized onto anti-Fc

: MAG-Fc



Fluorescent labelled neurons are incubated with immobilized MAG-Fc

: neurons vitally labelled with a fluoresecnt dye



Unbound neurons are washed off. Bound neurons are counted by a FluorImager

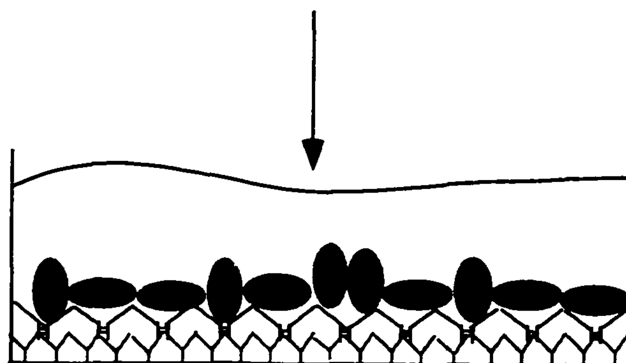


Fig. 3.6 Schematic diagram illustrating MAG-Fc solid-phase binding assay.
MAG-Fc molecules were immobilized in the correct orientation on a 96-well plate by precoating the plate with anti-Fc antibody. Isolated neurons were vitally labeled with the fluorescent dye calcein AM and incubated with the immobilized MAG-Fc. Unbound neurons were washed off and bound neurons were counted in a FluorImager

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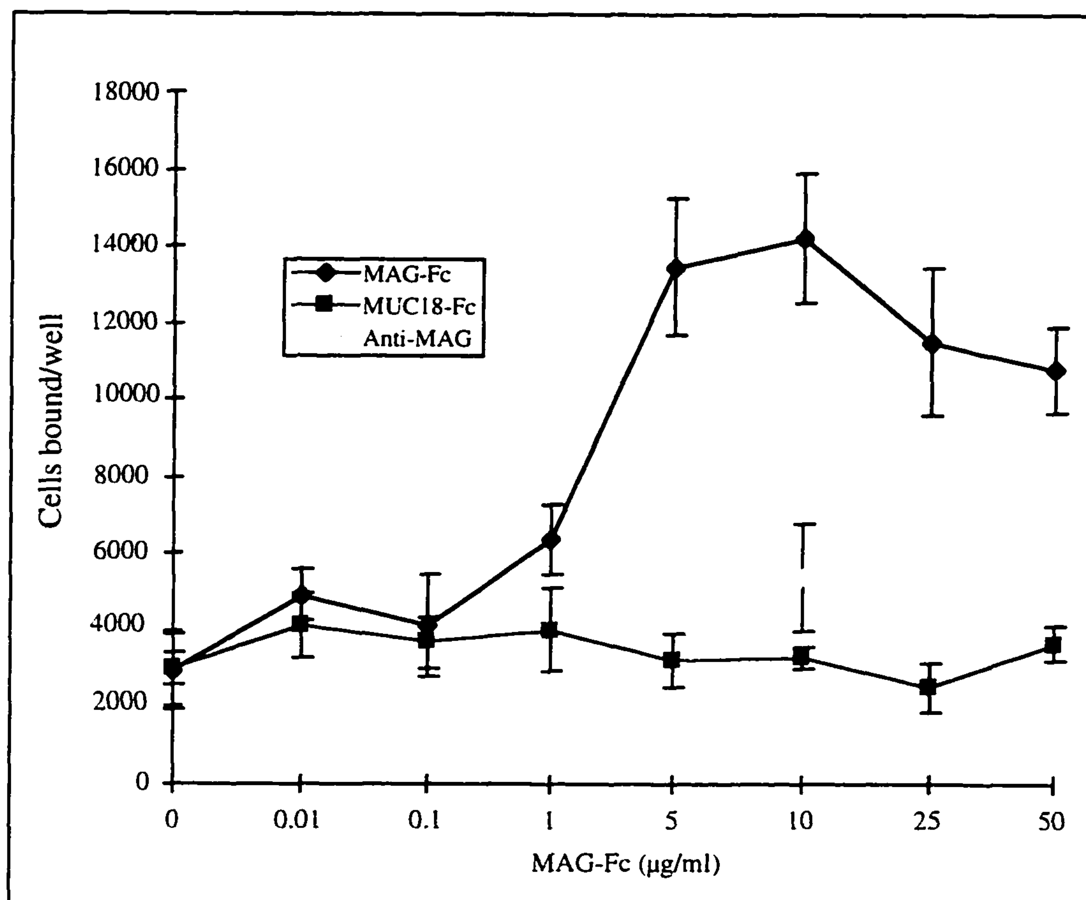

Fig. 3.7 Binding of cerebellar neurons to immobilized MAG-Fc. MAG-Fc and MUC18-Fc, 0.01-50µg/ml, were immobilized on an ELISA plate coated with anti-Fc. Cerebellar neurons, isolated from PND4 animals and vitally labeled with calcein AM, were incubated with immobilized Fc-chimeras, $1.5x10^5$ cells/well. After washing, bound cells were quantitated with a FluorImager. Where indicated, the 513 MAG monoclonal antibody was included at 1µg/ml (triangle). Results represent the mean ± SE.

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We showed previously, MAG inhibits neurite outgrowth from both cerebellar neurons and adult DRG neurons, but it promotes neurite outgrowth from newborn DRG neurons. The switch in response to MAG from promotion to inhibition of neurite outgrowth occurs sharply at postnatal Day (PND) 3 (Mukhopadhyay et al., 1994; DeBellard et al., 1996). To test the binding of MAG-Fc to neurons at different postnatal ages, the cerebellar and DRG neurons from PND1 and PND9 were plated on immobilized MAG-Fc in a solid-phase binding assay. MAG-Fc bound specifically to both cerebellar and DRG neurons from PND1 and PND9 as there was only background binding of MUC18-Fc to the neurons (Fig. 3.8). Importantly, desialylation of both cerebellar and DRG neurons from PND1 and PND9 dramatically reduced their binding to MAG-Fc (Fig. 3.8). Therefore, MAG binds specifically in a sialic acid-dependent manner to both cerebellar and DRG neurons at all postnatal ages, regardless of whether neurite outgrowth is promoted or inhibited by MAG (DeBellard et al., 1996).

When different neuronal populations, HN, SN, SCG and RN, isolated at PND1 and PND9, were incubated with immobilized MAG-Fc in the solid-phase binding assay, as with cerebellar and DRG neurons, MAG-Fc specifically bound to all the neuronal populations tested as only background binding was detected to MUC18-Fc (Fig. 3.9). Importantly, the binding of MAG-Fc to neurons was reduced to background by desialylation of neurons before the assay (Fig. 3.9). Therefore, for all the neuronal populations tested, MAG binds to each of them in a specific, sialic acid-dependent manner.

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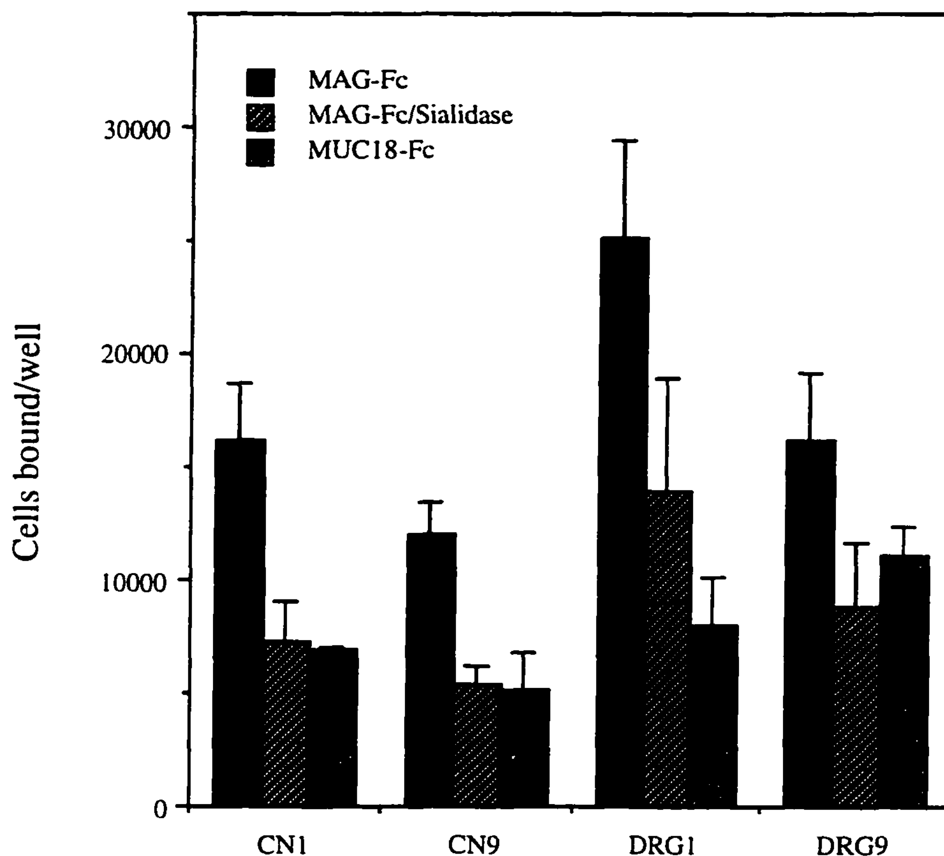

Fig. 3.8 Binding of MAG-Fc to desialylated cerebellar and DRG neurons at PND1 and PND9. Cerebellar and DRG neurons, isolated from PND1 and PND9 animals were desialylated before loading onto immobilized MAG-Fc, 10μg/ml in a solid-phase binding assay. 1.5×10^5 cells were added to each well. After incubation and washing, the number of cells bound to MAG-Fc was quantitated with a FluorImager. Results represent the mean ± SE.

Fig. 3.9 (Next Page) Binding of MAG-Fc to different neuronal populations at (a) PND1 (b) PND9. DRG, cerebellar (CN), SCG, spinal (SN), hippocampal (HN), and retinal (RN) neurons, isolated from PND1 and PND9 animals respectively and vitally labeled with calcein AM, were allowed to bind to either MAG-Fc or MUC18-Fc immobilized, at a concentration of 10μg/ml, on a 96-well ELISA plate, 1.5×10^5 cells/well. Where indicated, PND1 neurons were desialylated before loading onto immobilized MAG-Fc. Results represent the mean ± SE.

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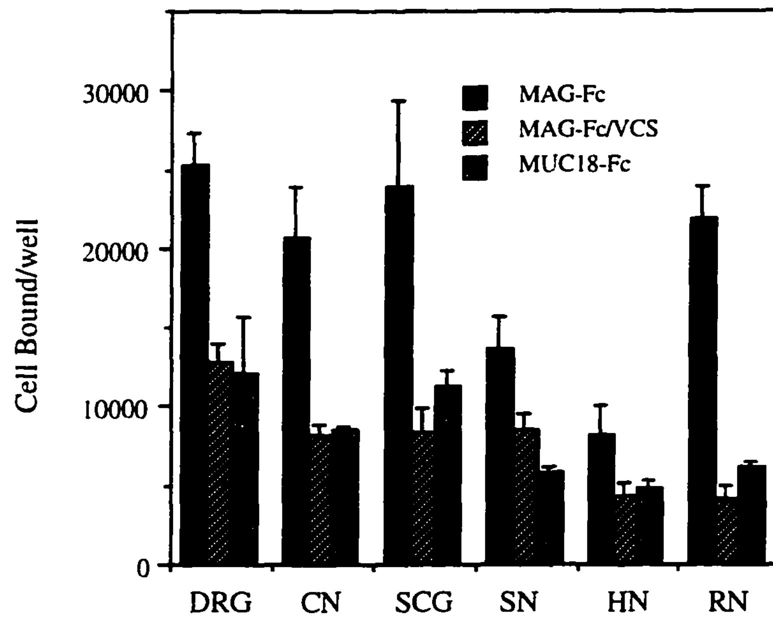
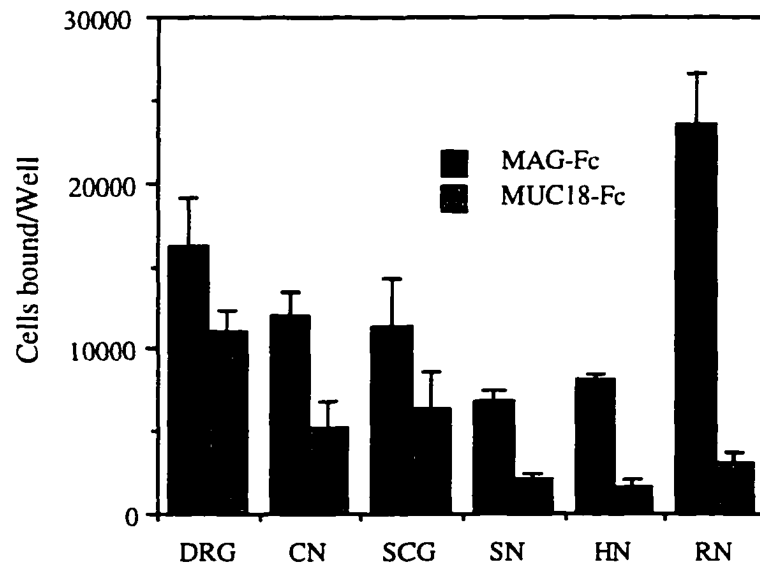
(a) PND1



(b) PND9



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In addition, to determine whether MAG binds to a neuronal sialo-glycoprotein or a sialo-glycolipid, PND1 cerebellar and DRG neurons were treated with a low concentration of trypsin (1.2% trypsin) before the solid-phase binding assay was carried out. The MAG binding to neurons decreased more than 50% if the neurons were trypsinized (Fig. 3.10). Under these conditions of trypsin treatment, 90% of the neurons excluded trypan blue and were still able to extend neurites if plated onto laminin, demonstrating that they were still viable. This strongly suggests that MAG binds to a sialo-glycoprotein on the surface of both PND1 DRG neurons from which it promotes axonal outgrowth, and PND1 cerebellar neurons from which it inhibits axonal growth (DeBellard et al., 1996).

3.3 Conclusion and Discussion

The specific binding of MAG to neurons was studied in two assays, a soluble binding assay and a solid-phase binding assay. The soluble binding assay was used to characterize the specific binding of MAG to cerebellar and DRG neurons. The solid-phase binding assay was applied to assess the binding of MAG to various neuronal populations. The conclusion derived from all our results is that the sialic acid of a sialoglycoprotein on the neuronal surface is sufficient to mediate the binding of MAG to a variety of neurons regardless of whether MAG promotes or inhibits neurite outgrowth from those neurons. Therefore, we suggest that the bifunctional effect of MAG on neurite outgrowth may be mediated via MAG's interaction with different sialoglycoproteins on different neurons to transduce signals through distinct signal pathways. Alternatively, the different effects of MAG may be via MAG's interaction with the same sialoglycoprotein on different neurons, but the downstream signaling pathways may be different (for review, see Filbin, 1995).

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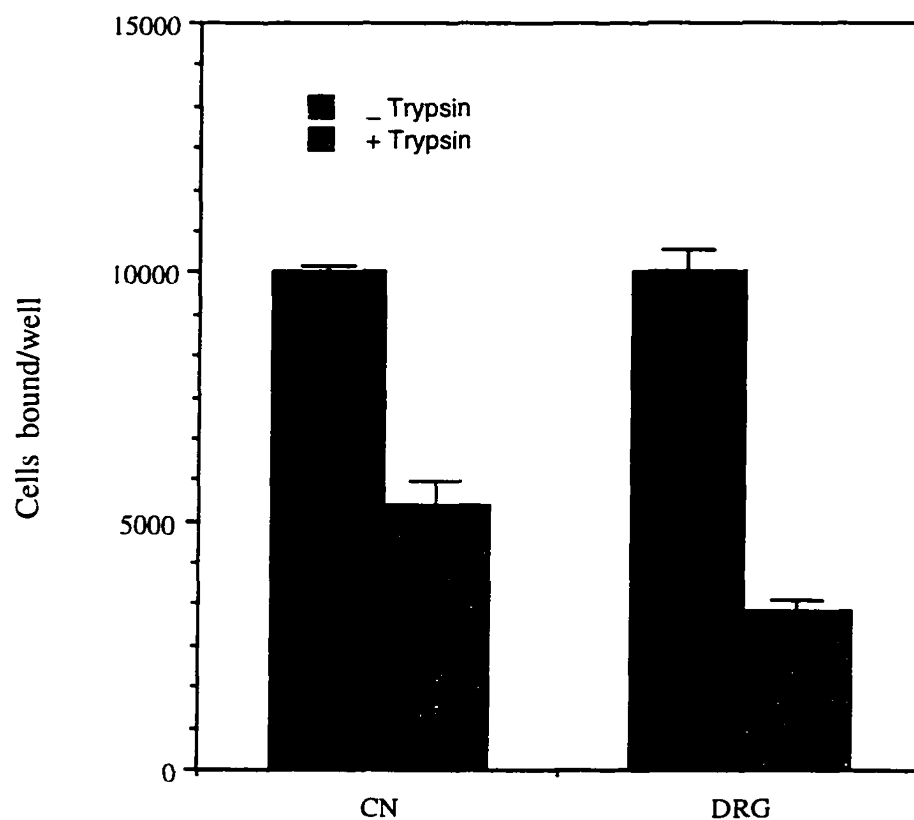

Fig. 3.10 Abolishing MAG-Fc binding to PND1 cerebellar and DRG neurons by trypsinization. Cerebellar and DRG neurons from PND1 animals were vitally labeled with calcein AM and one-half of the population was treated with 1.2% trypsin for 45min prior to addition to the immobilized MAG-Fc, 10μg/ml, in a solid-phase binding assay. 1.5×10^5 cells were added to each well. After incubation and washing, the number of cells bound to MAG-Fc was quantitated with a FluorImager. Results represent the mean ± SE.

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There are a number of advantages and disadvantages to each of the binding assays we used, the solid-phase binding assay and the soluble binding assay. The solid-phase binding assay does not require iodination and is faster than the soluble assay and more suitable to test binding of Fc-chimeras to different neuronal populations in a large scale. However, the numbers of neurons bound can not be compared among populations as the maximum number of neurons bound is dictated by the number that will fit in a monolayer on the MAG-Fc. Therefore, neuronal size contributes to apparent differences in binding among populations, although there could still be differences in affinity for MAG by different neurons. In other words, the solid-phase binding assay is qualitative. In contrast, the soluble binding assay provides a more quantitative assessment of binding of Fc-chimeras. The differences in binding in the soluble binding assay can also be used to distinguish between weak and strong binding.

As mentioned before, MAG belongs to a subgroup of the immunoglobulin superfamily, termed the sialoadhesins (Kelm et al., 1994). The five members of this family described so far are sialoadhesin, CD22, MAG, CD33 (Sylvie et al., 1995) and SMP (a protein similar to MAG but found only in avian species; Dulac et al., 1992). They share high sequence similarity (45 - 50%) among their first four Ig-like domains from the N-termini, and all bind sialic acid. However, the specificity of sialic acid for binding may differ from one member to another. For example, CD22 binds to α 2,6-linked sialic acid attached to N-linked glycoconjugates, sialoadhesin recognizes α 2,3-linked sialic acid attached to either N- or O-linked glycoconjugates, MAG binds to α 2,3-linked sialic acid attached to O-linked glycoconjugates (Kelm et al., 1994), and CD33 recognizes the same sialic acid linkage as sialoadhesin, α 2,3-linked sialic acid attached to either N- or O-linked glycoconjugates (Sylvie et al., 1995).

Recently, it was demonstrated that MAG expressed by Chinese hamster ovary (CHO) cells bound to isolated gangliosides (Yang et al., 1996; Collins et al., 1997). Whether neuronal gangliosides and sialoglycoproteins are both functional MAG binding partners or

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components of a MAG receptor remains to be determined. It is possible that different sialo-glycoconjugates may serve as MAG binding partners under different situations, just as MAG has different roles under different situations.

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Chapter IV

Inhibition of Axonal Regeneration by MAG-Fc

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4.1 Introduction

It has been shown that MAG expressed by Chinese hamster ovary (CHO) cells inhibits axonal regeneration (Mukhopadhyay et al, 1994). However, in order to prove that MAG is a true inhibitor of axonal regeneration and not merely a non-permissive substrate, we would like to show that it fulfills two important criteria: (1) MAG must induce growth cone collapse, (2) MAG in soluble form must inhibit axonal regeneration and its inhibition must be substrate independent (Luo and Raper, 1994). It has been shown that 60% of axonal growth cones of postnatal day (PND) 1 hippocampal neurons collapsed when they encountered recombinant MAG coated on polystyrene beads (Li et al., 1996). Therefore, demonstration that a soluble form of MAG, MAG-Fc, can inhibit neurite outgrowth on different promoting substrates will greatly strengthen the suggestion that MAG is a true inhibitor of axonal regeneration. In addition, as I described in the previous chapter that MAG-Fc binds to neurons in a sialic acid-dependent manner (Kelm et al, 1994 and DeBellard et al., 1996), it is important to determine if the sialic acid-dependent binding of MAG to neurons is critical for the inhibitory effect of MAG on axonal regeneration.

Furthermore, the studies on the possible inhibitory effects of MAG-Fc on neurite outgrowth take on an added importance because there is a soluble form of MAG found in vivo, termed dMAG. dMAG is a derivative of MAG formed by a calcium-activated, myelin-associated, neutral protease (Sato et al., 1982 and Sato et al., 1984a). It is soluble, quite stable, contains all five extracellular Ig-like domains and has a molecular weight of 90kD (Sato et al., 1982 and Quarles et al., 1992). dMAG is found in the cerebrospinal fluid of healthy human subjects and patients with demyelinating diseases at concentrations of 2-13 ng/ml (Yanagisawa et al., 1985). In addition, homogenates of white matter from multiple sclerosis (MS) brains show high levels of dMAG, and MAG is lost preferentially at the edges of some MS plaques (Sato et al., 1984b; for review, see Quarles et al., 1992). The rate of conversion

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of MAG to soluble dMAG is increased in myelin from MS patients compared to normal human controls (Sato et al., 1984b). Since dMAG, unlike MAG, may be released from its natural site in the membrane, it is likely that dMAG may possess chemorepellent potential and exerts its effect at a distance from its site of release. This may have importance in situations of axonal regeneration after spinal cord injury.

4.2 Results

4.2.1 MAG-Fc Binds Directly to Cerebellar Neurons in Suspension

We have demonstrated that MAG-Fc, when immobilized on an ELISA plate or added in solution as soluble immune complexes with anti-Fc, binds to cerebellar neurons in a sialic acid-dependent manner (Kelm et al., 1994; DeBellard et al., 1996). Here we extend that work to show that, when isolated cerebellar neurons are incubated with MAG-Fc or MUC18-Fc in suspension, the dimeric MAG-Fc can also bind to neurons without formation of immune complexes with anti-Fc. Fig. 4.1 shows binding of MAG-Fc to cerebellar neurons, visualized with a fluorescein-conjugated anti-Fc antibody. The fluorescent staining is evenly distributed over the surface of neurons in the presence of MAG-Fc, but there is no staining for neurons incubated with MUC18-Fc. This shows that when added as a dimer in solution, MAG-Fc can still bind specifically to cerebellar neurons (Tang et al., 1997a).

