

Fibronectin is a binding partner for the myelin-associated glycoprotein (siglec-4a)

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Abstract The myelin-associated glycoprotein (MAG) mediates cell–cell interactions between myelinating glial cells and neurons. Here we describe the extracellular matrix glycoprotein fibronectin as a binding partner of MAG. It has been identified by affinity precipitation with MAG-Fc from NG108-15 cells and by microsequencing of two peptides derived from a 210-kDa protein band. Western blot analysis showed that fibronectin is also present in MAG binding partners isolated from N2A (murine neuroblastoma) cells, rat brain and rat spinal cord. Different fibronectin isoforms have been isolated from brains of young and adult rats, indicating that the expression of MAG binding fibronectin changes during development. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Myelin-associated glycoprotein; Siglec; Sialic acid; Cellular interaction; Extracellular matrix; Fibronectin

1. Introduction

Interactions between neurons and glial cells are essential during the development of the nervous system. The myelin-associated glycoprotein (MAG), a molecule expressed by myelinating glial cells in the central and peripheral nervous systems [1], is one of the molecules involved in these processes. MAG is a member of the siglec family, a group of structurally related Ig-like proteins, which mediate cell adhesion in a sialic acid-dependent manner [2,3]. Several functions in glial cell neuron interaction have been proposed for MAG [4,5]. Although the location of MAG in the periaxonal myelin membrane and in vitro myelination studies have suggested that MAG is involved in the initiation of myelination [6], experiments with MAG knockout mice (MAG^{−/−} mice) provided evidence that MAG is not essential for this process. MAG appears to play an important role in maintaining a stable interaction between axons and myelin, since in aging MAG^{−/−} mice the integrity of myelin and myelinating cells in

the central and peripheral nervous systems deteriorates and the associated axons show clear signs of degeneration [4,7–10].

In addition, in vitro MAG can influence neurite outgrowth in a bifunctional manner. Whereas it promotes neurite outgrowth of neurons at early stages of development such as embryonic dorsal root ganglia, it inhibits neurite outgrowth of neurons at later stages, such as adult dorsal root ganglia or early postnatal cerebellum [11,12]. This neurite outgrowth inhibition activity of MAG has also been demonstrated with cell lines like NG108-15 neuroblastoma cells [13]. In vivo, MAG has been shown to inhibit axonal regrowth after a lesion of adult peripheral nerves [14]. Also in the central nervous system, a reduction in the inhibitory potency of myelin from MAG^{−/−} mice has been observed [15,16]. However, since the effects were relatively small, it is clear that other molecules besides MAG and/or mechanisms are sufficient to inhibit regeneration in the nervous system.

The capability of MAG to influence axonal outgrowth and to stabilize the axon/myelin interaction requires that MAG binds to receptors on the neuronal cell surface. MAG binding to neuronal cells is mainly mediated by *N*-glycans of glycoproteins since inhibition of *N*-glycosylation by swainsonine has been shown to reduce MAG binding to background levels [17]. Interactions between MAG and the extracellular matrix (ECM) proteins collagen [18] and tenascin-R [19] have been described. Furthermore, affinity precipitation experiments with MAG-Fc (recombinant extracellular domains of MAG fused to the Fc fragment of human immunoglobulin) from different neuroblastoma cells, isolated neurons and whole brain or spinal cord of mice and rat have demonstrated that MAG interacts with several glycoproteins which are likely to represent neuron-specific MAG binding partners [17,20] (Streng et al., submitted). By immunoblot analysis of these glycoproteins besides tenascin-R, tenascin-C has been identified as a MAG binding partner (Streng et al., submitted).

However, the molecular nature of the other MAG binding glycoproteins from neuronal sources still has to be identified. Here we demonstrate by microsequencing and immunoblot analysis that some isoforms of cellular fibronectin (Fn) act as MAG binding partners.

2. Materials and methods

2.1. Reagents

D-[6-³H]Glucosamine-HCl was from Amersham, Braunschweig, Germany; Pefablock SC was from Biomol, Hamburg, Germany; protein A-Sepharose beads were from Pharmacia Biotech, Uppsala, Swe-

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Abbreviations: ECM, extracellular matrix; FCS, fetal calf serum; Fn, fibronectin; MAG, myelin-associated glycoprotein; MAG-Fc, recombinant extracellular domains of MAG fused to the Fc fragment of human immunoglobulin

den; Super Signal West Dura Extended Duration Substrate was from Pierce, Rockford, IL, USA; glycopeptidase F (PNGase F) was from Roche, Mannheim, Germany.

