The Amino Acid Sequences of the Myelin-associated Glycoproteins: Homology to the Immunoglobulin Gene Superfamily

James L. Salzer,*‡ W. Patrick Holmes,* and David R. Colman*

Departments of *Cell Biology and ‡Neurology, New York University School of Medicine, New York 10016

Abstract. The myelin associated glycoproteins (MAG) are integral plasma membrane proteins which are found in oligodendrocytes and Schwann cells and are believed to mediate the axonal–glial interactions of myelination. In this paper we demonstrate the existence in central nervous system myelin of two MAG polypeptides with M_rs of 67,000 and 72,000 that we have designated small MAG (S-MAG) and large MAG (L-MAG), respectively. The complete amino acid sequence of L-MAG and a partial amino acid sequence of S-MAG have been deduced from the nucleotide sequences of corresponding cDNA clones isolated from a lambda gt11 rat brain expression library. Based on their amino acid sequences, we predict that both proteins have an identical membrane spanning segment and a large extracellular domain. The putative extracellular region contains an Arg-Gly-Asp sequence that may be involved in the interaction of these proteins with the axon. The extracellular portion of L-MAG also contains five segments of internal homology that resemble immunoglobulin domains, and are strikingly homologous to similar domains of the neural cell adhesion molecule and other members of the immunoglobulin gene superfamily. In addition, the two MAG proteins differ in the extent of their cytoplasmically disposed segments and appear to be the products of alternatively spliced mRNAs. Of considerable interest is the finding that the cytoplasmic domain of L-MAG, but not of S-MAG, contains an amino acid sequence that resembles the autophosphorylation site of the epidermal growth factor receptor.

THE myelin-associated glycoproteins (MAG)[1] are plasma membrane proteins of myelin-forming oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (reviewed in reference 40). Although the precise role of these proteins in the formation and maintenance of the myelin sheath is not known, it has been proposed that they are important in maintaining the apposition of the myelin sheath to the axon (40). Consistent with this idea is the localization of MAG to the periaxonal glial membrane, and its absence in compact myelin (50, 55, 56, 27). Furthermore, studies of the dysmyelinating mouse mutant, Quaking (the primary defect of which is not known, but which has only 15% of the normal levels of MAG [20]), have revealed an abnormally widened space between the axon and the innermost turn of the myelin sheath in discreet regions where MAG cannot be detected immunocytochemically (57). Lastly, MAG has an extensive extracellular exposure (35) and shares a carbohydrate determinant (HNK-1) with several other molecules that are proposed to mediate cell–cell interactions in the developing nervous system, including the neural cell adhesion molecule (N-CAM) and L1 (28, 21). Whether all of these presumptive nervous system adhesion molecules, including MAG, share general structural and amino acid sequence homologies has not yet been elucidated.

Two MAG polypeptides (M_r 72,000 and 67,000) are detectable in in vitro translation systems programmed with total brain mRNA (10). Presumably, these polypeptides, when glycosylated in vivo, co-migrate on SDS PAGE as the single, characteristically broad band (M_r 100,000) that corresponds to native MAG (40). The precise structural differences between the two MAG proteins are not known. Peptide maps of the two polypeptides are nearly identical (10) and suggest that the polypeptides differ by a single segment that is present only in the larger protein. Interestingly, the larger protein is expressed during the early rapid phase of myelination while the smaller protein is synthesized primarily in the adult, when myelination is nearly complete (10). This may reflect different functions for the individual MAG proteins in myelination.

In the present study we report the complete amino acid sequence of the large MAG polypeptide (L-MAG) and a partial amino acid sequence of the small MAG polypeptide (S-

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1. *Abbreviations used in this paper*: CNS, central nervous system; L-MAG, large myelin-associated glycoprotein; MAG, myelin-associated glycoproteins; N-CAM, neural cell adhesion molecule; nt, nucleotide(s); S-MAG, small myelin-associated glycoprotein.