4.2.2 MAG-Fc Inhibits Neurite Outgrowth from Cerebellar Neurons on L1-Fc

To determine if soluble MAG, like MAG expressed by CHO cells, can inhibit axonal growth, we investigated whether the recombinant soluble MAG, MAG-Fc, inhibits neurite outgrowth on different neurite-outgrowth promoting substrates. Initially we used L1 glycoprotein as a substrate in the neurite outgrowth assay. L1 glycoprotein has been shown

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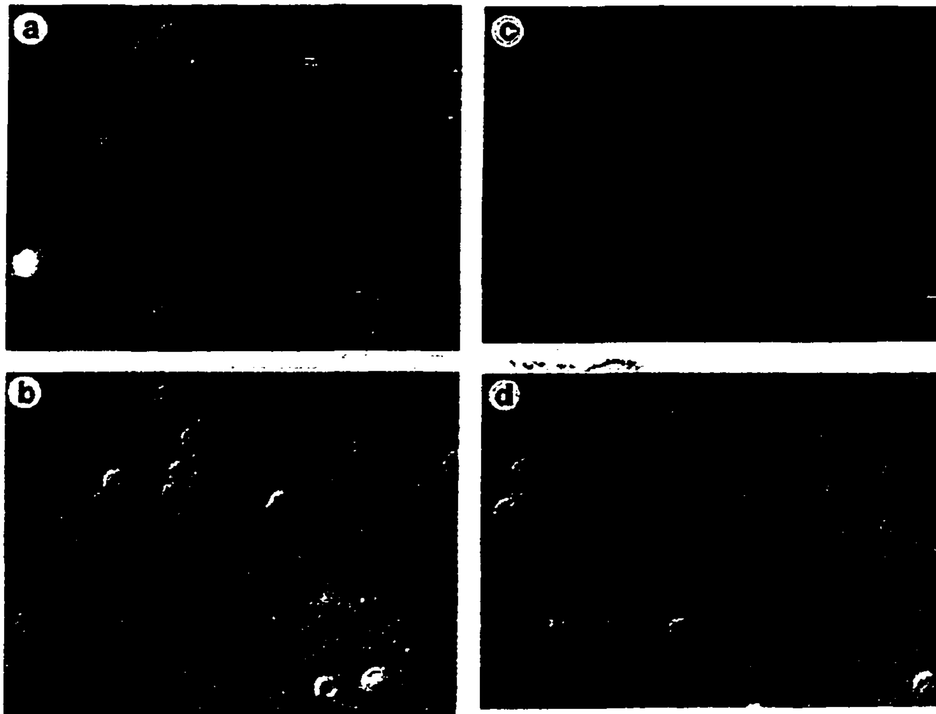

Fig. 4.1 Binding of MAG-Fc to dissociated cerebellar neurons in suspension. Dissociated cerebellar neurons from PND3 rats were plated into a 96-well plate and incubated with MAG-Fc (a and b) or MUC18-Fc (c and d) at a concentration of 25μg/ml. Bound Fc-chimera was visualized with a fluorescein-conjugated anti-Fc antibody. Cells were viewed under fluorescence (a and c) or Nomarski Dic contrast microscopy (b and d).

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to promote neurite outgrowth from a variety of neurons (for review, see Doherty and Walsh, 1992). The chimeric form of L1, L1-Fc, was immobilized on a 8-chamber slide precoated with anti-Fc (Doherty et al., 1995). Isolated cerebellar neurons from PND3 to PND7 rats were plated onto immobilized L1-Fc. Then MAG-Fc or control Fc-chimera, MUC18-Fc, was added into the cerebellar neuronal culture (Fig. 4.2). The neurons and neurites were fixed and stained with GAP43 after 16-18 hour incubation. The longest neurite from each GAP43 stained neuron was measured for at least a hundred neurons.

The results show that when cerebellar neurons were grown on L1-Fc with MUC18-Fc added as a control, neurons had long neurites, equivalent to when no Fc-chimera was added. However, when they were grown on L1-Fc with MAG-Fc added, the majority of neurons had short neurites (Fig. 4.3). After measuring neurite length of cerebellar neurons grown in presence of MAG-Fc or MUC18-Fc, it was found that increasing concentrations of MAG-Fc inhibited neurite outgrowth in a dose-dependent manner (Fig. 4.4). For the maximum concentration we tested, 25µg/ml, neurite outgrowth was inhibited by about 50% relative to neurite outgrowth in the presence of the same concentration of MUC18-Fc (Fig. 4.4). In addition, the neurite length was identical in the presence of MUC18-Fc or in absence of Fc-chimera (Fig. 4.5). This result suggests that MAG-Fc specifically inhibits neurite outgrowth from cerebellar neurons grown on L1 (Tang et al., 1996; Tang et al., 1997a).

To establish the specificity of the inhibition of neurite outgrowth by MAG-Fc, the 513 MAG monoclonal antibody (mAb513) was included in the neurite outgrowth assay, because it was previously shown that mAb513 blocks the specific binding of MAG-Fc to cerebellar neurons (Chapter III; Kelm et al., 1994; DeBellard et al., 1996). We demonstrated that simultaneous addition of mAb513 (25µg/ml) and MAG-Fc (25µg/ml) completely blocked the inhibition of neurite outgrowth by MAG-Fc (Fig. 4.5). In contrast, the antibody had no effect

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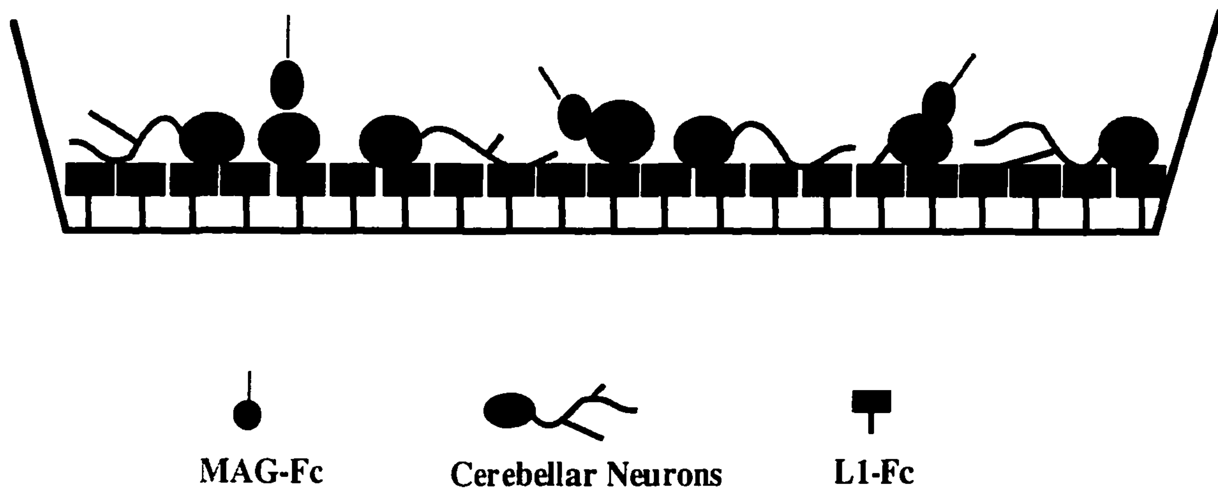

Fig. 4.2 Schematic diagram illustrating the neurite outgrowth assay for studying the inhibitory effect of MAG-Fc on neurite outgrowth. Isolated cerebellar neurons from PND3 to PND7 rats were plated onto immobilized L1-Fc. Then MAG-Fc or control Fc-chimera, MUC18-Fc, was added into the cerebellar neuronal culture. The neurons and neurites were fixed and stained with GAP43 after 16-18 hour incubation. The longest neurite from each GAP43 stained neuron was measured for at least a hundred neurons.

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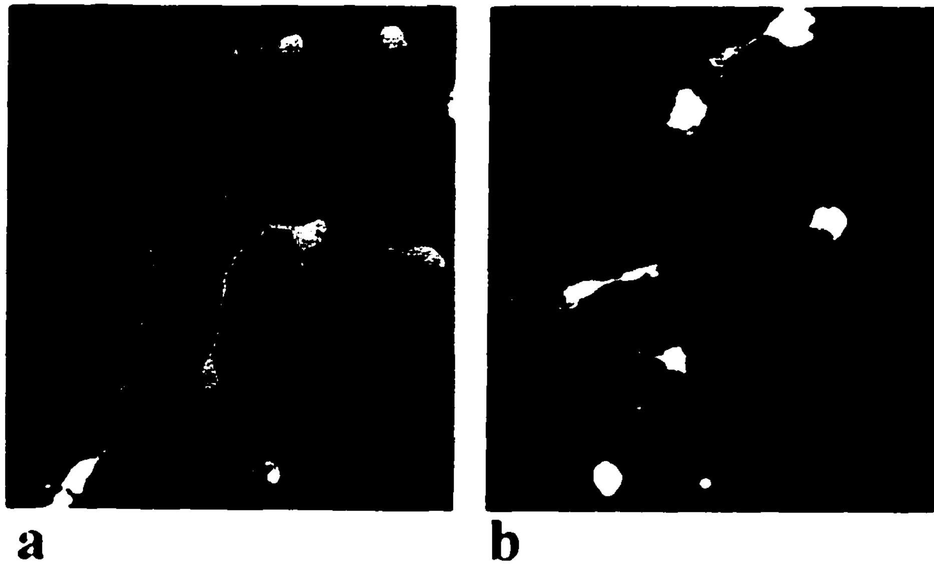

Fig. 4.3 The effect of MAG-Fc on neurite outgrowth from cerebellar neurons grown on immobilized L1. Dissociated cerebellar neurons (PND5) were cultured for 18hrs on immobilized L1-Fc, in the presence of 25ug/ml soluble MUC18-Fc (a) or MAG-Fc (b) before being fixed and immunostained for GAP43.

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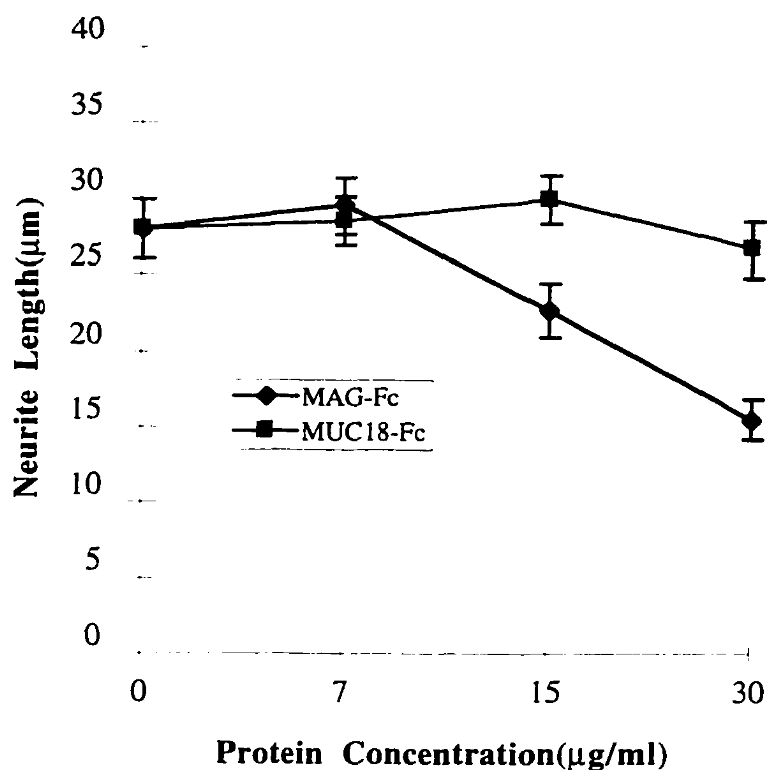

Fig. 4.4 The dose-dependent inhibition of neurite outgrowth by MAG-Fc. Dissociated cerebellar neurons (PND5) were cultured for 18hrs on immobilized L1-Fc, in the presence of various concentrations of MAG-Fc or MUC18-Fc before being fixed and immunostained for GAP43. Neurite length was then measured. Results represent the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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on neurite outgrowth in the presence of an equivalent concentration of MUC18-Fc (data not shown). In addition, as we also showed previously that MAG-Fc binds to neurons in a sialic acid-dependent manner (Kelm et al., 1994; DeBellard et al., 1996), to test if the inhibitory effect of MAG-Fc on neurite outgrowth is also sialic acid-dependent, we desialylated cerebellar neurons prior to the neurite outgrowth assay. To minimize replacement of sialic acid on the cerebellar cell surface during the culture period, neuraminidase, was included in the cultural medium. It was found that at the highest concentration used, 25μg/ml, MAG-Fc did not inhibit neurite outgrowth from the desialylated neurons; the desialylated neurons in the presence of MAG-Fc extended neurites of the same length as those from fully sialylated neurons grown in the presence of MUC18-Fc (Fig. 4.5). Therefore, we verified that MAG-Fc specifically inhibits the neurite outgrowth and that this inhibition is sialic acid-dependent (Tang et al., 1997a).

4.2.3 MAG-Fc Inhibits Neurite Outgrowth from Cerebellar Neurons on Cell Monolayers or Various Extracellular Matrix Components

Although we have shown that MAG-Fc inhibits neurite outgrowth from cerebellar neurons grown on L1, as mentioned before, for MAG to be a true inhibitor, the inhibitory effect of MAG-Fc on neurite outgrowth must be substrate-independent. Therefore, we investigated if MAG-Fc can inhibit neurite outgrowth from cerebellar neurons grown on different cell monolayers or various extracellular matrix components.

When cerebellar neurons were grown on 3T3 cell monolayers in presence of MAG-Fc at either 5μg/ml or 25μg/ml, the neurite length was 40% shorter than the neurites from cerebellar neurons grown in the presence of MUC18-Fc at the same concentrations (Fig. 4.6). This implies that the inhibition of neurite outgrowth by MAG-Fc for cerebellar neurons grown

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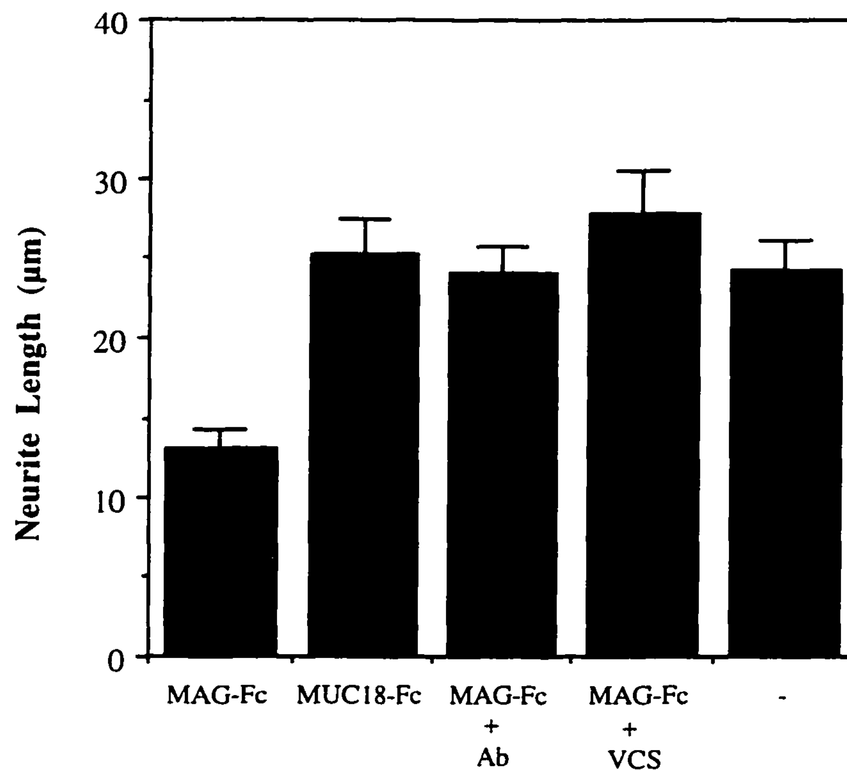

Fig. 4.5 The effect of MAG antibody and desialylation of the neurons on inhibition of neurite outgrowth by MAG-Fc. Dissociated cerebellar neurons (PND5), either desialylated or not, were cultured for 18hrs on immobilized L1-Fc, in the presence or absence of 25µg/ml MAG-Fc or MUC18-Fc, with or without the 513 MAG monoclonal antibody (mAb513, 25µg/ml), before being fixed and immunostained for GAP43. Neurite length was then measured. Results represent the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons. MAG-Fc, neurons cultured with MAG-Fc; MUC18-Fc, neurons cultured with MUC18-Fc; MAG-Fc+Ab, neurons cultured with MAG-Fc in the presence of mAb513 antibody; MAG-Fc+VCS, desialylated neurons cultured with MAG-Fc; -, neurons in the absence of Fc-chimera.

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on 3T3 cells reaches its maximum at the concentration of 5μg/ml. This inhibition was also completely reversed by the 513 MAG monoclonal antibody (Fig. 4.6). In addition, when cerebellar neurons were co-cultured on monolayers of transfected 3T3 cells (Williams et al., 1994) expressing growth-promoting molecules, L1, NCAM or N-cadherin, the neurite length was about twice as long as that from cerebellar neurons grown on untransfected 3T3 cells. However, if MAG-Fc was added at 5μg/ml to the cerebellar neurons co-cultured on the monolayers of transfected 3T3 cells, neurite length was approximately the same as that from the neurons grown on untransfected 3T3 cells in the presence of MAG-Fc (Fig. 4.6). After measurement, it was determined that MAG-Fc inhibited neurite outgrowth on transfected 3T3 cells by as much as 80%. Therefore, MAG-Fc inhibits the neurite outgrowth observed on control 3T3 cells as well as completely prevents the increase in the neurite length resulting from the expression of growth-promoting molecules on 3T3 cells (Tang et al., 1997a).

Fig. 4.6 (Next Page) The effect of MAG-Fc on neurite outgrowth from cerebellar neurons grown on monolayers of control and transfected 3T3 cells. Dissociated cerebellar neurons (PND5) were co-cultured in the presence of either MAG-Fc (solid and blank bars) or MUC18-Fc (stripped bars) on confluent monolayers of control 3T3 cells (3T3), NCAM-expressing 3T3 cells (NCAM-3T3), N cadherin-expressing 3T3 cells (Ncad-3T3) or L1-expressing 3T3 cells (L1-3T3) for 18hrs before being fixed and stained for GAP43. For the control 3T3 cells two concentrations of Fc-chimera were used, 5 μg/ml (solid bars) and 25 μg/ml (blank bar) and the 513 MAG monoclonal antibody was included at 25 μg/ml with MAG-Fc at 25 μg/ml (stippled bar). For all other cell lines, a concentration of Fc-chimera of 5 μg/ml was used. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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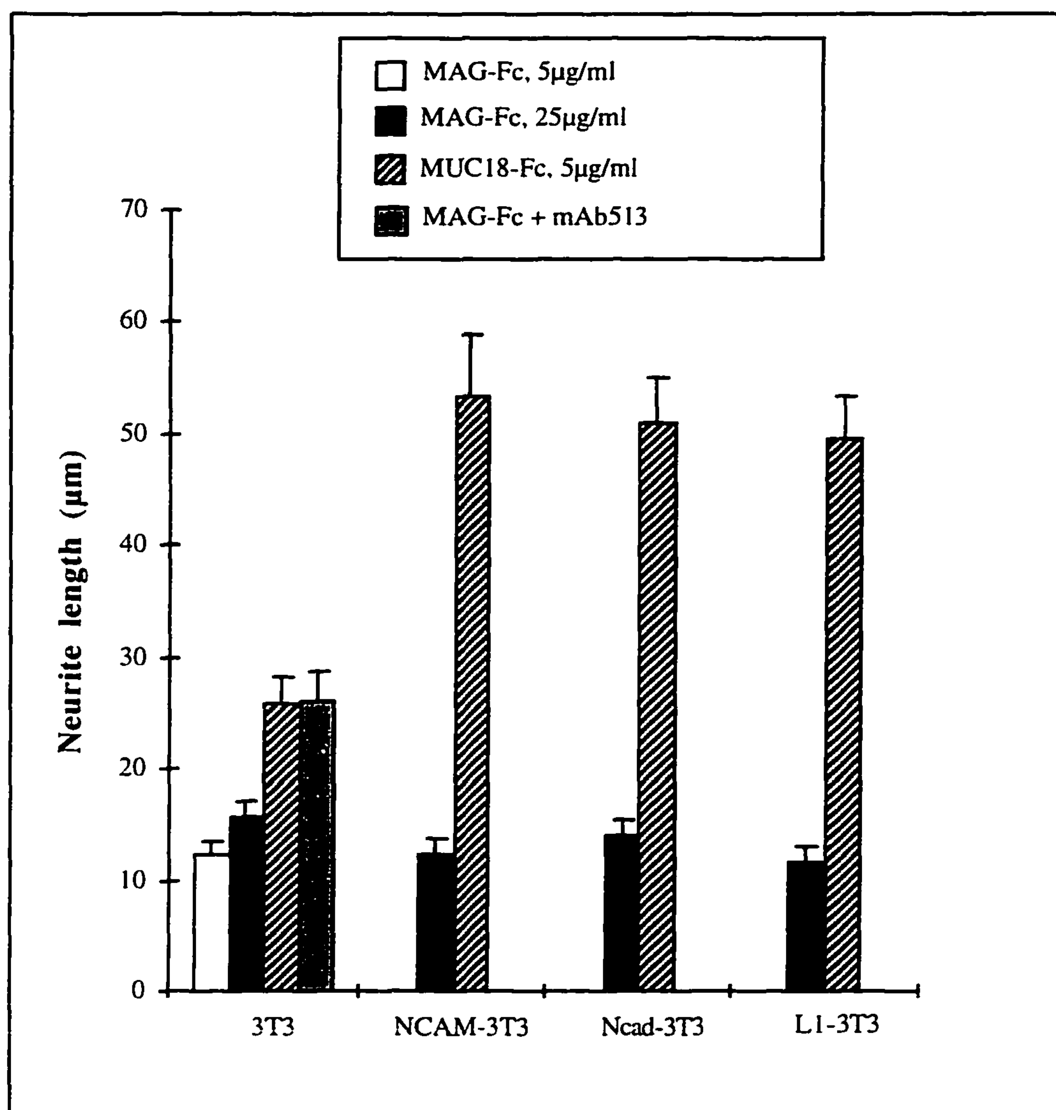

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In addition, it is well-known that Schwann cells which do not express MAG promote axonal regeneration in vivo, and we have shown previously that transfected Schwann cells expressing MAG inhibited neurite outgrowth by 40% (Fig. 4.7; Shen et al., 1996a). When MAG-Fc was included in the co-culture of cerebellar neurons on Schwann cells not expressing MAG, the inhibition on neurite outgrowth by MAG-Fc was equivalent to that from MAG-expressing Schwann cells (Fig. 4.7). Hence, soluble MAG-Fc can inhibit neurite outgrowth on the cells which are permissive for axonal regeneration in vivo.