2.2. Antibodies

Horse-radish peroxidase-conjugated affinity-purified donkey anti-mouse IgG antibody was from Dianova, Hamburg, Germany; monoclonal mouse antibody reacting with cellular Fn was from Transduction Lab, Lexington, KY, USA. In some control experiments, a rabbit polyclonal antibody against CASPR [21] was used.

2.3. MAG-Fc chimera

The plasmid encoding the Fc chimera containing the N-terminal three domains of MAG-Fc has been described [22]. Fc chimeras were produced by transient expression of the plasmid in COS cells followed by purification on protein A-Sepharose [23].

2.4. Metabolic labelling of glycans

N₂A (murine neuroblastoma) and NG108-15 (hybrid from murine neuroblastoma and rat glioma cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 200 mM L-glutamine. For labelling, they were cultured in 6.6 ml DMEM containing 200 mM L-glutamine, 37.5 µl FCS, 375 µl FCS dialyzed against 20 mM HEPES, pH 7.3, and 20 MBq D-[6-³H]-glucosamine-HCl for at least 3 days [17].

2.5. Preparation of detergent lysates from cultured cells, rat spinal cord or brain

Cultured neuroblastoma cells or homogenized brains or spinal cords were lysed with 20 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 1% NP-40 and protease inhibitors (500 µM Pefablock SC, 0.5 µg/ml leupeptin and 0.2 µg/ml trasylol) for 1 h on ice [17]. The lysate was cleared by centrifugation for 40 min at 100 000 × g.

2.6. Affinity precipitation of MAG binding partners

MAG binding partners were affinity precipitated with MAG-Fc immobilized to protein A-Sepharose beads and eluted from the beads with 20 mM synthetic sialic acid analogue methyl α-glycoside of 9-benzoylamino-9-deoxy-5-N-acetylneuraminic acid [24,25] as described (Strenge et al., submitted).

2.7. PNGase F-treatment

Proteins were denatured by adding SDS and 2-mercaptoethanol to final concentrations of 1% and 0.5%, respectively, and incubation at 100°C for 3 min. After cooling, an equal volume of 50 mM Tris buffer, pH 8.5, containing 1% NP-40 and 300 mU PNGase F was added and the mixture was incubated overnight at 37°C.

2.8. SDS-PAGE and Western blotting

SDS-PAGE was performed using 8% polyacrylamide gels [26]. Isolated MAG binding partners for sequencing were separated in preparative gels. Silver staining was performed as described [27]. Coomassie staining was performed with 0.05% Coomassie brilliant blue G-250 diluted in water with 10% methanol and 10% acetic acid. For Western blot analysis, proteins were transferred to nitrocellulose. In the case of [³H]glucosamine-labelled extracts glycoproteins were detected by phosphorimaging.

2.9. Microsequencing

In-gel trypsin digestion and mass spectrometric analysis of peptides were performed by WITA GmbH, Berlin, Germany.

2.10. Immunological analysis on Western blots

For immunodetection, blots were blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk (blocking buffer). Blots were washed five times for 10 min with Tris-buffered saline containing 0.1% Tween 20 at room temperature, incubated with anti-Fn antibodies (1:1000) in blocking buffer for 1 h at room temperature, washed as described above and incubated with horse-radish peroxidase-labelled anti-mouse IgG antibodies (1:20 000) in blocking buffer for 1 h at room temperature. After washing five times, they were incubated with Super Signal West Dura Extended Duration substrate for 5 min and signals were detected by autoradiography.