MAG), deduced from the nucleotide sequences of corresponding cDNA clones isolated from a rat brain lambda gtll expression library. Analysis of the primary amino acid sequences reveals several features of these proteins that may be related to their postulated function as glial–neuron recognition molecules. These include: (*a*) the tripeptide sequence Arg-Gly-Asp (RGD), which has been found to mediate binding in several receptor–ligand systems (42), (*b*) five tandem repeats of a highly conserved peptide domain within the extracellular portion of the protein, and (*c*) a cytoplasmically disposed region that is shorter in S-MAG than in L-MAG. The conserved extracellular domains, which are centered around cysteine residues, are significantly homologous to the variable regions of immunoglobulins and related membrane receptors, as well as to N-CAM. Finally, we report that the 3′ end of L-MAG is identical to a brain-specific cDNA clone, pIB236 (52), which has been well studied and was believed to be neuron-specific.

Materials and Methods

Generation of Anti-MAG Antibodies

MAG was isolated by the method of Quarles and Pasnak (39). In brief, purified rat brain myelin (32) was extracted with chloroform/methanol (2:1 vol/vol). The insoluble residue was treated with 0.25 M lithium diiodosalicylate and partitioned with phenol. The aqueous phase, which is enriched in MAG, was dialyzed and lyophilized. MAG was electrophoretically separated by preparative SDS PAGE (10% acrylamide) and the broad 100-kD MAG band was excised and electroeluted (16). Antibodies were raised by injecting purified MAG (50 μg) into rabbit popliteal lymph nodes in complete Freund's adjuvant and boosting every other week with 100 μg of MAG in incomplete adjuvant. Antiserum was affinity purified (26) against a lithium diiodosalicylate extract of myelin that had been endoglycosidase F-treated (New England Nuclear, Boston, MA) before coupling to cyanogen bromide–activated Sepharose CL-4B beads. The affinity-purified antibody was eluted with 4 M sodium thiocyanate, and dialyzed against several changes of sodium PBS (pH 7.5). By immunoblot analysis (54), this antibody detected a single broad band of 100 kD that was present in extracts of whole rat brain and was markedly enriched in a purified rat central myelin preparation. Immunocytochemical analysis of tissue sections of 4% paraformaldehyde-fixed adult rat brain demonstrated that the antibody specifically recognized myelinated fiber tracts.

Construction and Screening of a Rat Brain cDNA Library

10 μg of Poly(A)$^+$ RNA isolated from rat brain (postnatal day 20) was used as a template for cDNA synthesis. The first strand was synthesized using the protocol provided with M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), and second strand synthesis was performed as described (13). Double-stranded cDNA (2 μg) was treated (20 min, 37°C) with 5 U of mung bean nuclease (PL-Pharmacia, Piscataway, NJ), in 50 mM NaCl, 30 mM Na acetate, pH 5.5, 1 mM $ZnCl_2$, and 3% glycerol (100 μl final volume). The double-stranded cDNA was then methylated at internal Eco RI sites, and Eco RI linkers were attached in a standard ligation reaction. Redundant linker sequences were excised with Eco RI enzyme and the double-stranded cDNA was size-fractionated on a Sepharose CL-4B (PL-Pharmacia) column (10 ml). cDNAs larger than 1.5 kb were ligated to lambda phage gtll arms (Stratagene), that had been cleaved and dephosphorylated at the single Eco RI site, and packaged into bacteriophage with a commercial packaging extract (Stratagene Cloning Systems, San Diego, CA). This library contained 3 × 10^6 independent recombinants.

Affinity-purified anti-MAG antibodies were used to screen a portion (10^6 recombinants) of this library (66). Positive colonies were identified with a goat anti-rabbit antibody (Cappel Laboratories, Malvern, PA) conjugated to horseradish peroxidase in a standard reaction.

Nucleic Acid Blotting and Hybridization

RNAs [5–10 μg of total, 3 μg of Poly(A)$^+$] were subjected to electrophoresis on 1.7% agarose gels containing formaldehyde, and transferred by capillary blotting to filters (Genescreen, New England Nuclear). These filters were probed with cDNA inserts which were nick-translated with deoxycytidine 5′-triphosphate [α^{32}P] to a specific activity of 5 × 10^8 cpm/μg. Hybridization and washing were carried out as recommended by the manufacturer (New England Nuclear). DNA blotting was performed by the method of Southern (47, 7) and plaque hybridization by methods described in Maniatis et al. (25).