To assess if soluble MAG-Fc can inhibit neurite outgrowth from neurons grown on different components of extracellular matrix, the neurite outgrowth assay was carried out separately with laminin, fibronectin and poly-L-lysine as substrates. It was found that although the neurite length was equivalent for all the three extracellular matrix components in the presence of MUC18-Fc, MAG-Fc inhibited neurite outgrowth by about 40% when the cerebellar neurons grew on fibronectin or poly-lysine (Fig. 4.8). However, there was no inhibition of the neurite outgrowth by MAG-Fc for the neurons grown on laminin (Fig. 4.8). The possible reasons for lack of MAG inhibition on laminin will be discussed later.

All the results above indicate that for all the cellular or immobilized substrates we tested, soluble MAG-Fc effectively inhibited neurite outgrowth except when neurons grew on laminin. Therefore, this inhibitory effect by MAG-Fc on neurite outgrowth is substrate-independent except for laminin.

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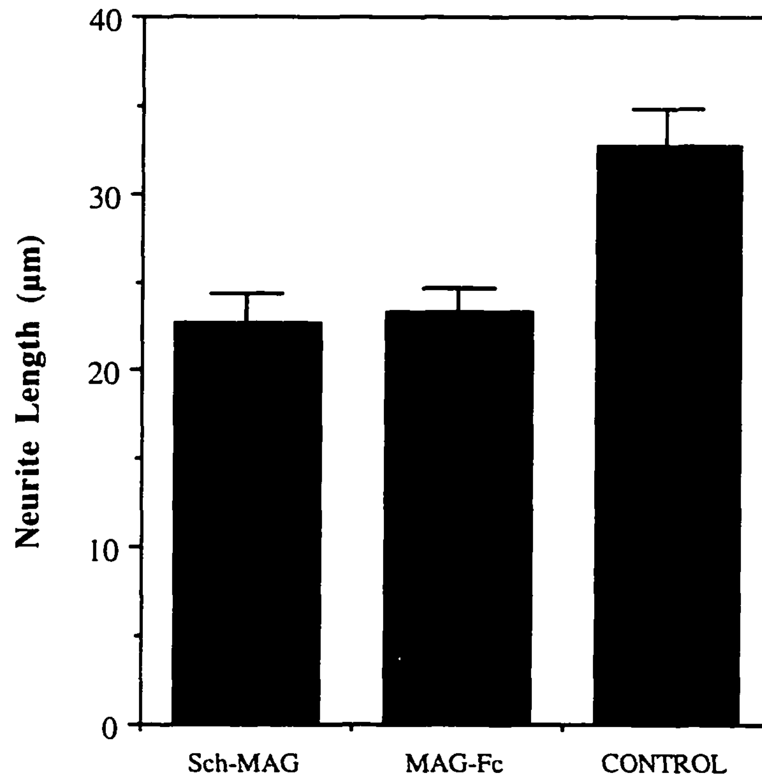

Fig. 4.7. The effect of MAG-Fc on neurite outgrowth from cerebellar neurons grown on Schwann cells. Dissociated cerebellar neurons (PND4) were co-cultured for 18hrs on confluent monolayers of Schwann cells transfected to express MAG (Sch-MAG), Schwann cells not expressing MAG in the presence of 25μg/ml MAG-Fc (MAG-Fc) or MUC18-Fc (Control) before being fixed and stained for GAP43. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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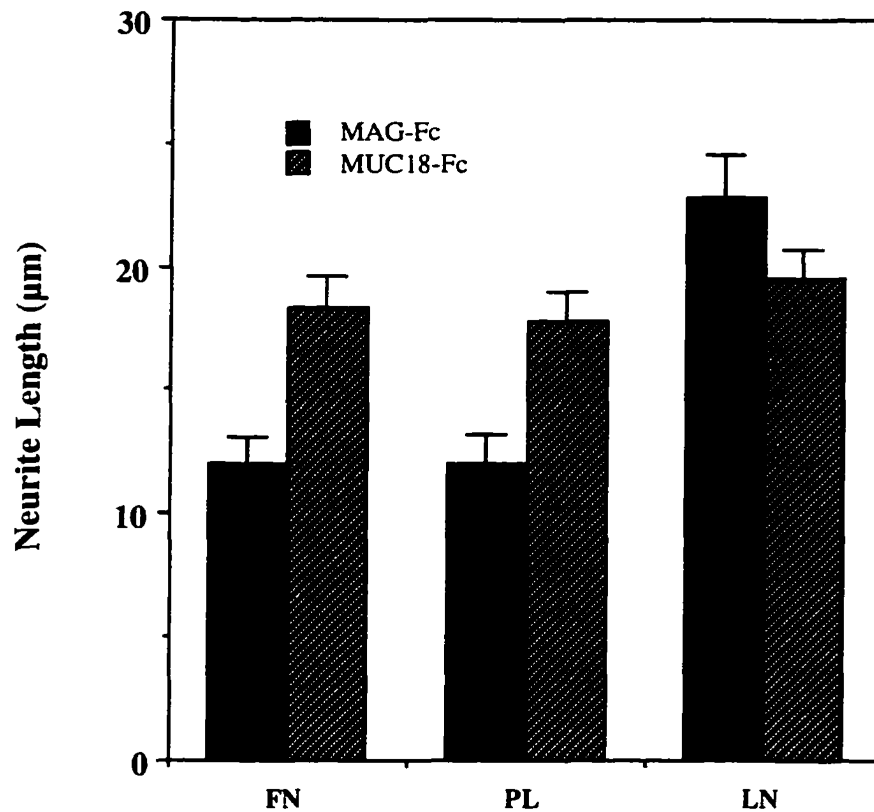

Fig. 4.8 The effect of MAG-Fc on neurite outgrowth from the cerebellar neurons grown on immobilized proteins. Dissociated cerebellar neurons (PND3) were cultured for 18hrs on immobilized fibronectin (FN), poly-L-lysine (PL) or laminin (LN) in the presence of 25 µg/ml of either MAG-Fc (solid bars) or MUC18-Fc (stripped bars) before being fixed and stained for GAP43. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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4.2.4 MAG-Fc Does Not Bind to a Variety of Substrates

Although we have provided strong evidence that the inhibitory effect of MAG-Fc on neurite outgrowth is substrate-independent, there is still a possibility that MAG-Fc binds to substrates and then alters the permissiveness of those substrates. To exclude this possibility, we used an ELISA assay to test whether there is any binding of MAG-Fc to the substrates used in our neurite outgrowth assays.

An ELISA plate was coated with L1-Fc or the extracellular matrix components described previously or plated with the cell monolayers which were used in our neurite outgrowth assays. As a positive control, anti-Fc was immobilized as a substrate at 15μg/ml. MAG-Fc was added at various concentrations, 0.5μg/ml - 25μg/ml, to the anti-Fc. It was found that MAG-Fc bound to immobilized anti-Fc in a dose-dependent manner until reaching saturation. However, there was no binding of MAG-Fc to any substrate tested when MAG-Fc was added at the maximum concentration, 25μg/ml (Table 4.1). These results provide evidence that MAG can not bind to any substrate used in our neurite outgrowth assays.

4.3. Conclusion and Discussion

In conclusion, the soluble form of MAG, MAG-Fc, can specifically inhibit neurite outgrowth from neurons grown on different permissive substrates. The inhibitory effect of MAG-Fc on neurite outgrowth is due to a direct interaction between MAG-Fc and neurons, not because MAG binds to the substrates and changes their permissiveness. In addition, it was shown previously that MAG caused growth cone collapse (Li et al., 1996), therefore, MAG satisfies our two criteria for a true inhibitor. In other words, MAG is a true inhibitor of axonal regeneration. Furthermore, we have also demonstrated that the inhibition of neurite outgrowth by MAG-Fc is sialic acid-dependent.

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Table 4.1 Binding of MAG-Fc to various substrates.

Substrate	MAG-Fc (μg/ml)	Relative Binding
Anti-Human Fc	25	100.0 ± 6.27
	10	90.13 ± 3.59
	5	87.0 ± 7.62
	1	39.01 ± 4.04
	0.5	30.04 ± 4.04
Poly-Lysine	25	6.48 ± 2.02
Fibronectin	25	2.43 ± 2.43
Laminin	25	6.48 ± 3.64
L1-Fc	25	6.99 ± 1.75
Schwann	25	5.21 ± 2.84
3T3	25	2.33 ± 1.66

Note: The proteins L1-Fc, fibronectin, poly-L-lysine, or laminin were immobilized or monolayers of control 3T3 cells (3T3) or Schwann cells grown to confluency in a 96-well ELISA plate. Anti-Fc was immobilized as a positive control. MAG-Fc was added at various concentrations to the immobilized anti-Fc and bound MAG-Fc was quantitated by an ELISA using the 513 MAG monoclonal antibody. A concentration of MAG-Fc at which binding to anti-Fc is saturated, 25 μg/ml, was used to assess binding to the other substrates. Results are relative absorbance units, presented as percentage, taking maximum binding of 25 μg/ml MAG-Fc to anti-Fc as 100% (±SEM).

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It is of note that MAG-Fc, without forming complexes with anti-Fc, can inhibit neurite outgrowth. This is important because we have shown previously that the binding of MAG-Fc to neurons in solution could only be measured after MAG-Fc formed soluble complexes with anti-Fc (Chapter III; Kelm et al., 1994). Here we demonstrate that although the multimeric interaction between MAG-Fc complexes and neurons is necessary for measuring MAG binding to neurons, dimeric MAG-Fc can also bind to neurons. The binding affinity of dimeric MAG-Fc to neurons may be too low to measure in available quantitative binding assays, but is enough to trigger specific MAG inhibition of neurite outgrowth.

In addition, although MAG-Fc inhibits neurite outgrowth on immobilized L1 and untransfected 3T3 cells (not expressing L1, N-CAM, or N-cadherin) to the same extent, about 40%, MAG-Fc inhibits neurite outgrowth on transfected 3T3 cells which express L1, N-CAM or N-cadherin up to 80%. Since neurons grown on the 3T3 cells expressing L1, N-CAM or N-cadherin have neurites twice as long as those neurons grown on untransfected 3T3 cells in the absence of MAG-Fc, MAG-Fc has the ability to completely abolish the neurite outgrowth promoting effect of L1, N-CAM and N-cadherin.

It was shown previously that the Schwann cells used in this study are very permissive for neurite outgrowth from cerebellar, DRG and retinal ganglion neurons (Shen et al., 1996a). However, when these same permissive Schwann cells are transfected to express MAG, neurite outgrowth from all these neurons is inhibited by 40%. Furthermore, MAG-Fc inhibits neurite outgrowth on Schwann cells (not-expressing MAG) to the same extent. In other words, soluble MAG and MAG expressed by Schwann cells have similar inhibitory effect on neurite outgrowth from neurons grown on Schwann cells. These results indicate that MAG alone is sufficient to change the permissiveness of Schwann cells for neurite outgrowth. Consistent with our results, Schäfer et al. (1996) reported that disruption of the gene for MAG improved axonal regeneration along the PNS myelin in C57BL/Ola mice. In C57BL/Ola mice, the PNS

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myelin is not rapidly removed, and the regeneration takes place very slowly if at all (Brown et al., 1991, 1994). But in the MAG-deficient C57BL/Ola mice, regeneration is much more robust (Schäfer et al., 1996). Therefore, it is suggested that down-regulation of MAG by Schwann cells as well as removal of myelin is essential for successful axonal regeneration in the PNS.

Furthermore, we have shown that, although MAG-Fc inhibits neurite outgrowth on the extracellular matrix components, poly-lysine and fibronectin, by about 40%, there is no inhibition of neurite outgrowth by MAG-Fc on laminin. One explanation is that laminin has stronger promoting effect than other substrates we tested, so the promoting effect from laminin outweighs the inhibitory effect from MAG on neurite outgrowth. It is also possible that the signal transduction pathway for MAG inhibition is interfered by a down-stream molecule in the signal transduction pathway for laminin promotion. Alternatively, perhaps laminin and MAG compete directly for the same neuronal receptor, when MAG binds to it, an inhibitory effect is transmitted, but laminin, binding to the same receptor, exerts a growth-promoting effect. If this is the case, laminin would be expected to have a higher affinity for the receptor than MAG.

Consistent with our observation with laminin as a substrate, David et al. (1995) reported that purified PNS myelin inhibited neurite outgrowth from cerebellar neurons and NG108-15 cells, but the inhibitory effect of purified PNS myelin could be masked by laminin. In addition, it was recently shown that when retinal explants were grown on freshly prepared myelin from normal adult rat sciatic nerves, no neurite outgrowth was detectable. In contrast, the presence of laminin resulted in a vigorous outgrowth of retinal axons on the myelin membranes (Bähr and Przyrembel, 1995). More notably, laminin is enriched in the PNS where axonal regeneration occurs after injury but is largely absent from the CNS, where there is little or no regeneration (Cohen et al., 1989; DeCurtis et al., 1991). Therefore, it is possible

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that the inhibitory effect of MAG on neurite outgrowth could be overridden by laminin in the PNS.

Here, we have shown for the first time that a recombinant soluble form of MAG (MAG-Fc) can potently inhibit axonal regeneration, and MAG is a true inhibitor for axonal regeneration. Thus, the next question we would like to ask is whether the endogenous soluble MAG, dMAG, which is found in vivo and released from purified myelin, also inhibits axonal regeneration.

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Chapter V

Inhibition of Axonal Regeneration by Soluble MAG in vivo

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5.1 Introduction

It is known that MAG is a 100kD glycoprotein with five extracellular immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic domain (for review, see Quarles et al., 1992). However, in the course of isolating MAG from human white matter for chemical characterization, it was observed that half the amount of MAG is converted to a smaller derivative after 30min incubation at neutral pH (Sato et al., 1982). Further studies indicated that the effect on MAG is mediated by a calcium-activated, myelin-associated, neutral protease in the CNS (Sato et al., 1982 and Sato et al., 1984a). The native proteolytic site in MAG is located extracellularly, near its transmembrane domain (Stebbins et al., 1997). The proteolytic fragment of MAG is soluble, stable and contains the entire extracellular domain with a molecular weight of 90kD, and is termed dMAG (Sato et al., 1982, Stebbins et al., 1997). Fig. 5.1 shows a schematic drawing of dMAG.

Recently, a species difference has been demonstrated in the proteolysis of MAG in isolated myelin. Under equivalent experimental conditions, the time required for a 50% conversion of MAG to dMAG is 18 - 24 hours in myelin from rodents and bovine, but only 2 hours for non-human primate samples. Human myelin samples need just 5min for a 50% conversion of MAG to dMAG (Möller, 1996).

In addition, it was found that MAG is substantially reduced at the periphery of some, but not all, acute multiple sclerosis (MS) plaques (for review, see Quarles et al., 1992). Homogenates of white matter from multiple sclerosis brains show high levels of dMAG. The rate of conversion of MAG to soluble dMAG is also increased in myelin from MS patients compared to normal human controls (Sato et al., 1984b). These observations suggest that proteolysis of MAG may be relevant to human disease.

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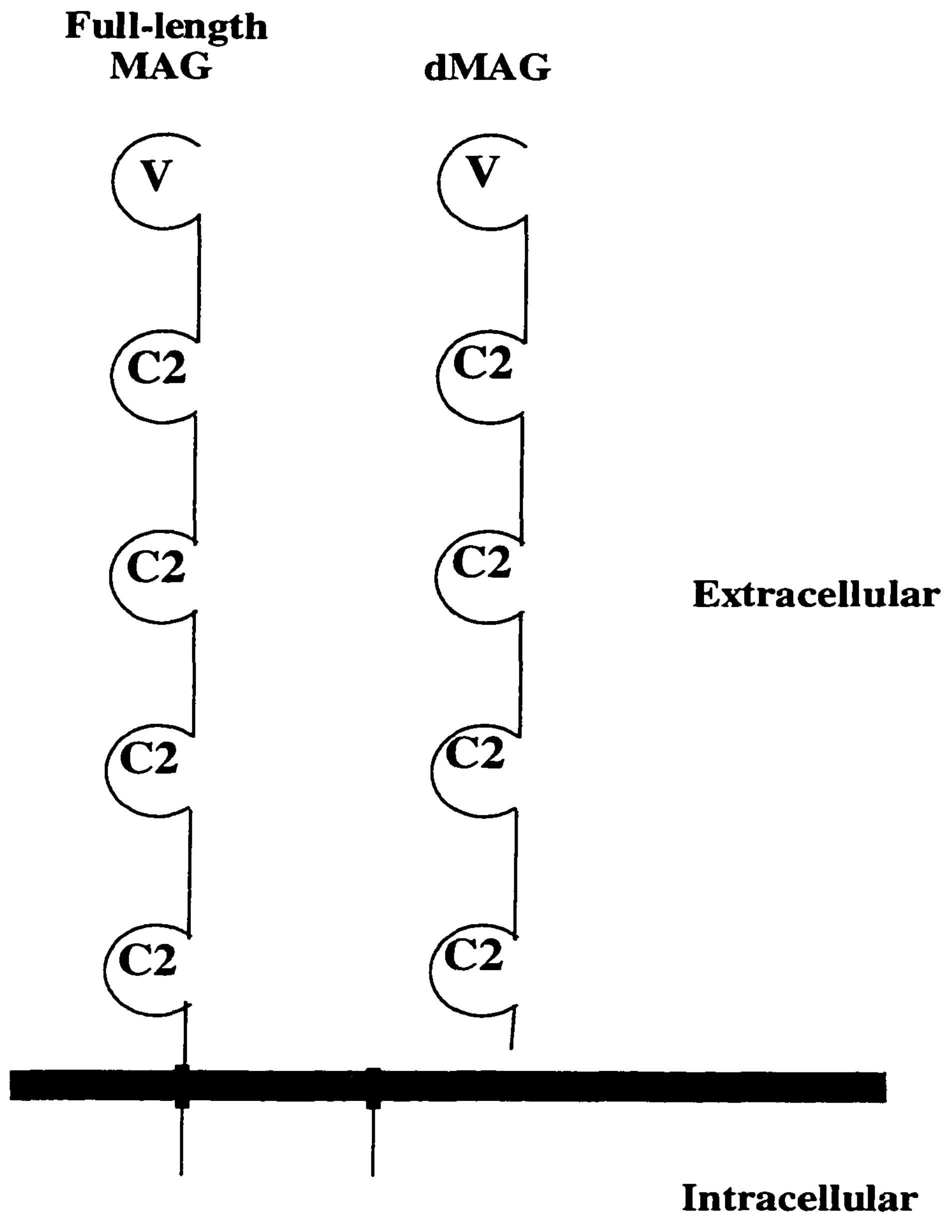

Fig. 5.1 The schematic drawing of dMAG. dMAG is soluble, stable and has a molecular weight of 90kD. dMAG contains of the entire extracellular domain of MAG, including all the five Ig-like domains, i.e. the V-like domain and the following four C2-type domains.

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dMAG is also found in the cerebrospinal fluid (CSF) of normal human subjects and patients with demyelinating diseases at the concentrations from 2 to 13ng/ml. There is no correlation of dMAG levels with active demyelinating diseases (Yanagisawa et al., 1985). The presence of dMAG in the CSF of healthy humans suggests that the proteolysis of MAG is a naturally occurring process in the CNS. However, the physiological significance of dMAG is not yet understood.

As dMAG is released from disrupted myelin, it is very likely to be released from damaged spinal cord. In addition, there may be an increase of dMAG in the CSF after spinal cord injury. Therefore, dMAG may have direct implications for spinal cord injury.

Since we have shown that the recombinant soluble form of MAG, MAG-Fc, as well as the full-length MAG expressed by transfected CHO or Schwann cells inhibits neurite outgrowth, we now want to know if the soluble form of MAG found in vivo, dMAG, can also inhibit neurite regrowth.

5.2 Results

5.2.1 An Abundant of dMAG is Released from Purified Myelin During Overnight Incubation

Myelin from rat white matter was isolated on a sucrose gradient as described previously (Norton and Poduslo, 1973), and resuspended in Sato medium for overnight incubation at room temperature. After pelleting myelin by centrifugation, the supernatant was collected and termed myelin-conditioned medium. Control medium was prepared in a similar manner from rat gray matter (GM) and termed gray matter-conditioned medium. The myelin-conditioned

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medium was either immunodepleted of dMAG, with the 513 MAG monoclonal antibody, or used directly in the neurite outgrowth assay (Fig. 5.2).

The Western blot, immunostained for MAG, in Fig. 5.3, shows that an abundance of dMAG is released from myelin into the medium after overnight incubation at room temperature (Fig. 5.3 Lane 2). dMAG appears at an apparent molecular weight just lower than that of the full-length, membrane-bound MAG on Western blotting (Fig. 5.3 Lane 1 and 2), which is consistent with previous observations (O'Shannessy et al., 1985; Möller, 1996). However, if this myelin-conditioned medium is incubated with the 513 MAG monoclonal antibody and the immune complexes are precipitated with protein A Sepharose and protein G Agarose slurry, no dMAG is detected in the medium (Fig. 5.3 Lane 3). This demonstrates that dMAG is effectively removed from the myelin-conditioned medium by immunodepletion. As expected, there is no dMAG detected in gray matter-conditioned medium (Fig. 5.3 Lane 4).

To quantitate the amount of dMAG released from myelin into the media, an ELISA assay was developed for the myelin-conditioned media. An ELISA plate was coated with the 513 MAG monoclonal antibody and then plated with diluted myelin-conditioned media. After overnight incubation, the plate was incubated with a rabbit, polyclonal MAG antibody (kindly provided by Dr. J. L. Salzer), followed with peroxidase-conjugated anti-rabbit IgG. Color reaction was developed and the absorbance values for myelin-conditioned media were measured on an ELISA reader. The absorbance values for MAG-Fc in the concentration range from 10ng/ml to 2μg/ml were used as a standard. When purified myelin with a starting protein concentration of 15mg/ml was incubated overnight, the concentration of dMAG released into the myelin-conditioned medium was measured as 36μg/ml. Thus, the dMAG concentration is about 1/400 of the starting concentration of the purified myelin, as shown in the Fig. 5.4.