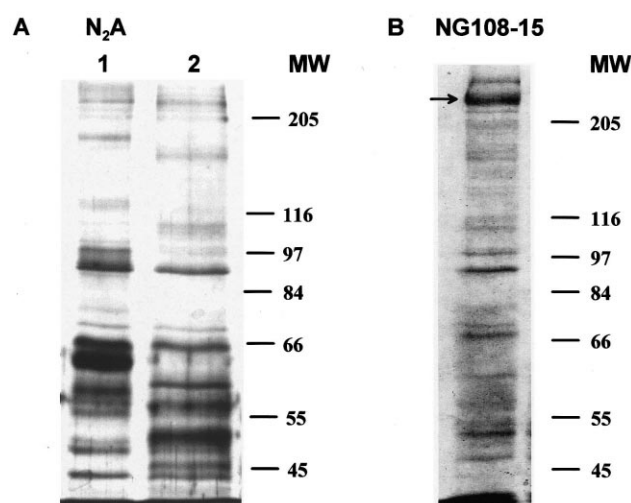


Fig. 1. Glycoproteins isolated by MAG-Fc affinity precipitations from neuroblastoma cells. A: Silver-stained SDS-PAGE of glycoproteins from N₂A cells. Lane 1: Untreated MAG binding partners, lane 2: PNGase F-treated MAG binding partners. Molecular weight markers are shown in kDa on the right. B: Coomassie-stained preparative SDS-PAGE of PNGase F-treated glycoproteins isolated by MAG-Fc affinity precipitation from NG108-15 cells. The arrow indicates the protein band which was used for peptide sequencing. Molecular weight markers are shown in kDa on the right.

3. Results

This study was performed to characterize one of the major isolated MAG binding partners, a 210-kDa protein, by microsequencing. MAG binding partners were prepared from N₂A neuroblastoma cells and separated by preparative SDS-PAGE followed by in-gel trypsin digestion. Although the amount of proteins was sufficient to be detected easily by Coomassie staining (data not shown), no peptides were obtained after in-gel trypsin digestion. This could be due to the glycosylation of the protein, since previous studies have indicated that binding of MAG is mainly mediated by N-glycans [17], and N-glycans are known to protect proteins against proteolytic cleavage. In order to analyze whether the isolated glycoproteins carry N-glycans and how much these contribute to their migration behavior in SDS-PAGE, we treated isolated MAG binding partners with the N-glycosidase PNGase F prior to SDS-PAGE side by side with untreated MAG binding partners (Fig. 1A). Significant changes in electrophoretic mobility of up to 20 kDa were observed for several proteins after glycosidase treatment, indicating that several N-glycans could be removed from MAG binding partners by PNGase F treatment. If Western blots of [³H]glucosamine-labelled MAG binding partners were treated with PNGase F, most of the proteins showed a significant loss of the radioactive label present in the glycans, whereas proteins on a control-treated blot remained unaffected (data not shown). This indicates that N-glycans contribute significantly to the overall glycosylation of these proteins.

Since neurite outgrowth of NG108-15 cells can be inhibited by MAG [13], it is likely that these cells express functional MAG binding partners. The protein patterns of isolated MAG binding partners derived from N₂A and NG108-15 cells are almost identical (Fig. 2), and PNGase F treatment of MAG binding proteins from NG108-15 cells led to the same

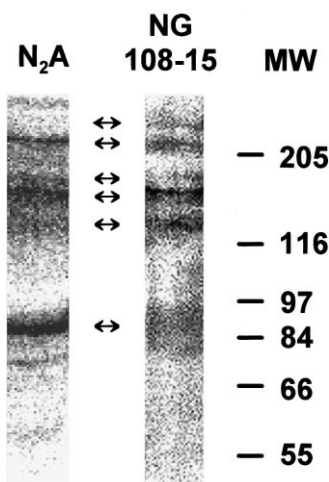


Fig. 2. Phosphoimages of Western blots of [3 H]glucosamine-labelled glycoproteins isolated by MAG-Fc affinity precipitation from N₂A or NG108-15 neuroblastoma cells. Molecular weight markers are shown in kDa on the right.

molecular weight shifts as found for N₂A cells (data not shown). Therefore, NG108-15 cells were used for further preparations. PNGase F-treated glycoproteins isolated from NG108-15 cells were separated by preparative SDS-PAGE and visualized with Coomassie blue staining (Fig. 1B). The 210-kDa protein band, indicated by an arrow, was cut out and peptides were analyzed after in-gel trypsin digestion. Amino acid sequences of two peptides were obtained from this protein (Table 1A). A search of the SwissProt database revealed that both peptides share 100% identity with murine Fn (aa 1960–1965 and aa 2241–2249). Fn is an ECM molecule which exists as an insoluble molecule (cellular Fn) and as a soluble form in extracellular fluids (plasma Fn). Therefore, it had to be considered that the isolated protein could represent a contamination carried over from the FCS in the culture medium. However, the amino acid sequence of peptide 2 is 100% identical to murine Fn but differs in one amino acid from the sequence of bovine Fn (Table 1B). This strongly suggests that the analyzed protein was derived from the murine cells and not from the FCS of the culture medium.