DNA Sequencing

DNA sequencing was performed by the dideoxy chain termination technique (45). Restriction endonuclease fragments were directionally subcloned into the M13 bacteriophages mp 18 and mp 19 (Pharmacia Fine Chemicals) and M13 um 20 (International Biotechnologies, Inc., New Haven, CT), which were digested to yield compatible cloning sites. In one case an oligonucleotide primer, synthesized with a DNA synthesizer (Applied Biosystems, Foster City, CA), was used to complete the sequence.

Iodination of Myelin and Immunoprecipitations

Aliquots (1 μg protein) of purified myelin (32) were washed three times by suspension in 1 ml of 50 mM Na borate buffer (pH 8), pelleted by centrifugation (100,000 *g*, 10 min), and resuspended. Iodination was carried out with the Bolton-Hunter reagent according to the manufacturer's instructions (ICN Biomedicals, Inc., Irvine, CA). The ^{125}I-labeled myelin was washed extensively (5 × 1 ml) in 400 mM Tris-HCl (pH 7.5) and then solubilized in 100 μl 2% SDS (100°C) before immunoprecipitation by the procedure of Goldman and Blobel (12).

Protein Blotting and Epitope Selection

Escherichia coli strain Y1089 was lysogenized with a recombinant bacteriophage clone (66). Approximately 10^{10} lysogenized bacteria were incubated at 42°C for 20 min and 10 mM isopropylthio-β-D-galactoside was added for 1.5 h to induce the β-galactosidase fusion protein. The bacteria were recovered by centrifugation, sonicated in 10% SDS, and the proteins were fractionated by preparative SDS PAGE. The fusion protein was identified by light staining with Coomassie Blue and was recovered from the gel by electroelution. 100 μg was injected into rabbits every other week to generate antibodies. These antibodies were affinity purified against the fusion protein before being used on Western blots of myelin. Alternatively, electrophoretically separated proteins extracted from the bacteria were transferred onto nitrocellulose paper and the fusion protein band was identified (by brief staining with 0.1% Fast Green), excised, and used as an adsorption matrix for the anti-MAG antiserum. This band was incubated overnight with a 1:10 dilution of the antiserum at 4°C. The nitrocellulose strip was then washed four times with PBS containing 0.1% Triton X-100 and 0.02% gelatin, and the bound antibody was eluted with 0.1 M glycine HCl (pH 3.0) for 1 min and rapidly neutralized with Tris HCl, pH 8.9. The eluted antibodies were used to probe myelin immunoblots and visualized with a goat anti-rabbit IgG conjugated to horseradish peroxidase in a standard reaction mixture.

In Vitro Transcription and Translation of L-MAG

Restriction mapping and sequence analysis of cDNA clone M10D (Fig. 5) revealed that two Apa 1 sites existed in the 5′ and 3′ untranslated regions, respectively. This allowed the subcloning of the entire coding region into the Bluescript vector (Stratagene). mRNA was transcribed with T3 polymerase from 2 μg of purified plasmid in a 10-μl reaction mixture, using the riboprobe system II (Promega Biotec, Madison, WI), according to the manufacturer's instructions. The reaction mixture also contained m^{7}G(5′)ppp(5′)G (Pharmacia Fine Chemicals) to cap the mRNA. 1 μl of the mixture was used to program a wheat germ translation system containing [^{35}S]methionine and incubated for 2 h at 28°C.

Computer Analysis of the Amino Acid Sequence of MAG

The protein data base of the National Biomedical Research Foundation was searched for homologous protein sequences by using the FASTP computer program (24). This program was also used to obtain optimized similarities

and the percent identity for each homology found. The significance of each homology was determined by the RDF program and expressed as a Z score (24). Internal segments of homology were visually aligned and also compared pairwise by the ALIGN program (49, 6). The ALIGN program was also used to optimize identities between N-CAM and MAG. The hydrophobicity analysis was performed by the ANALYSEP program (22).

Results

Anti-MAG Antibodies Recognize Two Polypeptides in Myelin

The polyclonal antibody was used to immunoprecipitate MAG from ^{125}I-labeled (CNS) myelin. SDS PAGE analysis of the immunoprecipitate revealed that, as expected, MAG migrates as a broad band of M_r 100,000 (Fig. 1, lane *a*). After enzymatic deglycosylation of the immunoprecipitate with Endo F, two polypeptides of M_r 72,000 and 67,000 were detected (Fig. 1, lane *b*). We have designated these proteins as L-MAG and S-MAG, respectively. This result directly demonstrates that adult rat CNS myelin contains two MAG proteins, whose presence had been previously inferred by in vitro translation studies of total brain mRNA (10). It is of interest that although L-MAG is apparently synthesized early and S-MAG is synthesized later in development (10), we have detected both proteins in equal abundance in the mature myelin sheath (Fig. 1, lane *b*).