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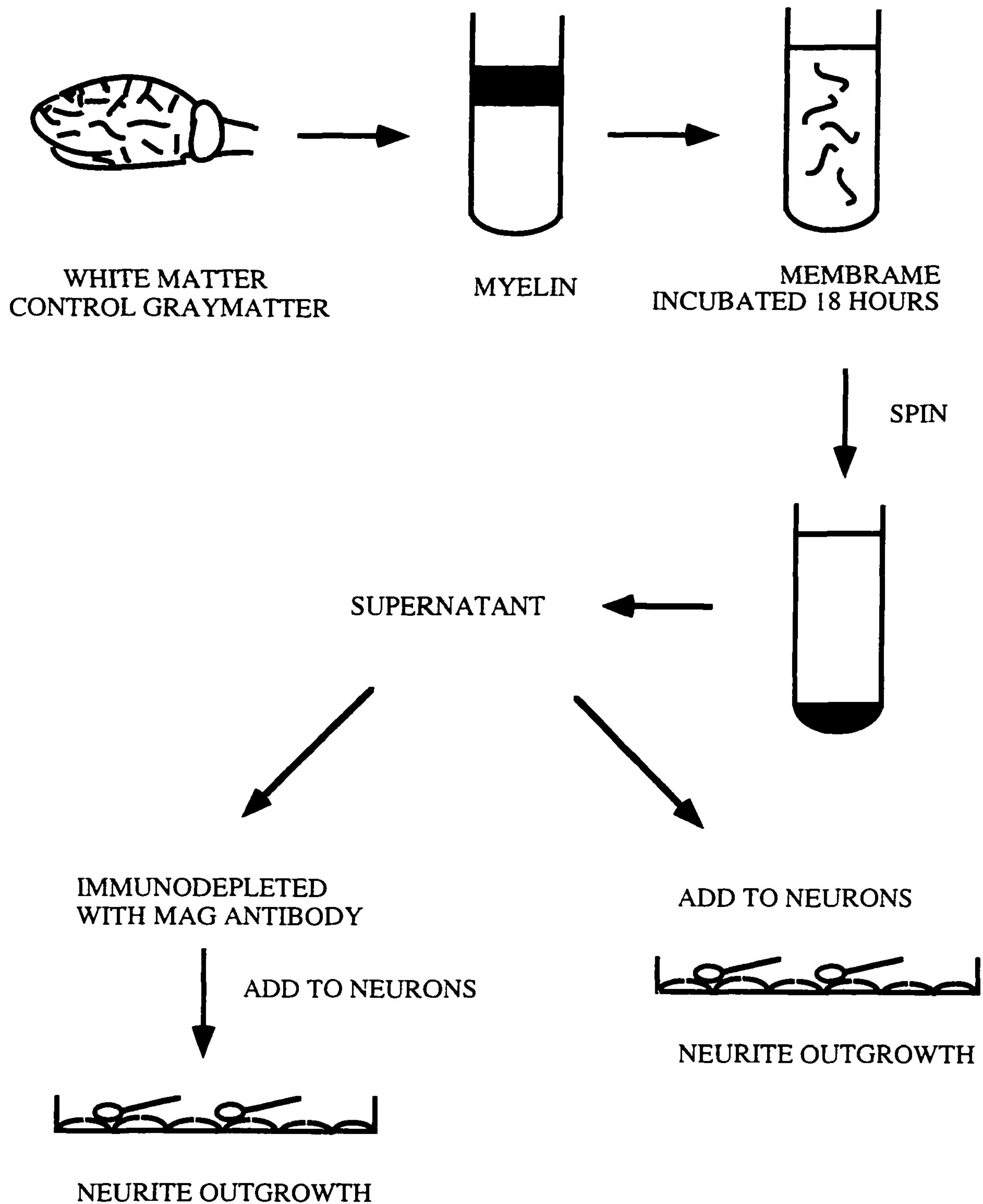

Fig. 5.2 Schematic diagram illustrating the strategy for studying the inhibitory effect of myelin-conditioned media on neurite outgrowth.

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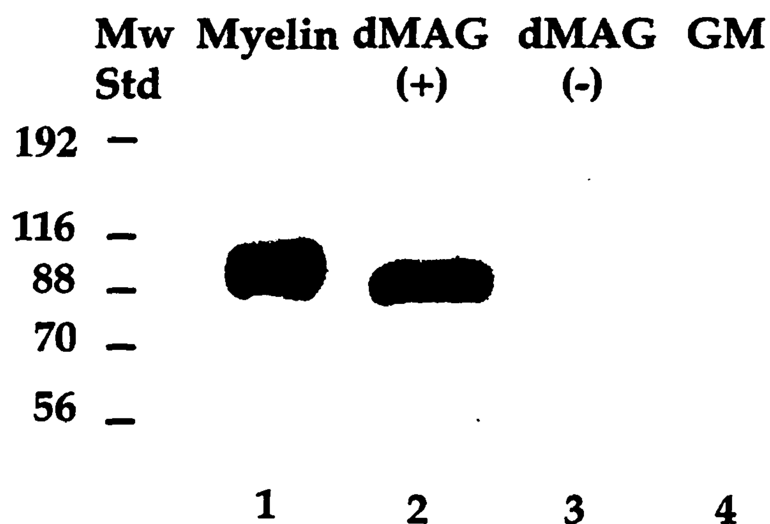

Fig. 5.3 Western blot immunostained for dMAG in myelin-conditioned media. Lane 1, myelin membranes (Myelin); lane 2, myelin-conditioned media (dMAG+); lane 3, myelin-conditioned media, immunodepleted of dMAG (dMAG-); and lane 4, gray matter-conditioned media (GM). Each lane represents approximately 100μg protein starting material from membrane-conditioned media. Molecular weight standards are shown on the left.

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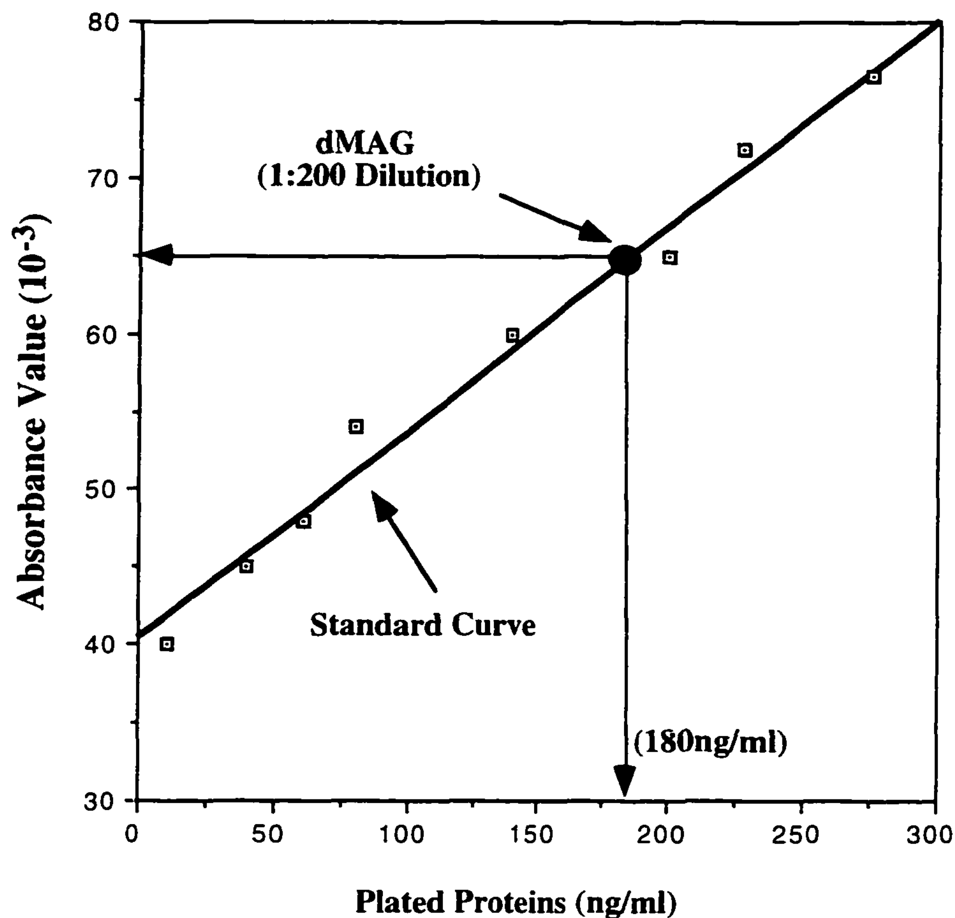

Fig. 5.4 Evaluation of dMAG concentration in myelin-conditioned media by ELISA. After coating an ELISA plate with the 513 MAG monoclonal antibody, MAG-Fc, at various concentrations (from 10ng/ml to 300ng/ml), was added and measured for absorbance value using a rabbit, polyclonal MAG antibody. The absorbance values of MAG-Fc at different concentrations were used to draw a standard curve. The absorbance value for diluted (1:200) myelin-conditioned medium (dMAG) was also measured, and the dMAG concentration in myelin-conditioned media was estimated by finding the corresponding concentration for its absorbance value on the MAG-Fc standard curve.

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5.2.2 dMAG in Myelin-Conditioned Media Inhibits Axonal Regeneration

The myelin-conditioned (dMAG(+)), myelin-conditioned and MAG-depleted (dMAG(-)), and the gray matter-conditioned (GM) media were tested for their effect on neurite outgrowth from cerebellar neurons grown on L1 (Fig. 5.2). As shown in Fig. 5.5, neurons extend very long neurites in gray matter-conditioned medium. In contrast, neurite outgrowth in myelin-conditioned medium is inhibited by 40% compared to that in gray matter-conditioned medium. However, when the myelin-conditioned and dMAG-depleted medium is used, there is no inhibition of neurite outgrowth. In addition, if the 513 MAG monoclonal antibody is added into the myelin-conditioned medium in a neurite outgrowth assay, the inhibitory effect of myelin-conditioned medium on neurite outgrowth is completely abolished (Fig. 5.5). Therefore, the inhibition of neurite outgrowth by myelin-conditioned media is because of the presence of dMAG.

To determine if dMAG is released from myelin in a time-dependent manner, myelin-conditioned media after 0, 1, 4, 7 and 18 hours of incubation time were tested for dMAG content by Western blotting. As shown in Fig. 5.6, dMAG concentration increases sharply from 0 to 4 hours, after which, release of dMAG levels of off. Coincident with an increase in dMAG content in myelin-conditioned media, there is a corresponding increase in inhibition of neurite outgrowth by myelin-conditioned media with incubation time from 0 to 4 hours. The inhibition by myelin-conditioned media reaches maximum level when the media are conditioned for longer than 4 hours (Fig. 5.7). Because we demonstrated previously that dMAG specifically inhibits neurite outgrowth, and we show here that the increase in inhibition by myelin-conditioned media parallels the release of dMAG from myelin, it is suggested strongly that the inhibition of neurite outgrowth by dMAG is dose-dependent.

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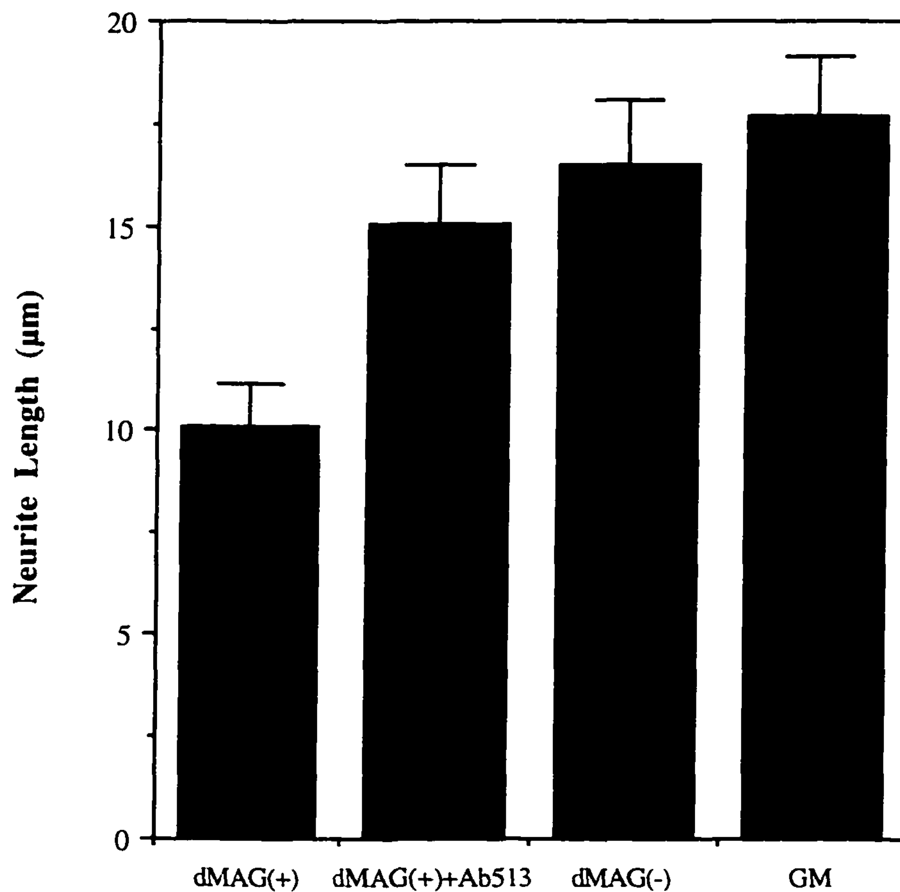

Fig. 5.5 The effect of dMAG on neurite outgrowth from cerebellar neurons. Dissociated cerebellar neurons (PND5) were cultured on immobilized L1-Fc for 18hrs in the presence of myelin-conditioned medium (dMAG+), myelin-conditioned medium with the 513 MAG monoclonal antibody (dMAG(+)+Ab513), myelin-conditioned medium immunodepleted of dMAG (dMAG-) or grey matter-conditioned medium (GM). Media were conditioned over night at room temperature. Where indicated, the 513 MAG monoclonal antibody was added to myelin-conditioned medium at 20μg/ml. Results show the mean length of the longest neurite per cell (±SEM) for 180-200 individual neurons.

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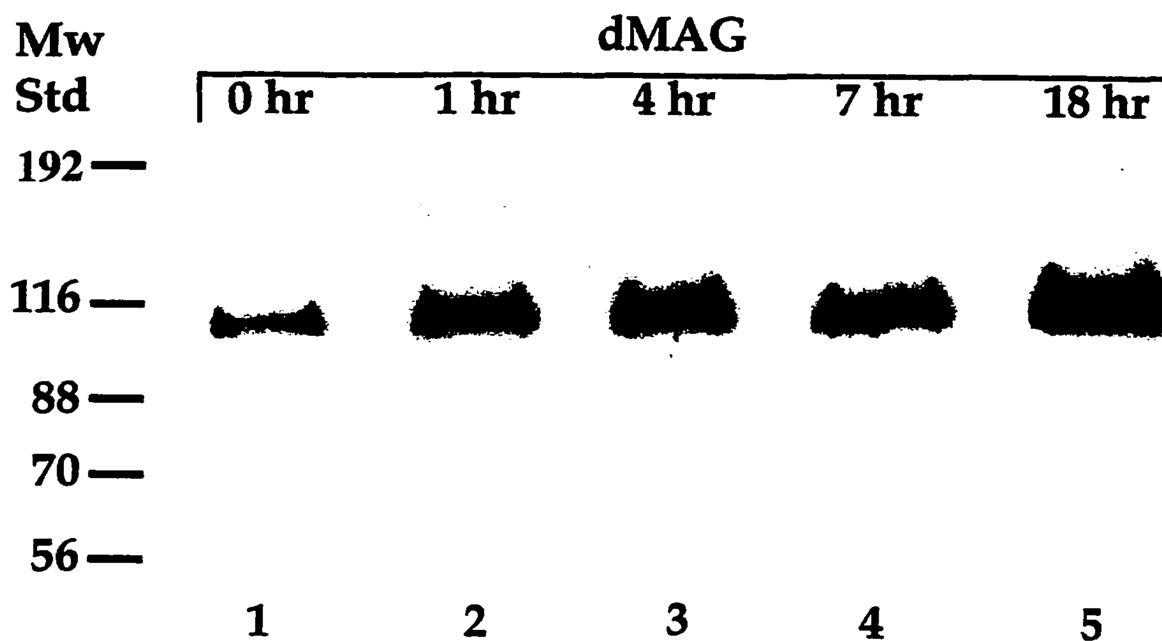

Fig. 5.6 Time-dependent release of dMAG from myelin. Aliquots of myelin-conditioned media were incubated for different times as indicated, and immunostained for MAG on a Western blot. Molecular weight standards are shown on the left.

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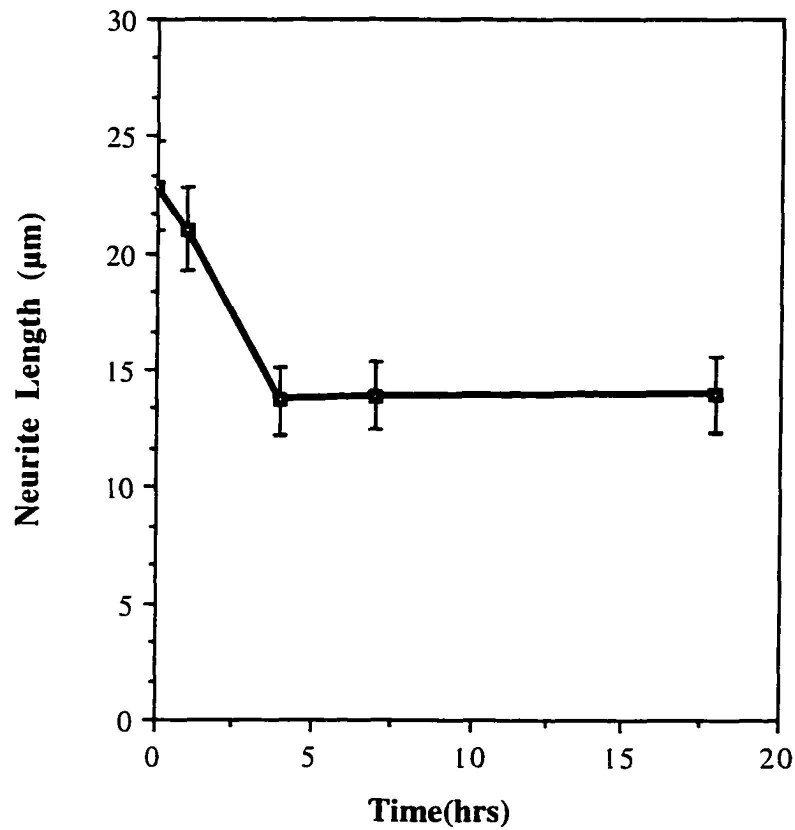

Fig. 5.7 The dose-dependent inhibition of neurite outgrowth by dMAG. Dissociated cerebellar neurons were cultured on immobilized L1-Fc for 18hrs in the presence of myelin-conditioned media that had been conditioned for various times as indicated above. Each point represents the mean length of the longest neurite per cell (±SEM) for 180-200 individual neurons.

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5.2.3 dMAG Exists as a Dimer in Myelin-Conditioned Media

We described in previous chapter (Chapter IV) that recombinant soluble MAG-Fc, as a dimer, can inhibit neurite outgrowth. In addition, we show above that the in vivo soluble form of MAG, dMAG, also inhibits neurite outgrowth. Hence, we want to know if dMAG is also a dimer.

To isolate dMAG from myelin-conditioned media, the myelin-conditioned media were dialyzed, concentrated, and subjected to gel filtration by passage through a Superose 12 FPLC column. The proteins in myelin-conditioned media were separated based on their molecular weights. The eluted fractions were collected, concentrated and subjected to SDS-PAGE and Western blotting for detection of dMAG. dMAG was only detected in the second elution fraction and appeared as a single MAG-positive band on a Western blot (Fig. 5.8 A and B). After calibrating the Superose 12 FPLC column with BioRad Molecular markers, we found that the second fraction has an estimated molecular weight of 180kD (Fig. 5.9). As mentioned previously, monomeric dMAG has a molecular weight about 90kD, the dMAG in the second elution fraction with a molecular weight of 180kD suggests that dMAG exists as a dimer in myelin-conditioned media.

Fig. 5.8 (Next Page) (a) Gel filtration chromatography of myelin-conditioned media. Elution profile of myelin-conditioned media resolved on a Superose 12 FPLC column in PBS buffer. Three elution fractions appears in the profile with high absorbance units. (b) Western blot immunostained for dMAG in the elution fractions. The three elution fractions (lane 1, 2 and 3) were immunostained for dMAG on a Western blot. MAG-Fc was included on the Western blot as a control (lane Ctrl). Molecular weight standards are shown on the left.