In order to further characterize the Fn from neuroblastoma cell extracts, MAG binding partners were isolated from N₂A or NG108-15 cells and 100 µg of the corresponding cell extracts were analyzed by staining of Western blots with antibodies directed against cellular Fn. Whereas only weak signals of two Fn isoforms were detected in both cell extracts, strong signals of three different Fn isoforms were detected in isolated MAG binding partners from both cell lines (Fig. 3). The bands detected represent Fn, since in control experiments without the primary anti-Fn antibody or with antibodies of unrelated specificity, these signals were not obtained (data not shown). This clearly demonstrates that Fn has been enriched by MAG-Fc affinity precipitation.

The next important question was whether cellular Fn from

Table 1A

Peptide sequences derived from a 210-kDa MAG binding partner isolated from the murine cell line NG108-15

Peptide 1	ITGLQP
Peptide 2	PGTSTSATL

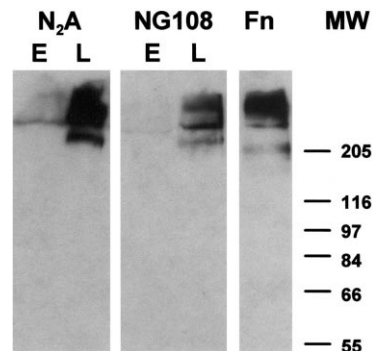


Fig. 3. Immunostaining of Western blots of 100 µg total cell extracts (E) and the corresponding isolated MAG binding partners (L) from N₂A and NG108-15 cells with antibodies against cellular Fn. Human cellular Fn (Fn) was used as positive control. The position of a molecular weight marker with 205 kDa is shown on the right.

primary tissues also interacts with MAG. As a bifunctional molecule, MAG can either promote or inhibit axonal regeneration depending on the age of neurons [11–13,28]. This may be due to developmentally controlled expression of individual MAG binding partners which then could mediate different intracellular signals. To investigate this possibility, MAG binding molecules were prepared from extracts of brains from 1- and 20-day-old rats or adult rats and analyzed by SDS-PAGE (Fig. 4). Significant changes in the patterns of MAG binding partners were observed (Fig. 4A). Compared to brains from adult animals, MAG binding proteins isolated from brains of 1-day-old rats showed fewer protein bands. In addition, some of the proteins from 1-day-old animals were not detected in precipitates from brains of adult animals. Examples are a diffuse strong protein band of apparently 300 kDa and proteins of 190, 110, 62 and 58 kDa. Furthermore, several proteins are more pronounced in preparations from adult animals or are only found in these preparations (36, 90, 100, 120, 130, 150, 170 and 210 kDa). Changes were also observed if MAG binding partners from spinal cords of different aged rats were analyzed (Fig. 4B). These results indicate for the first time that a development-dependent regulation of MAG binding proteins occurs in central nervous system of rats.

In order to investigate whether at all stages of neuronal development Fn isoforms are available as MAG binding partners, Western blots with preparations from different developmental stages were analyzed with antibodies directed against cellular Fn (Fig. 4). In isolated MAG binding partners from brains (Fig. 4A) of 1- and 20-day-old animals, a band of approximately 300 kDa represents the main Fn isoform. In preparations from 20-day-old animals, an additional Fn band barely entering the gel can be detected. In isolated MAG binding partners from brains of adult animals, this high molecular mass band was present only at very low levels, the 300-kDa Fn isoform was not detectable but two additional Fn bands of about 210 and 220 kDa appeared. In preparations from rat spinal cord (Fig. 4B), a clear signal for the 300-kDa

Table 1B

Comparison of the sequence of mouse peptide 2 with the corresponding murine and bovine sequences from Fn