Isolation of cDNA Clones that Encode Two MAG Polypeptides

A rat brain cDNA library constructed in the lambda gtll vector was screened with the affinity-purified antibody and six immunopositive clones were identified. Three of these six cDNAs were found to cross-hybridize, and one clone, designated M10, was selected for further study.

The insert of this clone was verified as a MAG cDNA by immunologic criteria. The M10 clone contains a cDNA insert of 663 bp; this clone expresses a β-galactosidase fusion protein of M_r 140,000. Since β-galactosidase has a molecular mass of ~117 kD, we estimated that the MAG portion of the fusion protein was ~23 kD, encompassing about one-third of the MAG polypeptide. This fusion protein was used in an epitope selection experiment (61), in which antibodies that specifically cross-reacted with the M10 fusion protein (immobilized on nitrocellulose paper) were isolated from the polyclonal MAG antiserum. The reactivity of the affinity-purified antibodies was then compared with GEN S-3, an anti-MAG monoclonal antibody (31), on immunoblots (Fig. 2). Both antibodies recognize the same 100-kD MAG band on Western blots of CNS myelin. In a control experiment, the fusion protein of a lambda gtll myelin basic protein clone (isolated from this library), failed to select any antibodies that reacted with MAG (Fig. 2, lane *d*). Finally, polyclonal antibodies were raised directly against the M10 fusion protein in several rabbits. These antibodies also specifically recognize MAG, as demonstrated on immunoblots of rat myelin (Fig. 2, lane *c*), and by immunoprecipitation of both native and the deglycosylated MAG polypeptides from iodinated myelin (data not shown).

We rescreened the lambda gtll library by plaque hybridization with the ^{32}P-labeled, M10 cDNA insert and identified two larger homologous clones. One of these clones, M10D, contains a cDNA insert of 2348 bp that is long enough to encode either MAG polypeptide. This cDNA was subcloned into the Bluescript plasmid vector and was further characterized by in vitro transcription and translation (Fig. 3). The synthetic mRNA transcribed with T3 polymerase was used to program a wheat germ cell-free translation system. The primary translation product had an M_r of 73,000 as measured on SDS PAGE (similar in size to the large MAG polypeptide), and was immunoprecipitable with anti-MAG antiserum (Fig. 3, lane *b*). The immunoprecipitation was completely inhibited by the addition of unlabeled MAG purified from myelin (Fig. 3, lane *c*), confirming the identity of the translation product and of the clone. The other large clone, M10E, contains a cDNA insert of 1083 bp. Sequence data, discussed below, reveals that it corresponds to an S-MAG cDNA.

Tissue and Temporal Expression of MAG mRNA

MAG mRNA levels were assessed by RNA blot analysis in

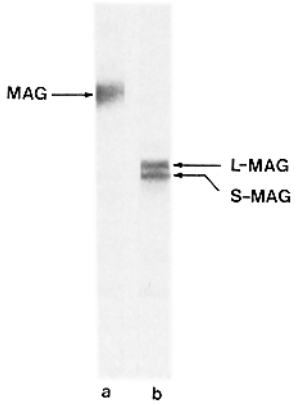

Figure 1. Two MAG polypeptides can be detected in myelin. An aliquot (1 μg protein) of purified osmotically shocked myelin was iodinated with Bolton-Hunter reagent, solubilized with detergents, and immunoprecipitated (12) with anti-MAG antiserum. Immunoprecipitates were subjected to electrophoresis directly on 10% polyacrylamide gels (lane *a*) or first treated with 2 U of endoglycosidase F for 10 h at 37°C before electrophoresis (lane *b*). Native MAG (lane *a*) has an M_r of 100,000; the L-MAG and S-MAG polypeptides (lane *b*) have M_r values of 72,000 and 67,000, respectively.