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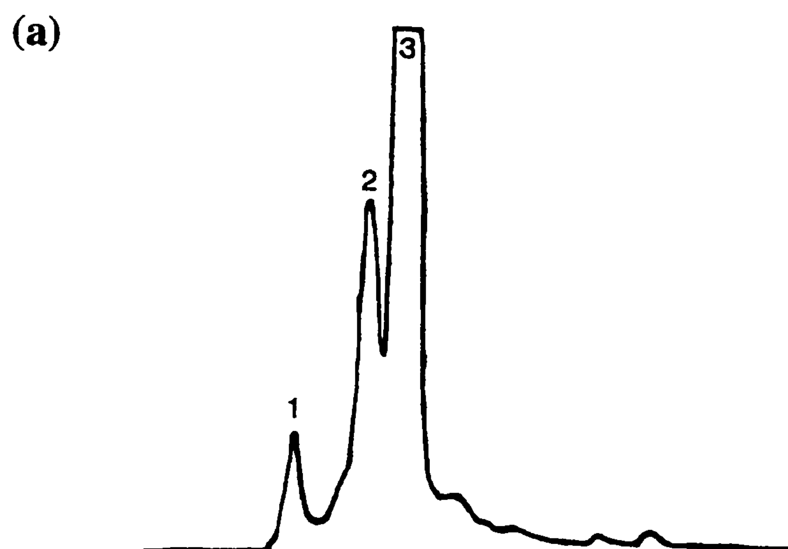
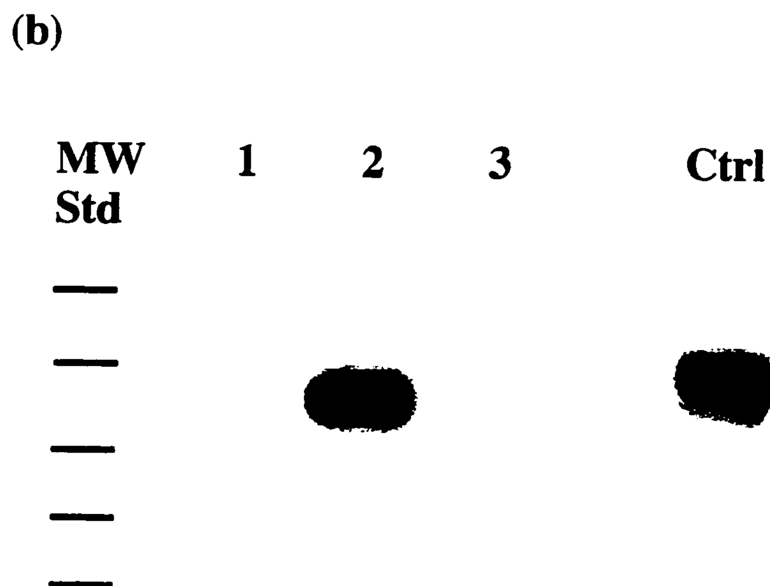
(a)



(b)



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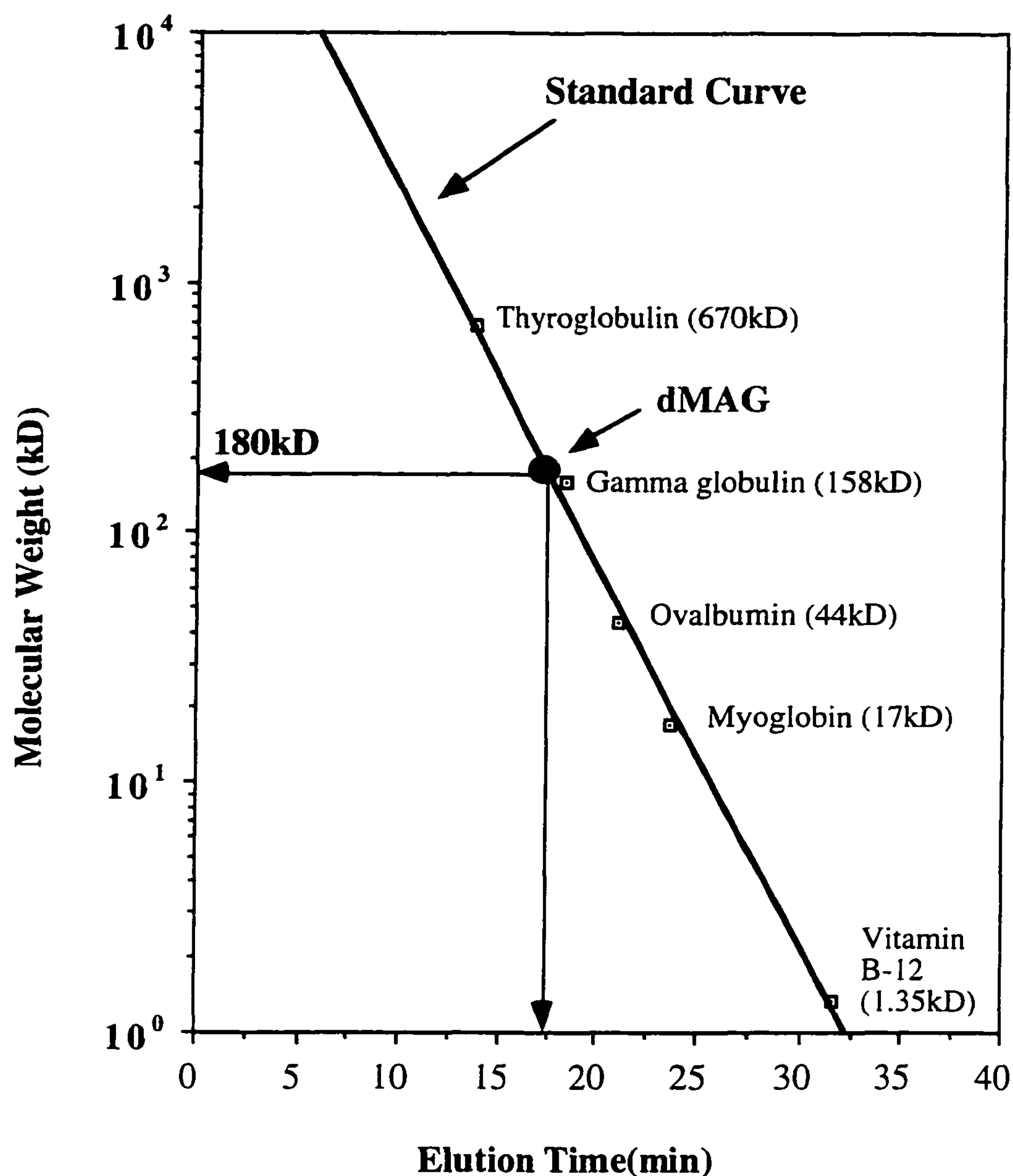

Fig. 5.9 Evaluation of molecular weight of dMAG isolated from myelin-conditioned media. The standard curve was derived from gel filtration standard proteins, Thyroglobulin (bovine, 670kD), Gamma globulin (bovine, 158kD), Ovalbumin (chicken, 44kD), Myoglobin (horse, 17kD) and Vitamin B-12 (1.35kD). The position of dMAG is also shown (●).

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5.2.4 dMAG is Released from Spinal Cord after Injury

Because dMAG is readily released from purified myelin (Möller, 1996) which in effect is disrupted myelin, it is conceivable that dMAG is also released from disrupted myelin after injury in vivo. To determine if dMAG is released from spinal cord after injury, spinal cords were removed from adult rats and placed in Sato media. Damage to the spinal cord was created by crushing at a number of points along its length. Both crushed and uncrushed spinal cords were incubated overnight in Sato media, and the media were collected and tested for dMAG by SDS-PAGE gel and Western blotting. The Western blot results show that the release of dMAG from crushed spinal cords is six-fold higher than that from uncrushed spinal cords (Fig. 5.10 A and B). The appearance of dMAG in the media of uncrushed spinal cords is most likely because removing spinal cords from the animals may cause damage to myelin and result in release of dMAG. However the amount of dMAG released after the spinal cords are crushed is much higher. Therefore, these results suggest that spinal cord injury causes a significant increase in release of dMAG from myelin.

Fig. 5.10 (Next Page) Release of dMAG after spinal cord injury. (a) Western blot immunostained for dMAG after spinal cord injury. Both crushed and uncrushed spinal cords were incubated overnight in Sato media, and the media were collected and tested for dMAG on a Western blot. Lane 1, uncrushed spinal cords; lane 2, crushed spinal cords. Molecular weight standards are shown on the left. (b) Quantitative analysis of release of dMAG from injured spinal cords by desitometry. The dMAG bands in the Western blot shown in (a) were measured for their intensity by desitometry. The band intensity of dMAG from crushed spinal cords is 6-fold higher than that from uncrushed spinal cords.

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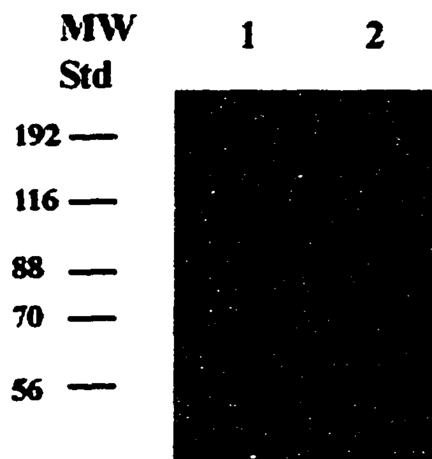
(a)



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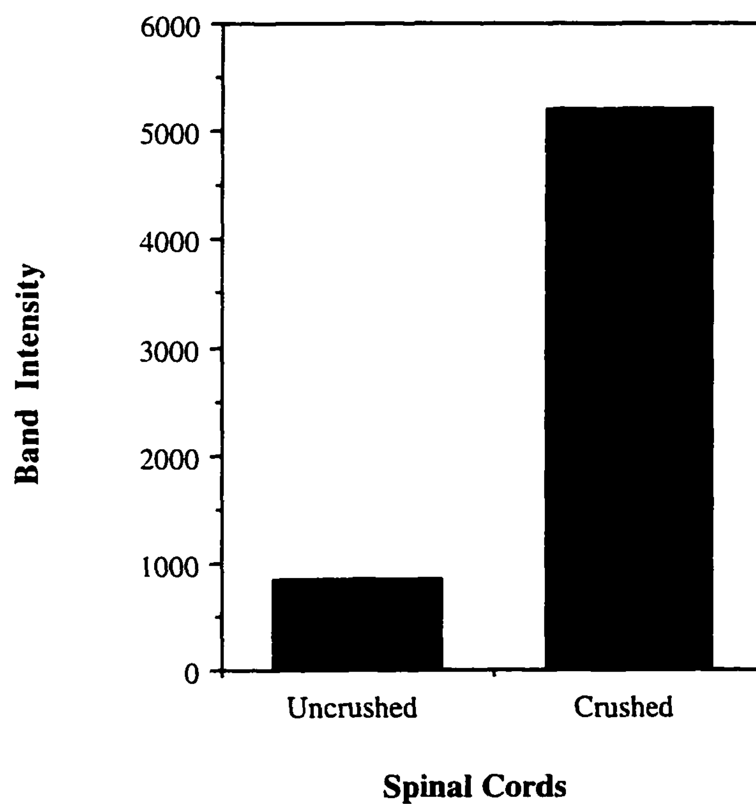

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5.3 Conclusion and Discussion

Here, we report that after overnight incubation of purified rat myelin there is an abundance of dMAG released into the media. When isolated cerebellar neurons are cultured in the myelin-conditioned media, neurite outgrowth is inhibited by 40% compared to neurons grown in gray matter-conditioned media. This inhibition is dMAG-specific as the 513 MAG monoclonal antibody abolishes the inhibition, and immunodepletion of dMAG from myelin-conditioned media completely eliminates the inhibition. In addition, we also demonstrated that dMAG is released from myelin in a time-dependent manner and dMAG inhibits neurite outgrowth in a dose-dependent manner. Therefore, along with MAG and other putative inhibitors remaining in the myelin membrane, dMAG may also contribute to the inhibition of axonal regeneration after injury.

A standard assay to monitor the inhibitory properties of CNS myelin is to culture neurons on purified myelin and assess neurite outgrowth (Caroni and Schwab, 1988b; Bartsch et al., 1995; Li et al., 1996). However, our studies as well as those from others (Möller, 1996) show that depending on how myelin is prepared, the content of MAG in purified myelin can vary considerably. Möller recently demonstrated that 50% of MAG is converted to dMAG in purified myelin from rodents and bovine after overnight incubation. A similar conversion of MAG to dMAG needs only 2 hour incubation for non-human primate samples and as short as 5min for human myelin samples (Möller, 1996). In other words, for the purified myelin used in neurite outgrowth assays, if myelin is freshly prepared, the MAG content is high in myelin, in contrast, if the myelin is stored and washed before use, the MAG may be lost as dMAG is washed away. Hence, the species difference in the rate for MAG to dMAG conversion and whether dMAG is washed away from purified myelin may cause variations in inhibition of neurite outgrowth by myelin samples.

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In addition, results from the gel filtration suggest that dMAG exists as a dimer in myelin-conditioned media. As it was shown previously that dimeric MAG-Fc binds specifically to neurons and both soluble forms of MAG (MAG-Fc and dMAG), with the same extracellular domain, inhibit neurite outgrowth, it is most likely that dimeric dMAG can also mediate specific binding to neurons.

We have noted that there is a sharp increase in dMAG released from spinal cords after injury. As it has been shown that both the membrane-bound MAG expressed on transfected CHO or Schwann cells, and the soluble forms of MAG, MAG-Fc and dMAG, inhibit neurite outgrowth (Tang et al., 1997a, b), MAG may exert its locally inhibitory effect via membrane-bound form, but when in its soluble form, it could act over a substantial distance from the site of release. Therefore, not only the membrane-bound MAG but also the endogenous soluble form of MAG, dMAG, must be considered when attempting to encourage axonal regeneration after spinal cord injury.

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Chapter VI

Mapping the Site on MAG for Sialic Acid Binding to Neurons

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6.1 Introduction

We have shown that MAG binds to neurons in a sialic acid-dependent manner and, based on the two criteria put forward by Luo and Raper (1994), MAG is a true inhibitor of neurite outgrowth because MAG induces growth cone collapse (Li et al., 1996) and the soluble forms of MAG inhibit neurite outgrowth (Chapter IV and V; Tang et al., 1997a). Here, we would like to identify the sialic acid binding site on MAG and assess its role in MAG's effect on neurite outgrowth.

The first clue to the identity of the site on MAG for sialic acid binding was from studies on two other sialoadhesin family members, CD22 and sialoadhesin. Through site-directed mutagenesis, residues involved in the sialic acid-dependent binding of CD22 and sialoadhesin were mapped to the GFCC'C" face of the N-terminal domains, centered on Arg130 on CD22 and Arg97 on sialoadhesin (Vinson et al., 1996; van der Merwe et al., 1996). By aligning the first Ig-like domain of MAG, sialoadhesin and CD22, it was noted that Arg118 (R118) in MAG's first Ig-like domain corresponds to the arginine involved in sialic acid-dependent binding of CD22 and sialoadhesin.

Here, we mutated Arg118 of MAG to either an alanine or an aspartate, changing the positive charge to neutral or negative respectively (Shen et al., 1996b). The mutated forms of MAG were expressed either by transfected Schwann or Chinese hamster ovary (CHO) cells, or in soluble forms, and tested for their sialic acid-dependent binding to neurons and their effect on neurite outgrowth. In addition, sialoadhesin, which recognizes the same sialic acid linkage as MAG, and a truncated form of MAG-Fc, consisting of the first three Ig-like domains of MAG, were also studied for their effect on neurite outgrowth. Based on the results from these studies, a model of MAG's interaction and effect on neurite outgrowth was proposed (Tang et al., 1997b).

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6.2 Results

6.2.1 Arg118 in First Ig-Like Domain of MAG is Critical for Sialic Acid-Dependent Binding of MAG to Neurons

When the first Ig-like domains of MAG, sialoadhesin and CD22 are aligned, an arginine corresponding to Arg97 in sialoadhesin, Arg130 in CD22 and Arg118 in MAG appear is conserved in all three proteins (Fig. 6.1). To determine if Arg118 in MAG is involved in sialic acid-dependent binding to neurons, the nucleotides coding for this arginine were mutated to the nucleotides coding for either alanine (R118A), a nondisruptive substitution, or aspartate (R118D), a disruptive substitution. The mutations were created as MAG-Fc molecules, and the full-length MAG which was then expressed by transfected CHO or Schwann cells.

To assess if the conformation of MAG is changed by mutation to Arg118, the ability of a conformation-dependent MAG monoclonal antibody (mAb513) to recognize R118A- and R118D-MAG-Fc was determined in an ELISA assay. mAb513 recognizes the extracellular domain of MAG in its native conformation (Poltorak et al., 1987). Fig. 6.2 shows that when wild-type MAG-Fc, R118A- and R118D-MAG-Fc were immobilized on an ELISA plate, mAb513 bound to both the wild-type and mutated forms of MAG-Fc in a dose-dependent manner. In addition, there was no difference in binding of mAb513 to either the wild-type or mutated forms of MAG-Fc (Fig. 6.2). This result indicates that the native conformation of MAG is maintained in R118A- and R118D-MAG-Fc (Shen et al., 1996b).

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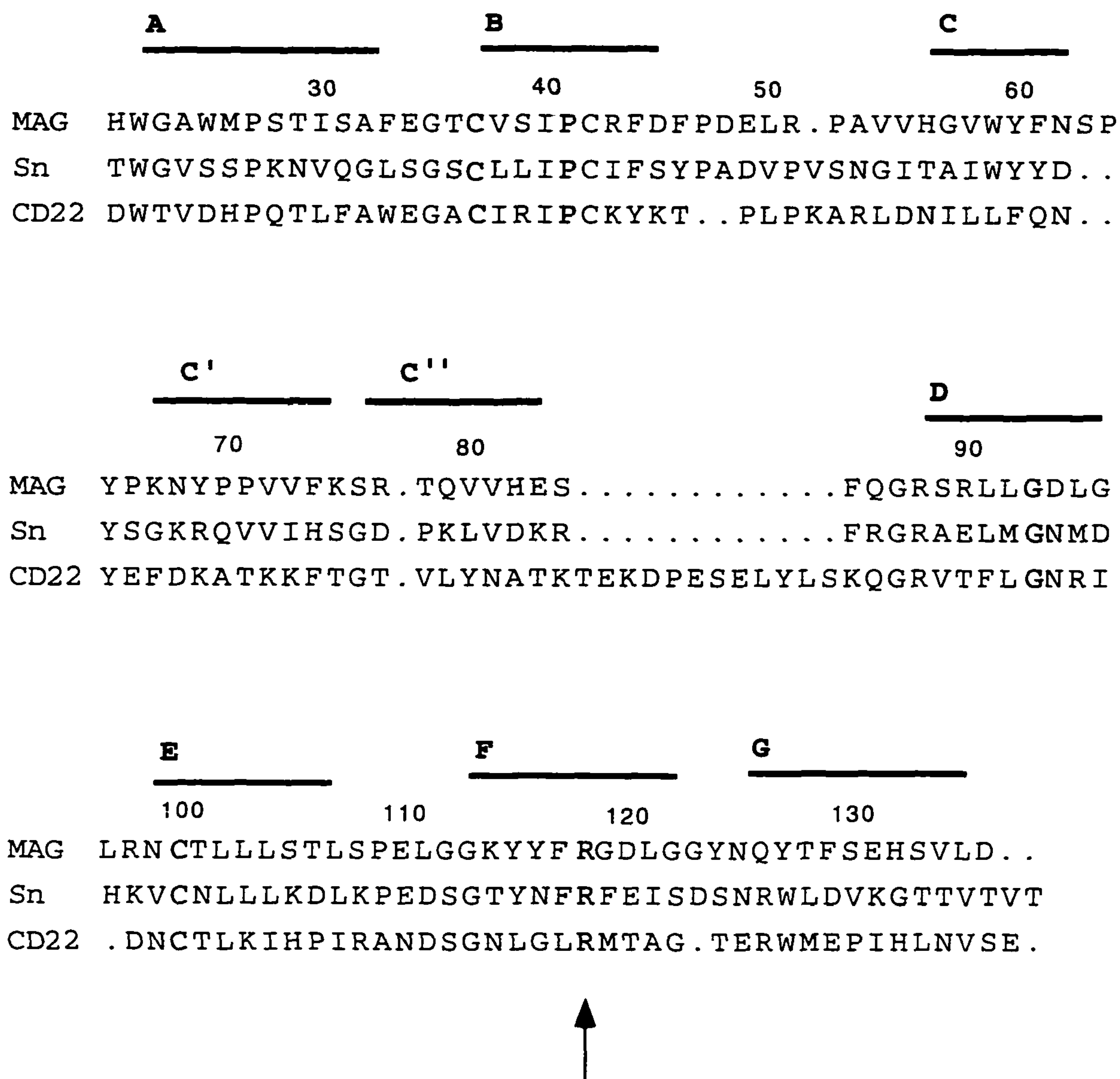

Fig. 6.1 Alignment of the first Ig-domains of sialoadhesin (SN), rat CD22 and MAG. Predicted β-strands are labeled as A, B, C, C', C", D, E and F. Amino acids involved in sialic acid binding of sialoadhesin and conserved in MAG are high-lighted. The arrow refers to Arg118 in MAG.

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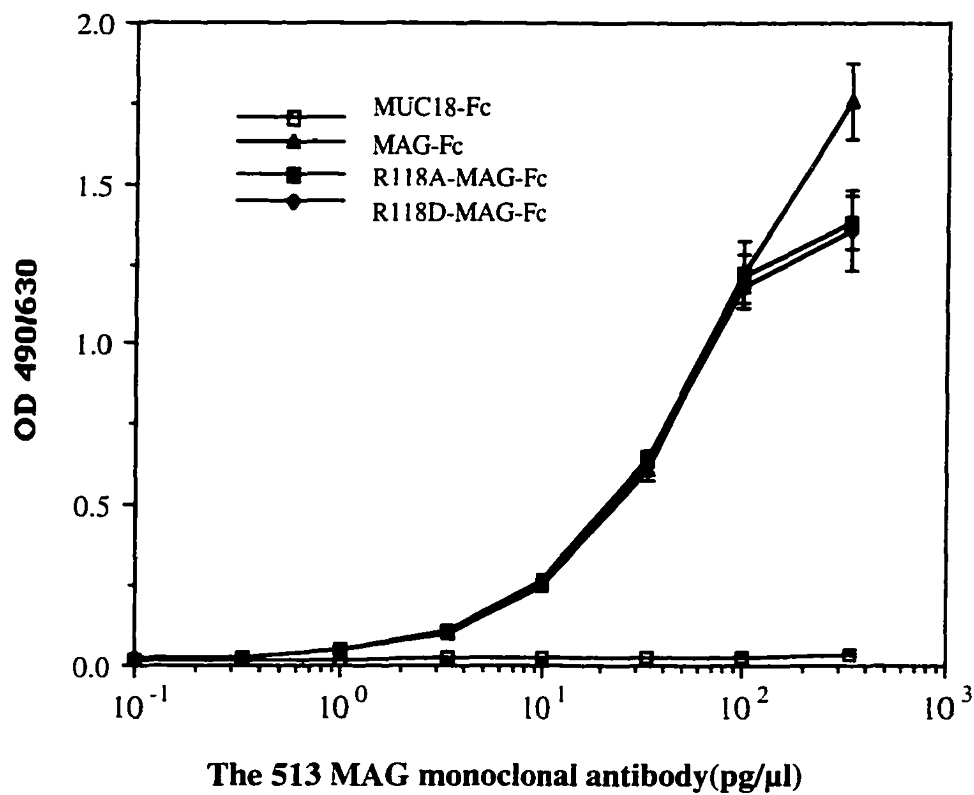

Fig. 6.2 Binding of conformation-dependent 513 MAG monoclonal antibody to mutated MAG-Fc. The Fc-chimeras were immobilized at a concentration of 1μg/ml on an ELISA plate already coated with anti-Fc antibody. The 513 MAG monoclonal antibody was added at various concentrations and bound antibody was detected by ELISA. MAG-Fc (closed triangles), R118A-MAG-Fc (closed squares), R118D-MAG-Fc (open rhombus) or MUC18-Fc (open squares). Results represent 3 experiments (±SEM).

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To determine if R118 is involved in the sialic acid-dependent binding of MAG to neurons, a solid-phase binding assay was performed with wild-type and mutated forms of MAG-Fc. Consistent with our previous results, the wild-type MAG-Fc, at a concentration of 10µg/ml, bound to isolated cerebellar neurons, and there was only background binding of the control chimera, MUC18-Fc. In contrast, at the same concentration, neither R118A- nor R118D-MAG-Fc bound to cerebellar neurons in the solid-phase binding assay (Fig. 6.3). Based on binding of the conformation-dependent MAG antibody (mAb513), both mutated forms of MAG-Fc retain the native conformation of MAG, so the loss of binding of MAG-Fc to cerebellar neurons by either a nondisruptive or disruptive substitution at R118 indicates that this residue is critical for sialic acid-dependent binding of MAG to neurons.

6.2.2 The R118 Binding Site on MAG is Required for Inhibition of Neurite Outgrowth by MAG-Fc

We have shown recently that wild-type MAG-Fc, at a concentration of 25µg/ml, when added to cerebellar neurons in culture, inhibits neurite outgrowth by about 50%, while the MUC18-Fc, at the same concentration, has no effect (Tang et al., 1997a). To determine if a mutation on R118 abolishes the inhibitory effect of MAG-Fc on neurite outgrowth, each mutated form of MAG-Fc, at a concentration of 25µg/ml, was added to cerebellar neurons growing on L1. As shown in Fig. 6.4, although wild-type MAG-Fc inhibited neurite outgrowth by 50%, neither R118A- nor R118D-MAG-Fc had an effect on neurite outgrowth. The neurite length in the presence of mutated forms of MAG-Fc was similar to that from neurons growing with MUC18-Fc (Fig. 6.4). Therefore, it appears that the inhibitory effect of soluble MAG-Fc is entirely dependent on its ability to bind to neurons in a sialic acid-dependent manner.