Fn mouse (2241)	PGTSTSATL
Fn bovine (2243)	PGTSASATL

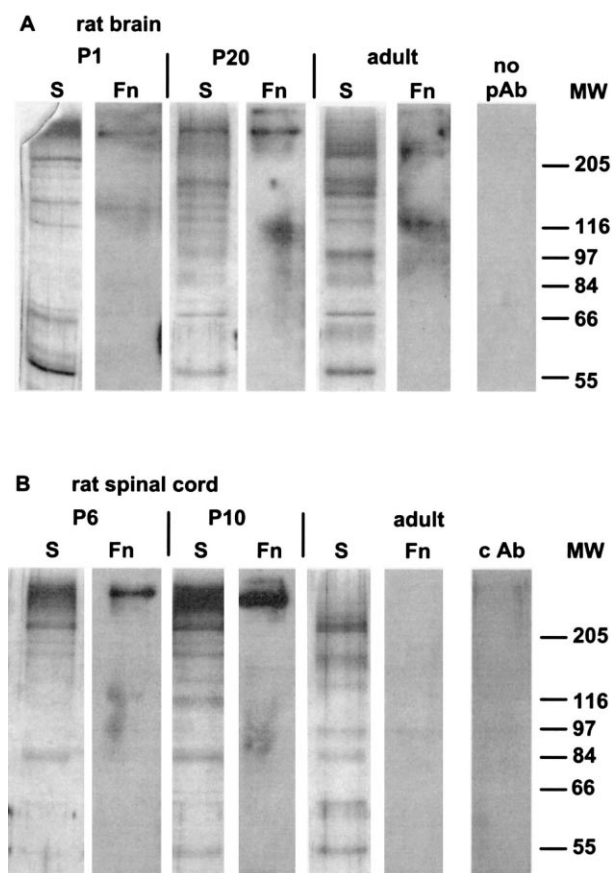


Fig. 4. MAG binding glycoproteins isolated from rat central nervous system. Glycoproteins isolated by affinity precipitation with MAG-Fc were separated by SDS-PAGE. Total proteins were visualized by silver staining (lanes S) and Fn (lanes Fn) by immunostaining of Western blots with anti-Fn antibodies. Molecular weight markers are shown in kDa on the right. A: Glycoproteins isolated by affinity precipitation with MAG-Fc from rat brain extracts (10 mg protein) of postnatal day 1 (P1), day 20 (P20) or adult animals as indicated. As a control, immunostaining without the primary antibody (no pAb) is shown for a preparation from adult brain. B: Glycoproteins isolated by affinity precipitation with MAG-Fc from total rat spinal cord extracts (1 mg protein) of postnatal day 6 (P6), day 10 (P10) or adult animals as indicated. As a control for the antibody specificity an immunostaining of a preparation from adult spinal cord with anti-CASPR antibody (cAb) is shown.

Fn isoform was detected in MAG binding partners isolated from 6-day-old animals which was even more pronounced in samples from 10-day-old animals, whereas no Fn was found in MAG binding partners isolated from adult rat spinal cord. None of the bands detected with the anti-Fn antibody appeared if the anti-Fn antibody was omitted (Fig. 4A) or an unrelated antibody was used (Fig. 4B).

The occurrence of these Fn isoforms is also reflected by the protein pattern of silver-stained gels of the corresponding samples. However, it should be noted that the two bands of apparently 210 and 220 kDa clearly visible on the silver-stained SDS gel of MAG binding partners from rat spinal cord apparently did not represent Fn.

4. Discussion

In the present study we have identified the ECM molecule Fn as a MAG binding partner by peptide sequencing and by

demonstrating direct binding of Fn to immobilized recombinant MAG-Fc. Fn is a ubiquitous, multifunctional glycoprotein which exists as a soluble form in extracellular fluids and as an insoluble form in the ECM, and plays crucial roles in many important physiological processes, such as embryogenesis, wound healing and cell adhesion [29,30]. It contains binding sites for other ECM proteins, cell surface receptors, circulating blood proteins, such as fibrin, and different glycosaminoglycans.

For the interaction between MAG and Fn the glycosylation of Fn plays a pivotal role, since MAG binds with high preference to α 2,3-linked sialic acids on *N*- and *O*-glycans [3,22]. Most of the carbohydrate moieties of Fn are complex *N*-glycans [31]. However, the structures of these glycans depend on the cell type producing the protein. Most critical for MAG binding are the sialic acid residues which are α 2,3-linked on cellular Fn, but basically only α 2,6-linked on plasma Fn [32]. This explains why in previous studies with plasma Fn, no interaction with MAG could be shown [33], whereas the experiments described here demonstrate direct interaction between MAG and cellular Fn from extracts of neuronal cells.

Besides Fn, several other glycoproteins have been isolated by MAG-Fc affinity precipitations. In principle, this is not surprising, since the specificity of MAG for sialylated glycans permits the binding of different glycoproteins. However, the presence of α 2,3-linked sialic acids alone seems not to be sufficient to mediate MAG binding, since only a subset of brain glycoproteins containing α 2,3-linked sialic acids on *N*-glycans bind to MAG. This has been shown by a comparison of MAG binding partners isolated from total brain extract with the pattern of all glycoproteins containing α 2,3-linked sialic acids present in the extract as detected by lectin overlays (Strenge et al., submitted). Furthermore, if Fc chimeras of other siglecs like sialoadhesin (siglec-1) [17] or CD22 (siglec-2) (Fig. 5) were used in these affinity precipitations, other band patterns were obtained. In particular, no band with the molecular mass of the Fn isoforms binding to MAG has been precipitated with other Fc chimeras. This indicates that additional structural features are involved in the recognition events between MAG and the isolated glycoproteins.

A similar pattern of glycoproteins has been isolated from different neuronal sources, suggesting that all these proteins

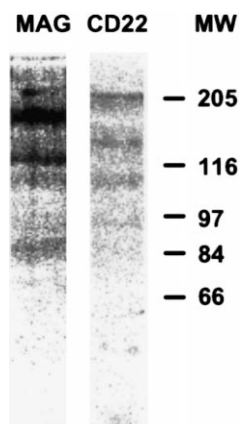


Fig. 5. Phosphorimages of Western blots of [3 H]glucosamine-labelled glycoproteins isolated by MAG-Fc or CD22-Fc affinity precipitation from rat spinal cord neurons. Molecular weight markers are shown in kDa on the right.

represent potential neuron-specific MAG binding partners. Since they can compete for MAG binding sites, this scenario would allow a relatively complex regulation of MAG-mediated processes involving several binding partners. Elucidating this regulatory network will be a crucial step towards the understanding of the functions of these glycoproteins as MAG binding partner *in vivo*.

It has recently been shown that the sialic acid binding site of MAG may only provide a docking function and that an additional binding site in the Ig domains 4 and 5 mediates the effects on neurite outgrowth [34]. In the study described here proteins binding to domains 4 or 5 would not have been precipitated, since Fc chimeras containing only the three N-terminal Ig domains of MAG were used. However, in other experiments with Fc-MAG_{d1–5}, no additional proteins could be identified in affinity precipitations from N₂A cell extracts (data not shown).

Of course it remains to be elucidated what role Fn plays as a MAG binding partner *in vivo*. In this context it is important to note that developmental changes in the expression patterns of MAG binding cellular Fn isoforms have been detected in the present study. It is most likely that these represent different splice variants of this molecule. In rat, 12 possible variants of Fn result from developmentally regulated alternative splicing in the Fn type III domains [35]. The isolation of larger Fn isoforms from young animals and smaller isoforms from adult animals by MAG-Fc affinity precipitation is in good agreement with the typical development-dependent splice patterns found for Fn. Whether this relates to the functional switch of MAG effects on neurite outgrowth from promotion to inhibition during development [11] remains to be investigated.

Interestingly, the repair of nerves in the peripheral nervous system is associated with the reappearance of embryonic forms of Fn mRNA [36]. This suggests that the re-expression of embryonic Fn may be important for successful repair in the peripheral nervous system and that a development- and tissue repair-dependent expression of different spliced Fn isoforms may be involved in regulation of growth processes or stability. It would be interesting to analyze whether such regulation of Fn mRNA splicing also occurs after injuries in the central nervous system.

Although MAG has always been proposed to interact directly with neuronal cell surface receptors, so far such binding partners have not been identified. Instead, different ECM molecules have been shown to interact with MAG. Previous *in vitro* studies have demonstrated that MAG also interacts with the ECM proteins collagen [18] and tenascin-R [19]. In addition, tenascin-R and tenascin-C have been identified in isolated MAG binding partners from mouse brain and rat spinal cord by immunoblot analysis (Streng et al., submitted). During recent years, increasing attention has been paid to the structural and functional complexity of ECM molecules in brain [37]. This has led to the concept that ECM components contribute significantly to molecular signals in the nervous system both as diffusible and as membrane-bound molecules [38]. It will be interesting to elucidate to what extent interactions between MAG and the different MAG binding ECM molecules influence interactions between MAG and neurons.

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