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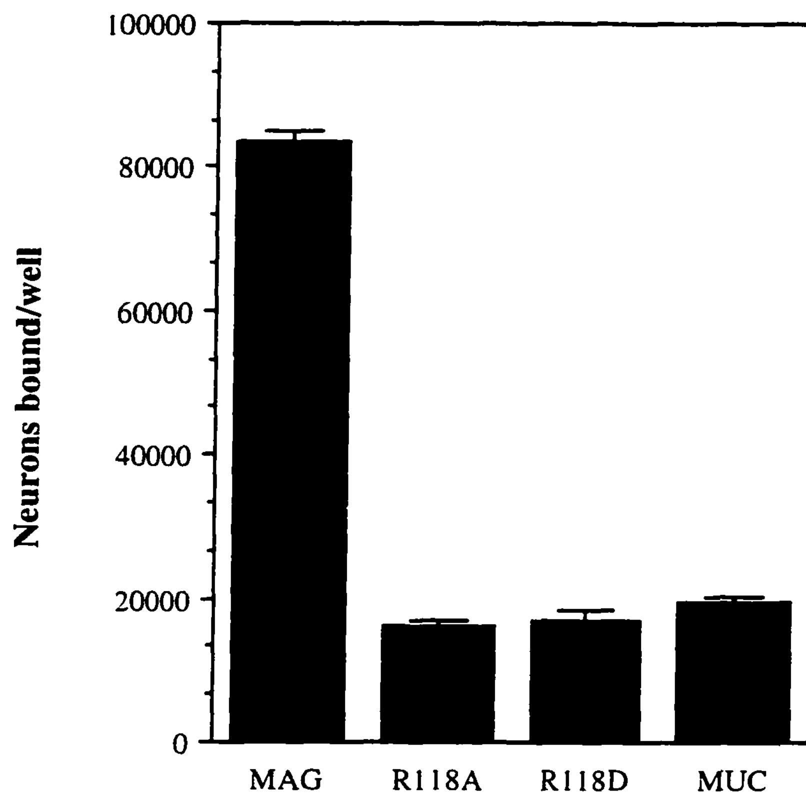

Fig. 6.3 Binding of R118-mutated MAG to cerebellar neurons. Dissociated PND4 cerebellar neurons, vitally labeled with calcein AM, were allowed to bind to MAG-Fc (MAG), R118A-MAG-Fc (R118A), R118D-MAG-Fc (R118D) or MUC18-Fc (MUC) immobilized at a concentration of 10μg/ml, on a 96-well dish. 200,000 neurons were added to each well and after incubation and washing, the number of cells bound was quantitated with a FluorImager. Results are from three experiments, each with 10 samples and represent the mean (±SEM).

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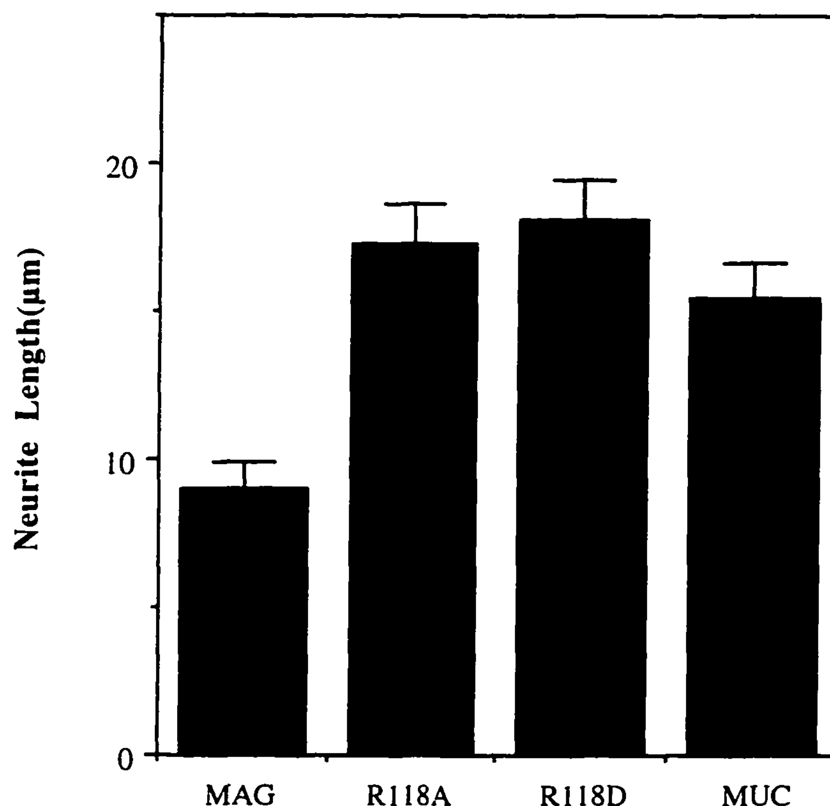

Fig. 6.4 The effect of R118-mutated MAG-Fc on neurite outgrowth. Dissociated PND4 cerebellar neurons were incubated with MAG-Fc (MAG), R118A-MAG-Fc (R118A), R118D-MAG-Fc (R118D), or MUC18-Fc (MUC) at a concentration of 25μg/ml before being plated onto a substrate of L1-Fc immobilized on wells coated with anti-Fc antibody. Neurons were cultured for 18hrs, fixed and immunostained for GAP43, and neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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6.2.3 Sialic Acid-Dependent Binding of MAG to Neurons is Necessary but Not Sufficient for Inhibition of Neurite Outgrowth by MAG-Fc

It has been demonstrated that members of the sialoadhesin family are all sialic acid binding proteins, but each recognizes sialic acid in a different linkage: CD22 prefers to bind α 2, 6-linked sialic acid attached to N-linked glycoconjugates, sialoadhesin (SN) prefers α 2, 3-linked sialic acid attached to either N- or O-linked glycoconjugates, and MAG prefers α 2, 3-linked sialic acid attached to O-linked glycoconjugates (Kelm et al., 1994). To assess if other members of the sialoadhesin family bind to neurons, like MAG, in a sialic acid-dependent manner, we performed a solid-phase binding assay with the chimeric forms of CD22, SN and MAG. Fig. 6.5 shows that binding of cerebellar neurons to MAG-Fc and SN-Fc is significant and equivalent, but there is no binding of CD22-Fc. The binding of SN-Fc to neurons is also in sialic acid-dependent manner, as desialylation completely abolishes this binding (Fig. 6.5). This result is expected as SN, but not CD22, binds the same sialic acid linkage recognized by MAG.

In addition, a truncated form of MAG-Fc, consisting of only the first three Ig-domains and termed MAG(d1-3)-Fc, was used in the binding assay. The binding of MAG(d1-3)-Fc is equivalent to that of full-length MAG-Fc, and this binding can also be eliminated by desialylation of the neurons (Fig. 6.5). This result is consistent with our previous observation showing that the R118 in the first Ig-like domain of MAG is critical for MAG binding to neurons in a sialic acid-dependent manner as MAG(d1-3)-Fc retains R118.

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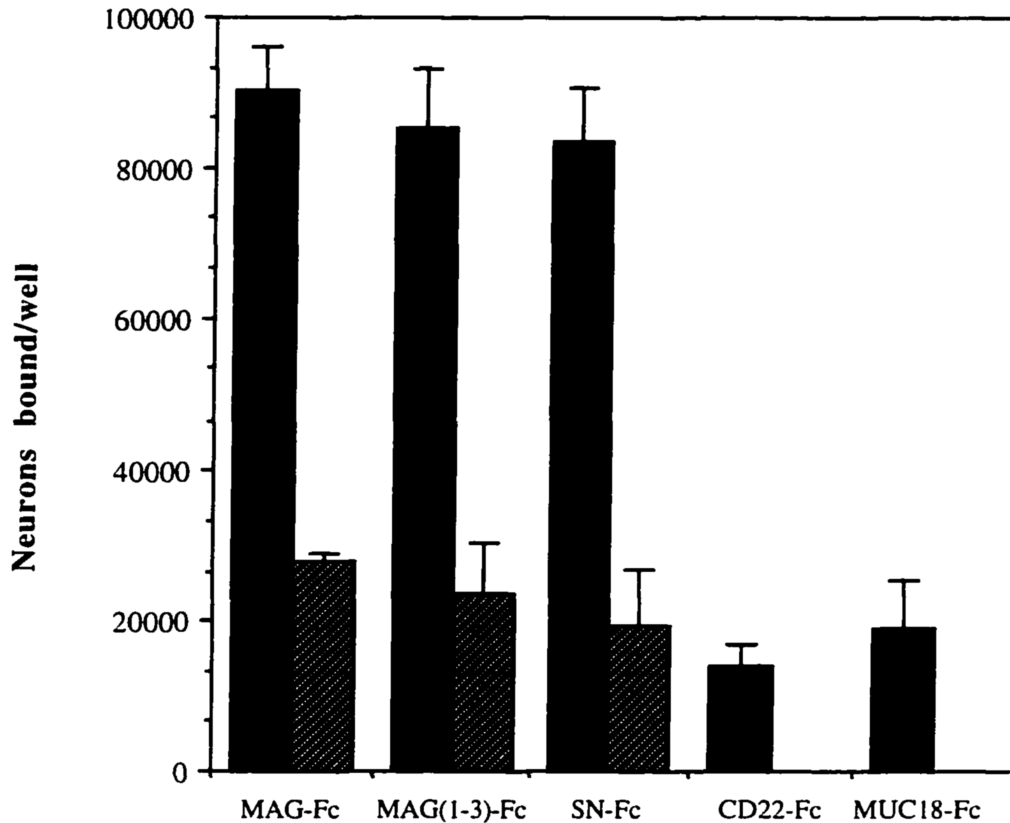

Fig. 6.5 Binding of sialoadhesin-Fc, CD22-Fc and MAG(d1-3)-Fc to cerebellar neurons. Dissociated PND4 cerebellar neurons, vitally labeled with calcein AM, were allowed to bind to MAG-Fc, MAG(d1-3)-Fc, sialoadhesin(SN)-Fc, CD22-Fc or MUC18-Fc immobilized at a concentration of 10 μg/ml, on a 96-well dish coated with anti-Fc antibody. 200,000 neurons, either pre-treated with sialidase (stippled bars) or not (solid bars) were added to each well and after incubation and washing, the number of cells bound was quantitated with a FluorImager. Results are from three experiments, each with 10 samples and represent the mean ±SEM.

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As both SN-Fc and MAG(d1-3)-Fc bind to cerebellar neurons in a sialic acid-dependent manner similar to MAG-Fc (Kelm et al., 1994), we assessed their effect on neurite outgrowth. When MAG-Fc, MAG(d1-3)-Fc, SN-Fc as well as MUC18-Fc, at a concentration of 25μg/ml, were each added to cultures of cerebellar neurons growing on L1, MAG-Fc inhibited neurite outgrowth by 50%, but there was no inhibition of neurite outgrowth by MAG(d1-3)-Fc or SN-Fc (Fig. 6.6a). A similar result was obtained, when cerebellar neurons were grown on a 3T3 cell monolayer in the presence of MAG-Fc, MAG(d1-3)-Fc or SN-Fc: MAG-Fc inhibited the neurite outgrowth by 40%, but neither MAG(d1-3)-Fc nor SN-Fc had any effect on neurite outgrowth (Fig. 6.6b). Therefore, although mutation of R118 in MAG-Fc indicates that sialic acid-dependent binding is required for full-length MAG-Fc to inhibit neurite outgrowth, the results from the neurite outgrowth assay with both MAG(d1-3)-Fc and SN-Fc suggest that the sialic acid-dependent binding alone is not sufficient to mediate MAG's inhibition of neurite outgrowth.

Fig. 6.6 (Next Page) The effect of sialoadhesin-Fc, CD22-Fc and MAG(d1-3)-Fc on neurite outgrowth from cerebellar neurons grown on (a) L1-Fc or (b) a 3T3 cell monolayer. Dissociated PND4 cerebellar neurons were incubated with MAG-Fc, sialoadhesin(SN)-Fc, MAG(d1-3)-Fc, or MUC18-Fc (MUC-Fc) at a concentration of 25μg/ml before being plated onto (a) immobilized L1-Fc or (b) a 3T3 cell monolayer. Neurons were cultured for 18hrs, fixed and immunostained for GAP43, and neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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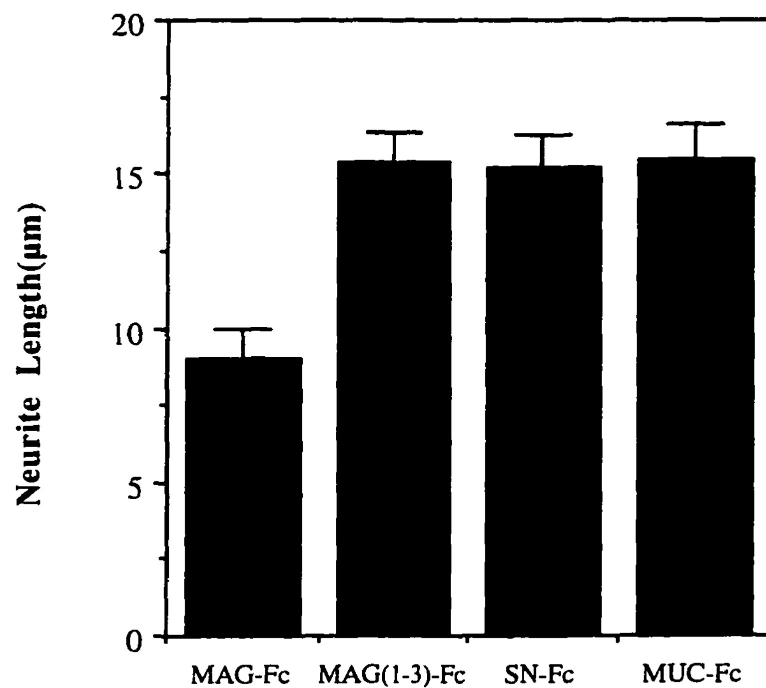

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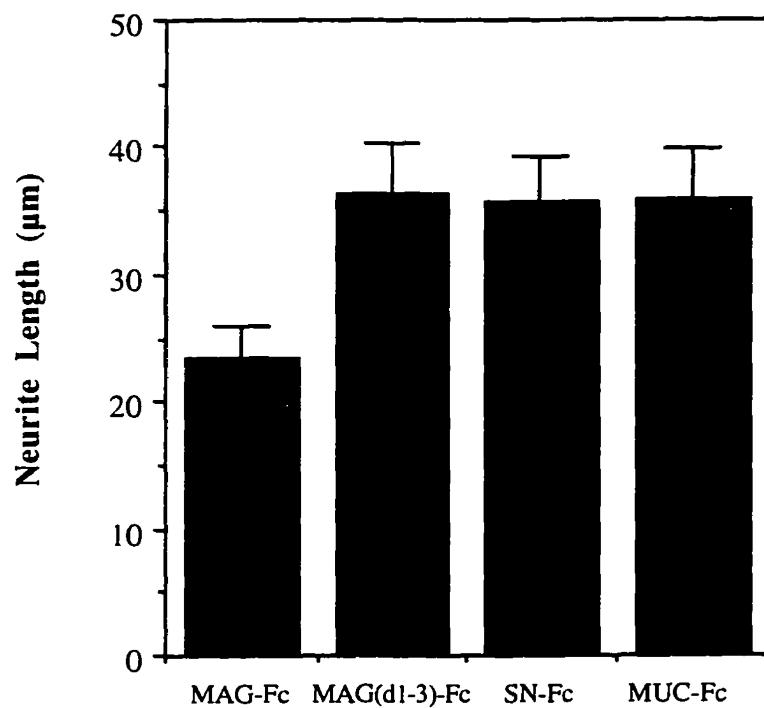

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6.2.4 Characterization of Transfected CHO and Schwann Cells Expressing High Levels of Mutated MAG

To test the effect of mutation at R118 in MAG on inhibition of neurite outgrowth by full-length MAG expressed by cells, cDNAs coding for full-length, mutated MAG in the pSJL vector were transfected into CHO and Schwann cells as described previously (Mukhopadhyay et al., 1994; Tang et. al., 1997b).

The transfected CHO cells were single-cell cloned, and a number of colonies were screened for expression of the mutated forms of MAG (Tang et al., 1997b). For the Schwann cells, a spontaneously transformed Schwann cell line, which does not express MAG and is permissive for neurite outgrowth, was used (Porter et al., 1987; Owens et al., 1990). After transfection and selection with G418, resistant colonies of Schwann cells were pooled, and the Schwann cells were immunofluorescently stained with the 513 MAG monoclonal antibody. Then the stained Schwann cells were sorted by FACS for the transfected cells expressing high levels of MAG which were then expanded and further selected with G418 (Tang et al., 1997b).

The expression of wild-type and mutated MAG by transfected CHO and Schwann cells was characterized (1) by immunostaining for MAG with a MAG monoclonal antibody (B11F7, O'Shannessy et al., 1985) on Western blots of cell lysates, and (2) by immunofluorescence of live cells with the 513 MAG monoclonal antibody (mAb513, Poltorak et al., 1987).

Immunostaining of a Western blot of the lysates from two clones of transfected CHO cells shows that both mutated forms of MAG, R118A- and R118D-MAG, have a molecular weight of about 100kD, similar to that of wild-type MAG expressed by CHO cells or sciatic nerve (Fig. 6.7). It was also observed from an immunostained Western blot of transfected Schwann cells that R118A-MAG expressed by Schwann cells has a similar molecular weight to

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the wild-type MAG expressed by Schwann cells (Fig. 6.8). In addition, the amount of R118A-MAG and wild-type MAG expressed by Schwann cells were approximately equivalent (Fig. 6.8). These results suggest that both R118A-MAG expressed by CHO and Schwann cells and R118D-MAG expressed by CHO cells are glycosylated to the same extent as wild-type MAG.

It should be noted that no MAG expression was detected by Western blotting in the Schwann cells transfected with R118D-MAG (Fig. 6.8). A small portion of transfected Schwann cells expressed high level of R118D-MAG upon first FACS sorting. After expansion of the first FACS-sorted R118D-MAG transfected cells, the cells were sorted again. There were still only a few transfected Schwann cells expressing high level of R118D-MAG after the second FACS sorting. However, when the double FACS-sorted cells were expanded, there was no MAG detected by Western blotting. These results suggest that the Schwann cells expressing high level of R118D-MAG stopped expressing this protein with passage. A possible explanation is that the Schwann cells expressing R118D-MAG may die after several passages.

To determine whether mutated MAG reaches the surface of transfected CHO and Schwann cells, live cells were immunostained with mAb513. As shown in Fig. 6.9, the transfected CHO cells expressing R118A- and R118D-MAG were immunofluorescently stained with mAb513 and the staining was evenly distributed on the cell surface. Similarly, the transfected Schwann cells expressing R118A-MAG and the Schwann cells expressing wild-type MAG also showed the same distribution of staining with mAb513 (Fig. 6.10). As expected, no MAG was detected on surface of the Schwann cells transfected with R118D-MAG (data not shown). Hence, these results indicate that both mutated forms of MAG expressed by CHO cells and the R118D-MAG expressed by Schwann cells all reach the cell surface and maintain the native conformation of MAG which is recognized by mAb513.

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Fig. 6.7 Western blotting of both mutated forms of MAG expressed by transfected CHO cells. Lysates of transfected CHO cells were immunostained for MAG (arrow). Lane a, control transfected CHO cells; lane b, MAG-expressing cells; lane c, R118A-MAG-expressing cells; lane d, R118D-MAG-expressing cells; and lane e, rat sciatic nerve (lane e). Each lane was loaded with 50ug of protein except for lane e, which was loaded with 30ug. Proteins were separated by polyacrylamide gel electrophoresis (8%), transferred to PVDF membrane, and immunostained for MAG with a monoclonal rat anti-MAG antibody (1:100) (Gift from Dr. Richard Quarles). Molecular weight standards are shown on the left, from top to bottom, 192, 116, 88, 70 and 56kD.

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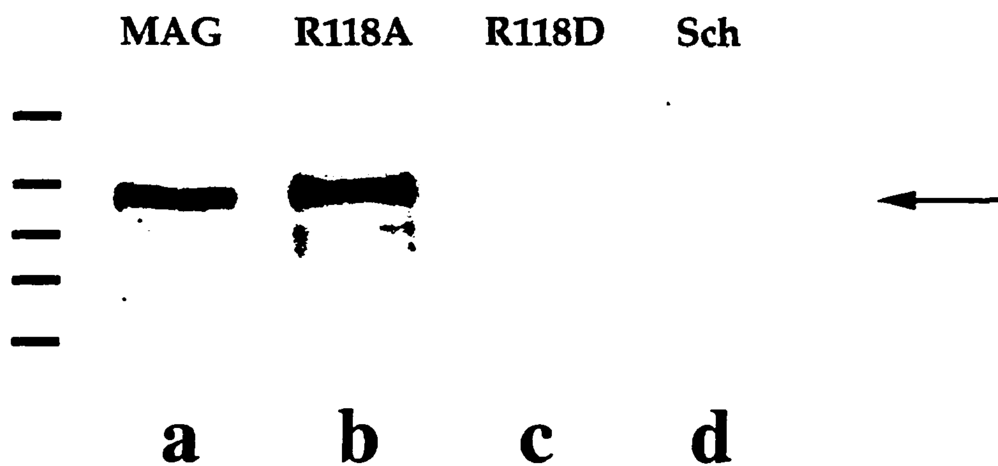

Fig. 6.8. Western blotting of both mutated forms of MAG expressed by transfected Schwann cells. Lysates of transfected Schwann cells were immunostained for MAG(arrow). Lane a: wild-type MAG-transfected Schwann cells (MAG); lane b, R118A-MAG-transfected Schwann cells (R118A); lane c, R118D-MAG-transfected Schwann cells (R118D) and lane d, control transfected Schwann cells (Sch). Each lane was loaded with 20μg of protein. Molecular weight standards are shown on the left, from top to bottom, 192, 116, 88, 70 and 56kD.

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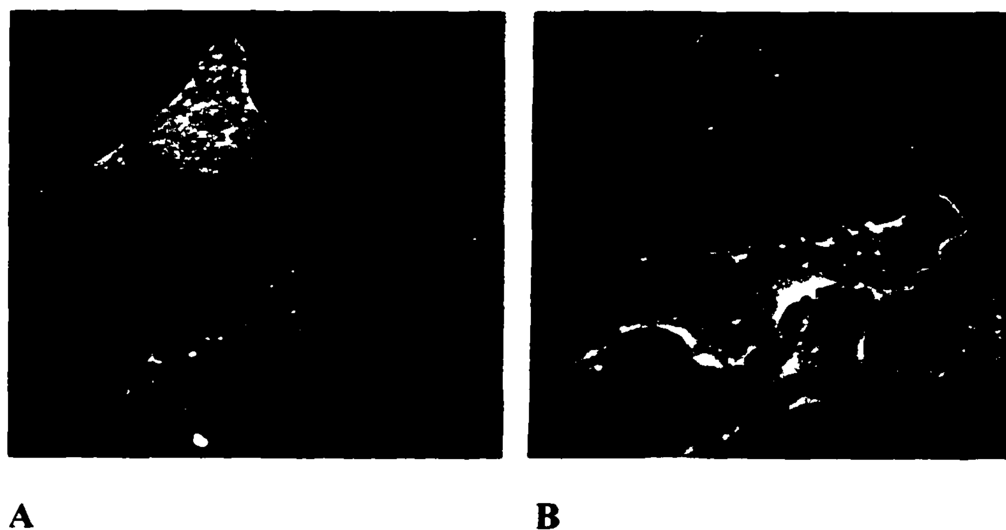

Fig. 6.9 Surface detection of mutated-MAG on transfected CHO cells by immunofluorescent staining. Live CHO cells expressing R118A-MAG (panel A) or R118D-MAG (panel B) were incubated with the 513 MAG monoclonal antibody (5ug/ml), then fixed with 4% paraformaldehyde and incubated with phycoprobe-conjugated, goat anti-mouse IgG (1:50).

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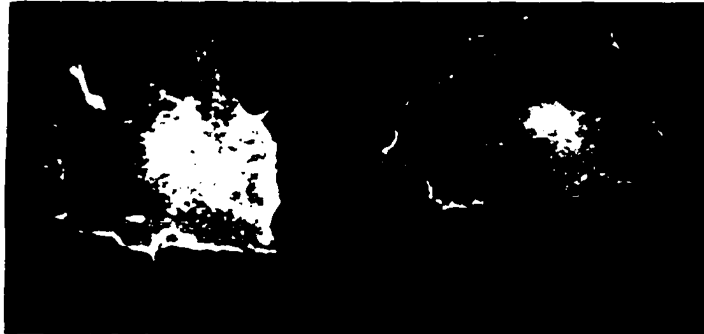

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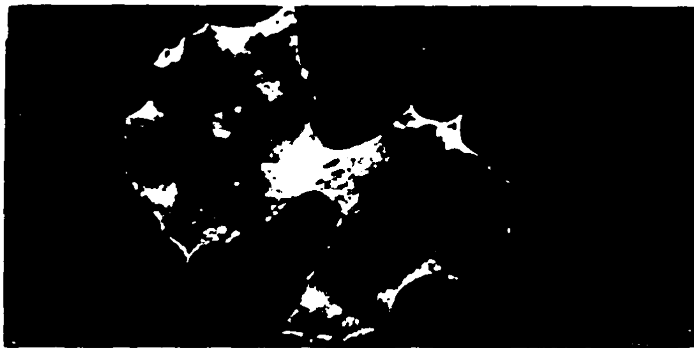

B

Fig. 6.10 Detection of MAG on the surface of transfected Schwann cells by immunofluorescent staining. Live Schwann cells expressing wild-type-MAG (panel A) or R118A-mutated MAG (panel B) were incubated with the 513 MAG monoclonal antibody (5ug/ml), then fixed with 4% paraformaldehyde and incubated with phycoprobe-conjugated, goat anti-mouse IgG (1:50).

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6.2.5 Mutated MAG Expressed by CHO or Schwann Cells Fails to Mediate Sialic Acid-dependent Adhesion

Before carrying out the neurite outgrowth assay with transfected CHO or Schwann cells expressing mutated forms of MAG, we wanted to find out if the mutated forms of MAG expressed by transfected CHO or Schwann cells lost their sialic acid-binding capacity. To do this, we monitored the binding of human erythrocytes to these transfected cells. Human erythrocytes provide a useful model system for studying sialic acid-dependent binding as they contain well-characterized O- and N-linked sialylated glycans (for review, see Crocker and Kelm, 1997). Recently, human erythrocytes were used to determine the specificity of sialic acids recognized by different members in the sialoadhesin family (Kelm et al., 1994; Sylvie et al., 1995).

Kelm et al. (1994) has demonstrated that the binding of human erythrocytes to the wild-type MAG transiently expressed by COS cells is indicated by formation of rosettes by human erythrocytes on a monolayer of the transfected COS cells (Kelm et al., 1994). Hence, the appearance of rosettes by human erythrocytes on the monolayer of transfected CHO cells expressing wild-type MAG suggests that the wild-type MAG expressed by CHO cells binds to human erythrocytes (Fig. 6.11a). However, high levels of binding of human erythrocytes to MAG-expressing CHO cells were only detectable if the CHO cells were desialylated before the binding assay (Fig. 6.11a). This finding suggests that MAG may bind to endogenous sialoglycoconjugates on the same transfected CHO cell in a cis manner, reducing its availability for sialic acid-dependent interaction with an exogenous target.

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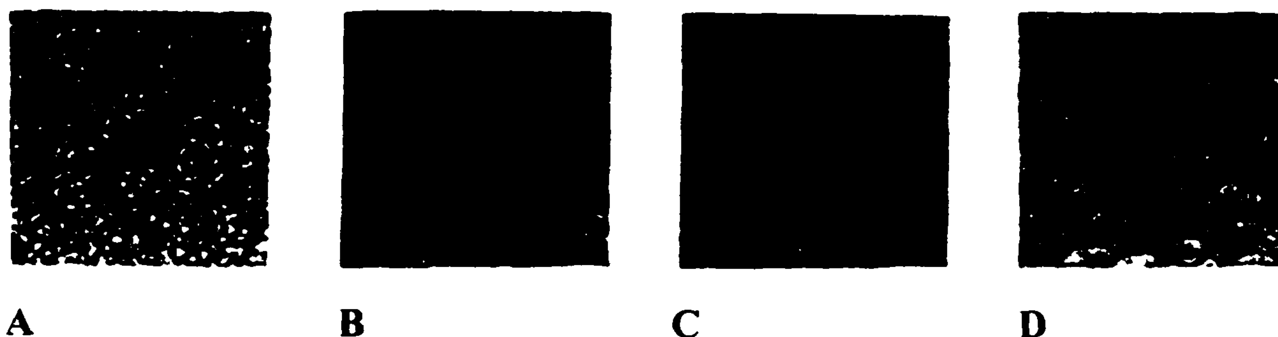

Fig. 6.11 Binding of human red blood cells to transfected CHO cells. Confluent monolayers of MAG-expressing (A) and (B), R118A-MAG-expressing (C) and R118D-MAG-expressing CHO (D) cells were desialylated by incubation with sialidase. A single-cell suspension of human red bloods was added to each monolayer and allowed to bind. For (B) the red blood cells were desialylated before being added to the monolayer. Binding was assessed by appearance of rosettes of human red blood cells bound to the monolayers under phase microscopy.

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Importantly, there was no rosette formed by human erythrocytes on monolayers of transfected CHO cells expressing either of the mutated forms of MAG, regardless of whether the CHO cells were desialylated or not (Fig. 6.11c and d). In addition, the binding of MAG-expressing CHO cells to human erythrocytes was abolished by desialylation of human erythrocytes (Fig. 6.11b). Therefore, the MAG-expressing CHO cells bind to human erythrocytes in a sialic acid-dependent manner, and this binding can be abolished by mutation of R118 to either R118A or R118D, same as we had already established for MAG-Fc.

Similarly, the appearance of rosettes by human erythrocytes was also observed on a monolayer of Schwann cells expressing wild-type MAG again if the monolayer was desialylated prior to the binding assay (Fig. 6.12a). However, no rosette was formed if the human erythrocytes themselves were desialylated (Fig. 6.12b). Notably, no rosette formation was seen on the Schwann cells expressing R118A-MAG, regardless of whether the Schwann cells were desialylated or not (Fig. 6.12c). Therefore, these results indicate that the sialic acid-dependent binding of MAG when expressed by transfected Schwann cells is also abolished by mutation of R118.

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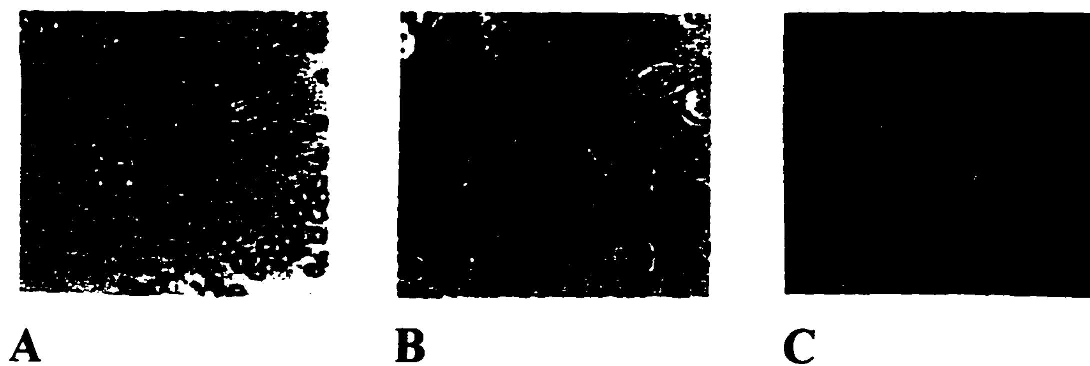

Fig. 6.12 Binding of human red blood cells to transfected Schwann cells. Confluent monolayers of MAG-expressing (A) and (B), R118A-mutated MAG-expressing (C) Schwann cells were desialylated by incubation with sialidase. A single-cell suspension of human red bloods was added to each monolayer and allowed to bind. For (B) the red blood cells were desialylated before being added to the monolayer. Binding was assessed by appearance of rosettes of human red blood cells bound to the monolayers under phase microscopy.

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6.2.6 Mutated MAG Expressed by CHO or Schwann Cells Still Inhibits Neurite Outgrowth

To test the effect of mutated MAG expressed by CHO and Schwann cells on neurite outgrowth, isolated cerebellar neurons were grown overnight on monolayers of CHO cells expressing wild-type MAG, R118A- or R118D-MAG, or Schwann cells expressing wild-type MAG or R118A-MAG. As reported before and shown here (Fig. 6.13), neurite outgrowth was inhibited by about 70% when grown on CHO cells expressing wild-type MAG (Mukhopadhyay et al., 1994; DeBellard et al., 1996), and by about 40% when grown on Schwann cells expressing wild-type MAG (Shen et al., 1996a). Surprisingly, a similar inhibition of neurite outgrowth was apparent when cerebellar neurons were grown on monolayers of transfected CHO cells expressing either of the mutated forms of MAG or Schwann cells expressing R118A-MAG (Fig. 6.13). These results indicate that even though mutation at R118 abolishes the sialic acid-dependent binding of MAG to neurons, the mutated MAG expressed by CHO or Schwann cells is still capable of inhibiting neurite outgrowth.

Fig. 6.13 (Next Page) The effect of R118-mutated-MAG expressed by CHO or Schwann cells on neurite outgrowth from cerebellar neurons. Dissociated PND4 cerebellar neurons were cultured for 18hrs on confluent monolayers of (a) CHO cells expressing wild-type MAG(MAG), R118A-MAG(R118A), R118D-MAG (R118D) or control CHO cells not expressing MAG (Control), or (b) Schwann cells expressing wild-type MAG(MAG), R118A-MAG(R118A) or control Schwann cells not expressing MAG(Sch). Co-cultures were fixed and immunostained for GAP43, and neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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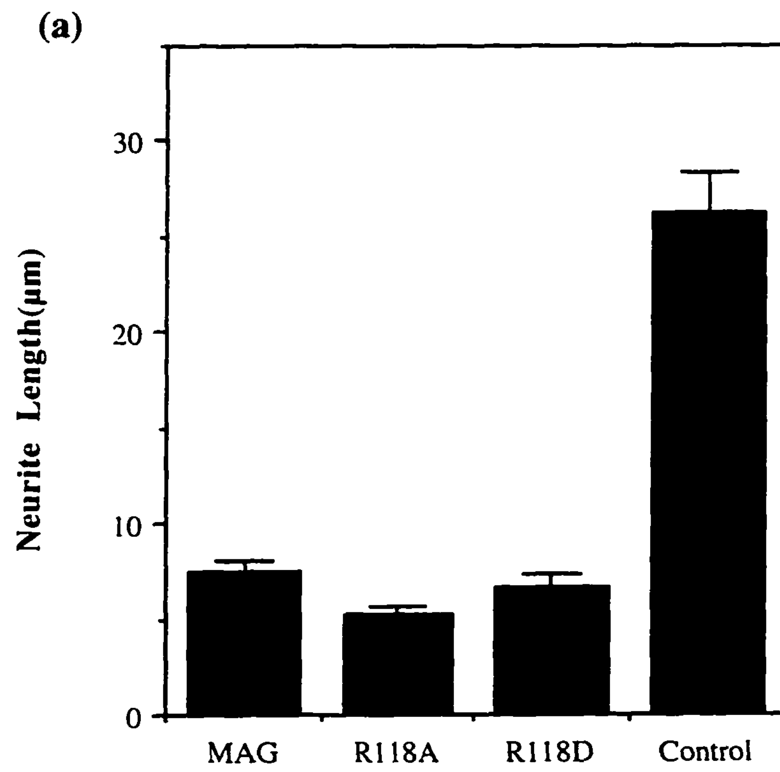

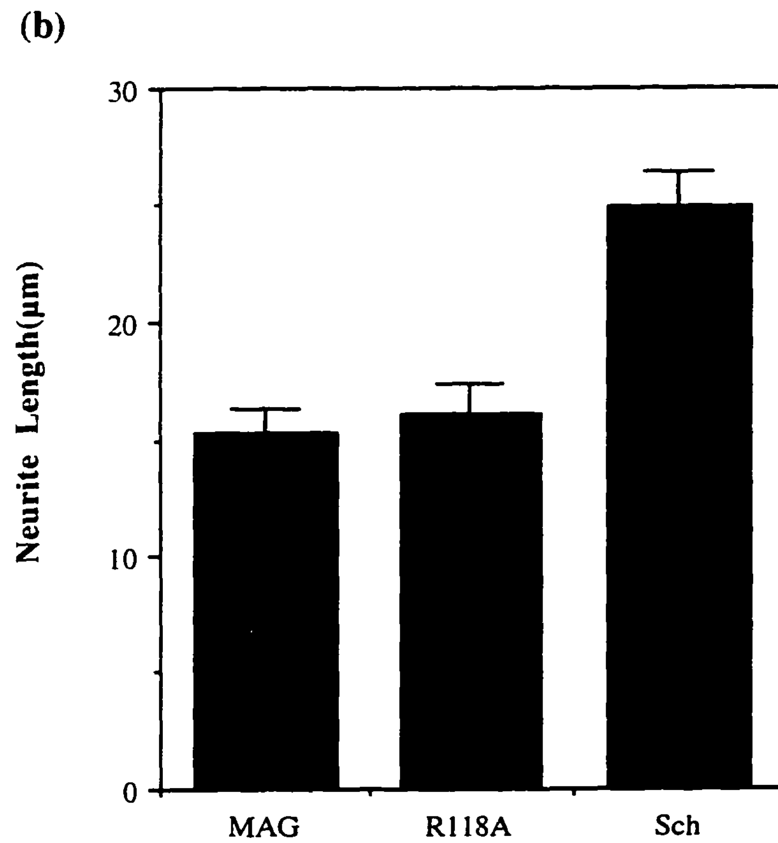

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6.2.7. Sialoadhesin Expressed by Transfected CHO Cells Dose Not Inhibit Neurite Outgrowth

As we have already shown that SN-Fc has no effect on neurite outgrowth even though it can bind to neurons in a sialic acid-dependent manner, we wanted to assess if SN expressed by CHO cells had any effect on neurite outgrowth. The SN-transfected CHO cells used in our neurite outgrowth assays express either a truncated form of SN consisting of Ig-like domains 1-6 or full-length SN with the entire 17 Ig-like domains. As shown before, neurite outgrowth was inhibited by about 70% when cerebellar neurons were grown on a monolayer of transfected CHO cells expressing MAG. In contrast, when cerebellar neurons were grown on the CHO cells expressing either the truncated SN or full-length SN, there was no difference in neurite length compared to neurite outgrowth on the control CHO cells (Fig. 6.14). These results demonstrate that, regardless of whether the sialoadhesin is expressed by transfected CHO cells or in a soluble chimeric form, the sialic acid-dependent binding of sialoadhesin to neurons is not sufficient to inhibit neurite outgrowth. Therefore, it is suggested strongly that MAG must carry a specific site responsible for inhibition of neurite outgrowth, which is different from the sialic acid-binding site at R118 in the first Ig-like domain of MAG.

6.3 Conclusion and Discussion

Through site-directed mutagenesis, R118 in the first Ig-like domain was mutated to either a nondisruptive amino acid, alanine, or a disruptive amino acid, aspartate. This mutation did not drastically affect the native conformation of MAG because the mutated forms of MAG are still recognized by the conformation-dependent MAG monoclonal antibody, mAb513, but it abolished the sialic acid-dependent binding of MAG to neurons. Therefore, R118 in the first Ig-like domain of MAG is critical for the sialic acid-dependent binding of MAG to neurons.

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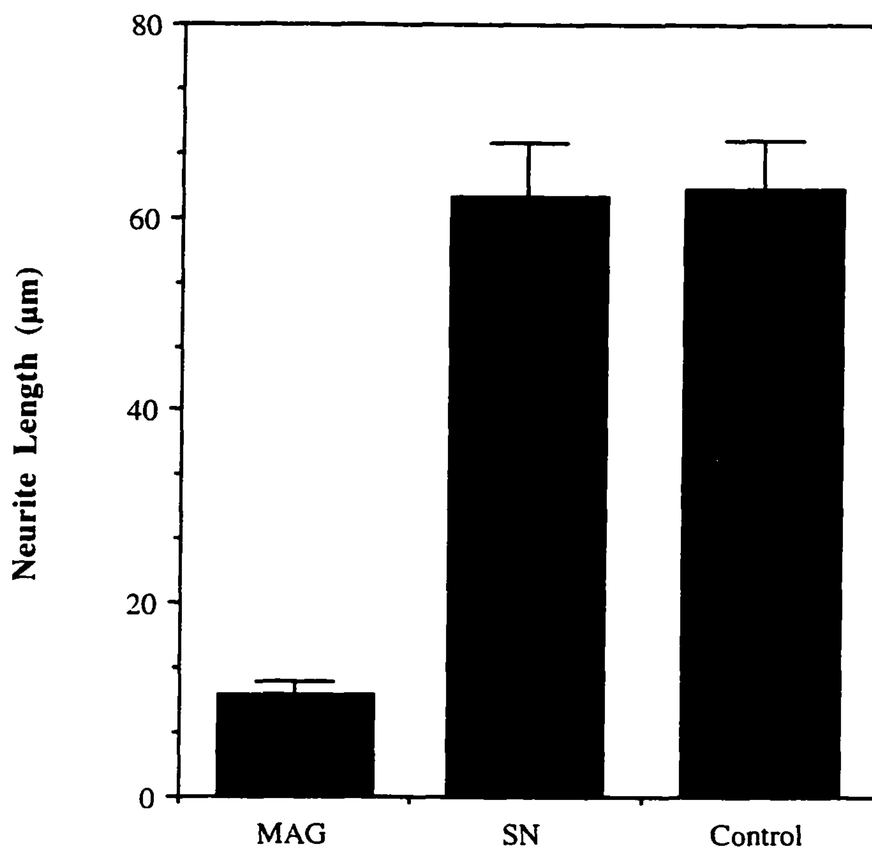

Fig. 6.14 The effect of Sialoadhesin expressed by CHO cells on neurite outgrowth. Dissociated PND4 cerebellar neurons were cultured for 18hrs on confluent monolayers of MAG-expressing(MAG), sialoadhesin-expressing(SN) or control CHO cells (Control). Co-cultures were fixed and immunostained for GAP43 and neurite length was measured. Results are the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

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R118 in MAG was chosen because mutation of an arginine conserved at the same location in two other sialoadhesin family members, sialoadhesin (R97) and CD22 (R130), abolished the sialic acid-dependent binding of these proteins (Vinson et al., 1996; van der Merwe et al., 1996). To aid selection of amino acids for mutagenesis, the V-like domain (the first Ig-like domain) of sialoadhesin was aligned with the V-like domain of CD8, whose structure is known. It was found that disruptive substitutions at six amino acid residues in the first Ig-like domain of sialoadhesin abolished sialic acid-dependent binding with little effect on protein folding. When conservative amino acid changes were introduced at these amino acids, only substitution at R97 led to loss of binding. Based on these results and the studies on the crystal structure of CD8, it was proposed that the six amino acids on sialoadhesin, which are not consecutive, form a sialic acid binding site centered on R97 (Vinson et al., 1996). Similar analysis indicated that R130 in CD22 is the key amino acid in sialic acid-dependent binding of CD22 (van der Merwe et al., 1996). Therefore, it is most likely that R118 in MAG, which is the conserved arginine in the first Ig-like domain of MAG, plays an important role in sialic acid-dependent binding of MAG to neurons. Out results here support this hypothesis (Tang et al., 1997b). Interestingly, the residue R118 in the first Ig domain of MAG is the first residue in the Arg-Gly-Asp (RGD) motif which is known as a sequence that interacts with integrins (Ruoslahti and Pierschbacher, 1987). However, it has been known that the RGD motif is not exposed at the surface of MAG molecule and consequently is not available for interaction (Pedraza et al., 1990; Quarles et al., 1992). Therefore, it is possible that only R118 from RGD motif is involved in the sialic acid-dependent binding of MAG to neurons and this binding is distinct from integrin-mediated binding.

It should be noted that high levels of sialic acid-dependent binding of human erythrocytes to either MAG-expressing CHO and Schwann cells can only be measured if the CHO or Schwann cells are desialylated prior to the binding assay. This is most likely a result of a large portion of MAG expressed by these cells engaging in cis interactions with molecules

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on the same cell, and not available for trans interactions with sialoglycoconjugates on neurons. Similar cis interactions have also been found in other sialoadhesin family members such as CD22 (Braesch-Anderson and Stamenkovic, 1994) and CD33 (Sylvie et al., 1995). However, both MAG-expressing CHO and Schwann cells inhibit neurite outgrowth without being desialylated before the neurite outgrowth assay. Hence, it is suggested that high levels of sialic acid-dependent binding are not essential for inhibition of neurite outgrowth by MAG expressed by CHO or Schwann cells.

Previously, we showed that the inhibitory effect of soluble MAG-Fc on neurite outgrowth is completely dependent on the sialic acid-dependent binding of MAG-Fc to neurons (Tang et al, 1997a), while only a portion of the inhibition exerted by MAG expressed by CHO cells is sialic acid-dependent (DeBellard et al., 1996). Consistent with these observations, mutation of R118 completely abolishes the ability of MAG-Fc to inhibit neurite outgrowth. On the other hand, R118-mutated MAG expressed by CHO or Schwann cells still inhibits neurite outgrowth to the same extent as wild-type MAG even though the cells expressing mutated MAG have lost their sialic acid binding capabilities. In addition, another sialoadhesin family member, sialoadhesin, either expressed by CHO cells or in a chimeric, soluble form, does not inhibit neurite outgrowth even though it binds neurons in a sialic acid-dependent manner and recognizes the same sialic acid linkage as MAG. Furthermore, a truncated form of MAG-Fc, MAG(d1-3)-Fc, also binds to neurons in sialic acid-dependent manner and recognizes the same sialic acid linkage as full length MAG-Fc (Kelm et al., 1994), but, surprisingly, has no effect on neurite outgrowth. Together, these results suggest that (1) the sialic acid-dependent binding of MAG to neurons is necessary, but not sufficient, for soluble MAG to inhibit neurite outgrowth; (2) the sialic acid-dependent binding of MAG to neurons is not required for the inhibitory effect of membrane-inserted MAG on neurite outgrowth. Therefore, another, distinct site on MAG is required to mediate the inhibitory effects of MAG on neurite outgrowth.

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Taking all these results into consideration, we suggest a two site model for MAG's inhibition of neurite outgrowth. As shown in Fig. 6.15, this model suggests that MAG has not only a sialic acid binding site which is at Arg118 in the first Ig-like domain of MAG, but also a neurite outgrowth inhibition site which is distinct from R118. We suggest that, (1) for soluble form of MAG, the binding of MAG to a sialoglycoconjugate on cerebellar neurons initiates the interaction of the neurite outgrowth inhibition site with neurons. This interaction may also be stabilized by the sialic acid-dependent binding, resulting in inhibition of neurite outgrowth by MAG (Fig. 6.15a). (2) Mutation of the sialic acid binding site on MAG-Fc abolishes the sialic acid-dependent binding of MAG-Fc to neurons, therefore the interaction between the neurite outgrowth inhibition site and neurons can not be initiated or maintained (Fig. 6.15b). (3) On the other hand, when MAG is expressed by CHO or Schwann cells, the binding of MAG to a sialoglycoconjugate on cerebellar neurons may facilitate the interaction of

Fig. 6.15 (Next Page) Schematic diagram illustrating the model for MAG inhibitory effect on neurite outgrowth. There are two recognition sites on MAG, a sialic acid binding site (SA, square symbol) and a neurite outgrowth inhibition site (NOG, triangular symbol). When soluble MAG-Fc is added to neurons, both the sialic acid binding and the neurite outgrowth inhibition sites engage the neuron and neurite outgrowth is inhibited (Panel A). However, when R118-mutated MAG-Fc is added to neurons, it cannot bind to neurons and consequently the inhibition site cannot engage the neuron and there is no inhibition of neurite outgrowth (Panel B). When MAG is expressed by CHO cells, both sites engage the neuron and neurite outgrowth is inhibited (Panel C). But when MAG mutated at its sialic acid binding site is expressed in CHO cells, another cell adhesion molecule (CAM) on the CHO cell surface engages the neuron along with the inhibition site and neurite outgrowth is still inhibited (Panel D). In contrast, when MAG(d1-3)-Fc is added to neurons it binds via its sialic acid binding site but does not inhibit axonal growth bccause the inhibition site is absent (Panel E).

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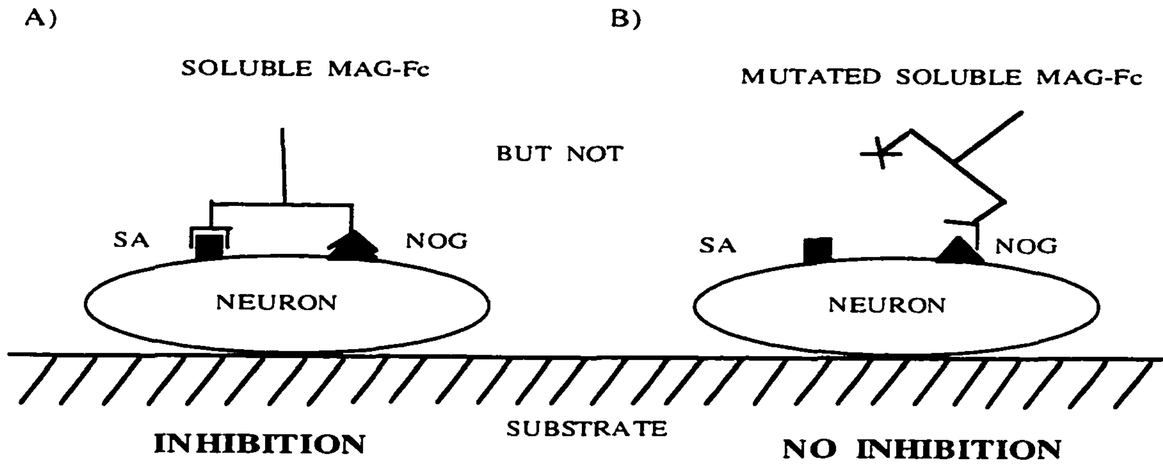

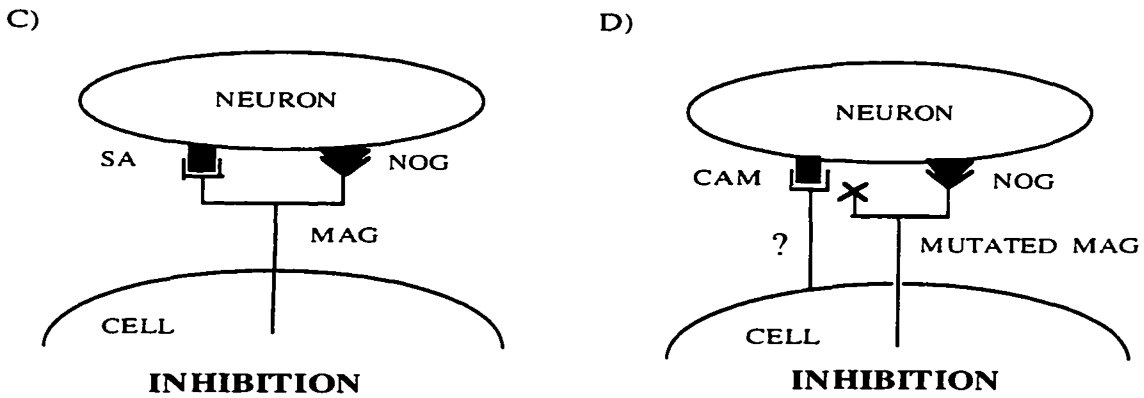

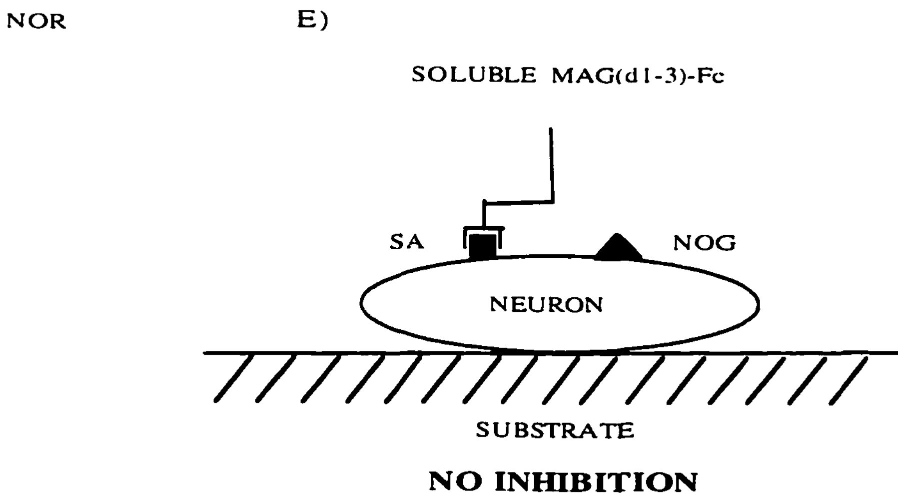

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the neurite outgrowth inhibition site with neurons (Fig. 6.15c). However, this sialic acid-dependent binding is not necessary for the inhibitory effect of membrane-inserted MAG, when R118-mutated MAG is expressed by CHO or Schwann cells, loss of sialic acid-dependent binding of MAG to neurons can be compensated by interaction of other adhesion molecule(s) on these cells. This adhesive interaction allows the neurite outgrowth inhibition site on MAG to interact with, and maintain, its effect on neurons, hence, the neurite outgrowth is still inhibited by CHO or Schwann cells expressing mutated forms of MAG (Fig. 6.15d).

In addition, we have demonstrated that the chimeric form of a truncated MAG, MAG(d1-3)-Fc, consisting of only the first three Ig-like domain of MAG, binds neurons in a sialic acid-dependent manner but does not inhibit neurite outgrowth. Therefore, MAG(d1-3)-Fc carries only one of the two sites, the sialic acid binding site (Fig. 6.15e). As MAG(d1-3)-Fc consists of only the Ig domains 1 - 3 of MAG, the neurite outgrowth inhibition site is most probably located in domains 4 - 5 or requires domains 4 or 5 to function (Tang et. al., 1997b).

In addition to the two-site model for MAG's inhibition of axonal regeneration, the idea of synergistic interaction of two distinct, neuronal recognition sites with neurons has also been proposed for some guidance molecules involved in neurite outgrowth during neuronal development. For example, semaphorins contain a 500 amino acid family "signature" semaphorin domain which specifies the biological activity of each family member (Koppel et al., 1997). Similar to the two-site model for MAG, it is suggested that semaphorins interact with their receptors, neuropilins, through two independent binding sites: one site, at the positively charged carboxy terminus of semaphorins, binds to their receptors with high affinity, while the other site, which is inside the semaphorin domain, mediates biological specificity of each molecule (Koppel et al., 1997; Feiner et al., 1997).

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In conclusion, we provide strong evidences that, (1) R118 is critical for the sialic acid-dependent binding of MAG to neurons, (2) the sialic acid-dependent binding is necessary, but the sialic acid binding alone is insufficient, to induce inhibition of neurite outgrowth by soluble MAG. Therefore, we propose a model that MAG interacts with neurons via two sites, a sialic acid binding site and a neurite outgrowth inhibition site.

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Chapter VII

Summary and Further Directions

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7.1 Summary of my work

The work reported in this thesis has provided strong evidences leading to three important conclusions: (1) MAG binds specifically to neurons in a sialic acid-dependent manner, (2) the soluble forms of MAG, MAG-Fc and dMAG, inhibit neurite outgrowth, and MAG is a true inhibitor of axonal regeneration, and (3) Arg118 in the first Ig-like domain of MAG is the sialic acid binding site on MAG.

7.1.1 MAG Specifically Binds to Neurons in a Sialic Acid-Dependent Manner

To identify the neuronal MAG-binding component, we used a soluble chimeric form of MAG, termed MAG-Fc. MAG-Fc is constructed by fusing the extracellular region of MAG to the Fc region of human IgG. Two binding assays were developed with MAG-Fc, termed the soluble binding assay and the solid-phase binding assay. It was shown in the soluble binding assay that MAG-Fc binds specifically to isolated cerebellar and DRG neurons since this binding can be blocked by a MAG monoclonal antibody. More importantly, MAG-Fc binds neurons in a sialic acid-dependent manner as desialylation of the neurons abolishes the binding (Kelm et al., 1994; Tang et al., 1995). In addition, it was shown in the solid-phase binding assay that MAG-Fc binds to a variety of neurons (retinal, spinal, hippocampal, and superior cervical ganglion neurons) in the same sialic acid-dependent manner as it binds to cerebellar and DRG neurons (DeBellard et al., 1996). Therefore, it is suggested that sialic acid-dependent binding may be a general mechanism mediating MAG:neuron interaction in vivo.

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7.1.2 The Soluble Forms of MAG, MAG-Fc and dMAG, Inhibit Neurite Outgrowth and MAG Is a True Inhibitor on Axonal Regeneration

Using a neurite outgrowth assay, we showed that the soluble, chimeric form of MAG, MAG-Fc, inhibits neurite outgrowth from cerebellar neurons grown on a variety of permissive/promoting substrates, including cell monolayers and immobilized proteins. MAG-Fc inhibits neurite outgrowth in dose-dependent manner. In addition, the inhibition is MAG-specific and sialic acid-dependent as the inhibition can be blocked completely by the 513 MAG monoclonal antibody or by desialylation of the neurons prior to the assay (Tang et al., 1997a). Together with MAG's ability to induce growth cone collapse, MAG is believed to be a truly inhibitory molecule and not merely non-permissive. Importantly, we demonstrated that the soluble form of MAG found in vivo, termed dMAG, also inhibits neurite outgrowth and its inhibition is dose-dependent and MAG-specific. Furthermore, it was found that an abundance of dMAG is released from purified myelin or after spinal cord injury. Taken together, we conclude that the soluble form of MAG released from myelin inhibits axonal outgrowth and may, along with MAG in the membrane, contribute to the lack of axonal regeneration after spinal cord injury.

7.1.3 The Arg118 in the First Ig-like Domain of MAG Is the Sialic Acid Binding Site on MAG

Finally, the sialic acid binding site on MAG was mapped and a functional model for MAG's inhibitory effect on axonal regeneration was proposed. By aligning the amino acid sequences of sialoadhesin family members, a subset of conserved residues was identified in which arginine 118 (R118) in the first Ig-like domain of MAG, is conserved in all three members, sialoadhesin, CD22 and MAG (Vinson et al., 1996; van der Merwe et al., 1996). Mutation of R118 of MAG to either alanine or aspartate abolishes its sialic acid-dependent

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binding and the inhibitory effect of MAG-Fc on neurite outgrowth. Therefore, the sialic acid-dependent binding of MAG to neurons is necessary for MAG-Fc to inhibit neurite outgrowth. However, when full-length MAG-Fc, a truncated form of MAG-Fc missing Ig-like domains 4 and 5, termed MAG(d1-3)-Fc, and another sialic acid binding protein, sialoadhesin are tested in our solid-phase binding assay and neurite outgrowth assay, they all can bind to neurons in a sialic acid-dependent manner, but only full-length MAG-Fc inhibits neurite outgrowth. Therefore, sialic acid binding alone is insufficient to inhibit neurite outgrowth. In addition, when expressed on the surface of either Chinese hamster ovary or Schwann cells, R118-mutated MAG retains the ability to inhibit neurite outgrowth. Taking all these results into consideration, we suggest a function model for MAG's inhibition of neurite outgrowth: MAG has two recognition sites for neurons, the sialic acid binding site at R118 and a distinct neurite outgrowth inhibition site which is absent from the first three Ig-like domains (Tang et al., 1997b).

7.2 Further Directions

The work described in this thesis has laid a solid foundation for continuous work in the following two areas: (1) to study the effect of dMAG on axonal regeneration after spinal cord injury, (2) to map the inhibition site for axonal regeneration on MAG.

7.2.1 To Study the Effect of dMAG on Axonal Regeneration After Spinal Cord Injury

We have demonstrated that dMAG, a soluble form of MAG found in vivo, is a potent inhibitor of axonal regeneration. In addition, we have also shown that the release of dMAG increases sharply after spinal cords are injured in vitro. Hence, it is believable that soluble dMAG released from spinal cords, along with MAG and other inhibitors still in the membrane,

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contributes to the inhibition of axonal growth. We will characterize the effect of dMAG on axonal regeneration after spinal cord injury in the following two experiments:

(1) To assess the inhibitory effect of dMAG released from injured spinal cords in a collagen gel assay. Explants of injured spinal cords will be plated next to rat dorsal root ganglia, and cultured in a three-dimensional collagen matrix or Matrigel (Collaborative Research). Whether dMAG diffused from the explant of injured spinal cords inhibits neurite extension on the proximal side of the ganglia will be observed.

(2) To reverse the inhibitory effect of dMAG after spinal cord injury. It has been reported that axonal regeneration can be encouraged by either transplantation of pieces of peripheral nerves (David et al., 1981), or application of IN-1 antibody, to lesioned rat corticospinal tract (CST) fibers (Schnell and Schwab, 1990). However, only a limited amount of axonal regeneration has been observed. Thus, to investigate blocking of the inhibitory effect of dMAG can further encourage axonal regeneration in addition to transplantation of peripheral nerves or application of IN-1 antibody, we will implant hybridoma cells producing the 513 MAG monoclonal antibodies, or the liposomes incorporated with sialidase or laminin to the lesioned sites of completely transected corticospinal tracts. The neurons and neurites in longitudinal sections of treated or untreated spinal cords will be fluorescent labeled and quantitated for axonal regeneration.

7.2.2 Map the Inhibition Site for Axonal Regeneration on MAG

According to our two-site model of MAG's function, the sialic acid-binding site on MAG is distinct from its neurite outgrowth inhibition site. The sialic acid binding site is at Arg118 in the first Ig-like domain of MAG, but the neurite outgrowth inhibition site probably

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locates to domain 4 and/or 5 (Tang et al, 1997b). Therefore, we will begin to map the neurite outgrowth inhibition site in two steps:

(1) To confirm the location of the neurite outgrowth inhibition site on MAG by domain-swapping analysis. It has been shown that sialoadhesin binds to neurons, but does not inhibit neurite outgrowth. In addition, sialoadhesin has its sialic acid binding site at Arg97 in its first Ig-like domain. To provide more evidence in supporting of the suggestion that the neurite outgrowth inhibition site of MAG locates to domain 4 and/or 5 of MAG, domains 4 and 5 of MAG will be fused to the domains 1 - 3 of sialoadhesin. In addition, another chimeric protein can be constructed by fusing domain 5 of MAG to domain 1 - 4 of sialoadhesin as the neurite outgrowth inhibition site may locate in MAG domain 4 or 5, or require both domain 4 and 5. All the new chimeric proteins, used as Fc-chimeras and expressed by CHO cells, will be tested in our binding assays for sialic acid-dependent binding and in our neurite outgrowth assays for their inhibitory effect on axonal regeneration.

(2) To locate the neurite outgrowth inhibition site on MAG by site-directed mutagenesis. After the neurite outgrowth inhibition site of MAG is located to certain domain on MAG, disruptive substitutions will be carried out at different amino acid residues, termed non-conservative mutation. The disruptive substitution(s) which abolishes the inhibitory effect of MAG on neurite outgrowth with little effect on protein folding will be selected. Then non-disruptive substitutions will be carried out on these selected amino acids, termed conservative mutation. Therefore, the amino acid(s), at which the introduced conservative mutation leads to loss of the neurite outgrowth inhibition effect of MAG, will be denoted as the neurite outgrowth inhibitory site on MAG. The mutated forms of MAG, used as Fc-chimeras and expressed by CHO cells, will be tested in our binding assays for sialic acid-dependent binding and in our neurite outgrowth assays for their inhibitory effect on axonal regeneration.

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Chapter VIII

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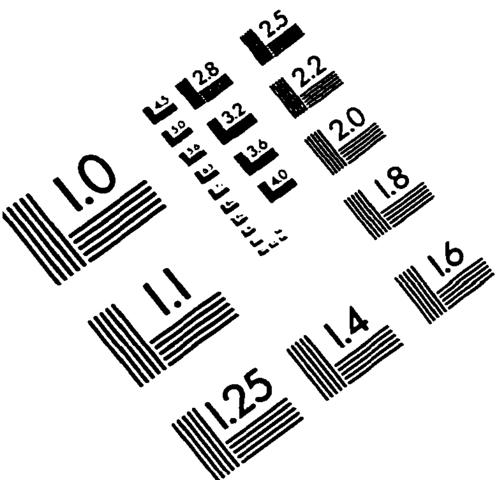
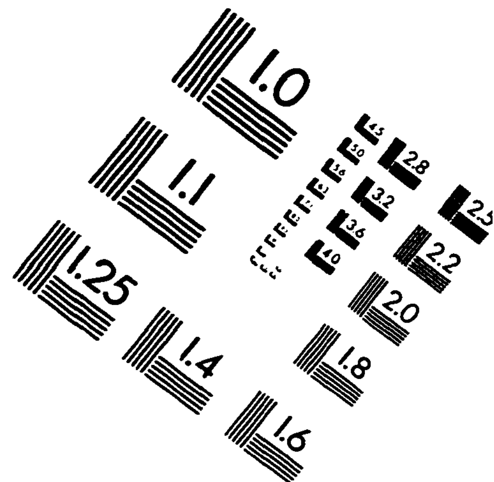
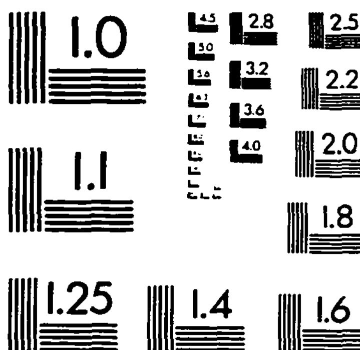
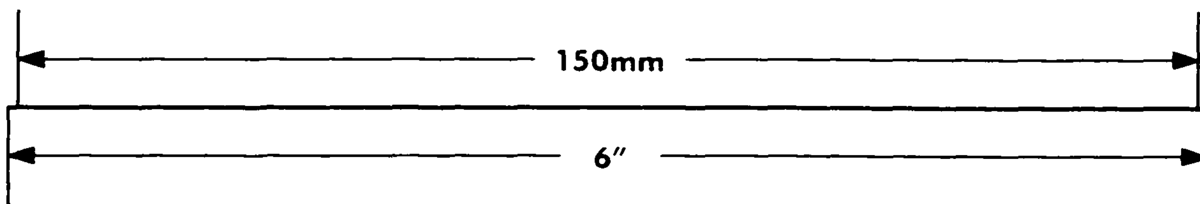
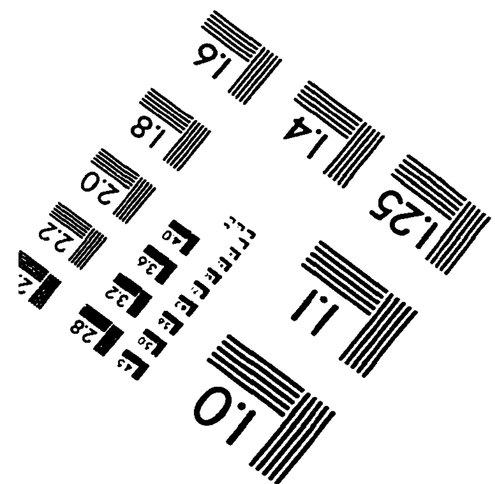
IMAGE EVALUATION TEST TARGET (QA-3)



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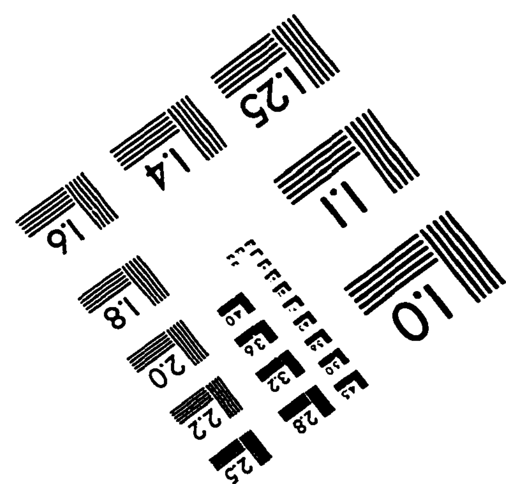

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