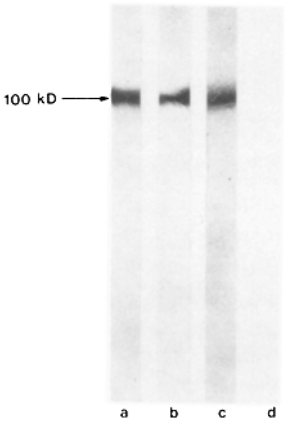

Figure 2. Characterization of the M10 fusion protein by immunoblot analysis. Each lane corresponds to 10 μg of purified myelin that was fractionated by SDS PAGE (10% acrylamide), electrophoretically transferred to nitrocellulose paper, and processed for immunoblotting. Nitrocellulose strips were incubated with the following antibody preparations: (lane *a*) a mouse anti-MAG monoclonal (31); (lane *b*) polyclonal MAG antiserum adsorbed to and eluted from the M10 fusion protein; (lane *c*) antibody raised and affinity purified against the M10 fusion protein; and (lane *d*) polyclonal anti-MAG antiserum adsorbed and eluted from a myelin basic protein fusion protein.

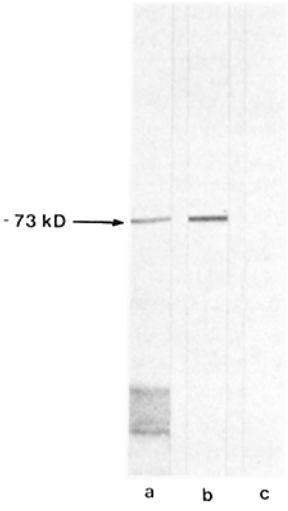

Figure 3. Characterization of the M10D translation product. The complete coding region of the M10D cDNA insert was subcloned into the Apa I site of the plasmid vector Bluescript and mRNA was transcribed with T_3 polymerase using the riboprobe system II according to the manufacturer's instructions. (Lane *a*) ~0.3 μg of RNA transcribed in vitro was translated in a wheat germ cell-free system with [^{35}S]methionine. (Lanes *b* and *c*) Immunoprecipitations of the translated products with anti-MAG antiserum in the absence (*b*) or presence (*c*) of 10 μg of unlabeled MAG purified from myelin. Immunoprecipitates were separated by SDS PAGE (8% acrylamide), and treated for fluorography with EN^{3}HANCE (New England Nuclear).

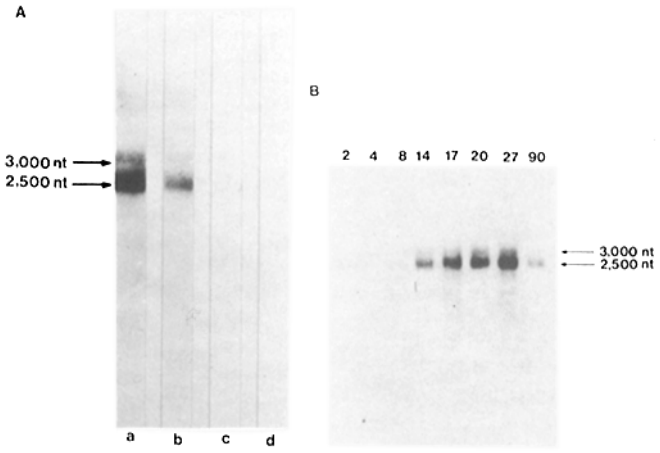

Figure 4. RNA blot analyses of MAG mRNA expression. (*A*) Tissue distribution of MAG mRNA. 3 μg of Poly (A)$^+$ RNA was isolated from various tissues of 20-d-old rats and subjected to electrophoresis in a 1.7% agarose gel containing formaldehyde. The RNA was transferred to GeneScreen membranes and hybridized to 0.5 μg of the nick-translated, ^{32}P-labeled M10 cDNA. (Lane *a*) Brain; (lane *b*) sciatic nerve; (lane *c*) thymus; (lane *d*) liver. The mRNA sizes indicated were estimated from the position of the 18S and 28S RNA bands. (*B*) Expression of MAG mRNA during development. Total brain RNA was prepared from rats killed on the postnatal day indicated and 5 μg were electrophoretically separated on formaldehyde containing agarose gels, transferred to GeneScreen, and hybridized as above.

different tissues, using the nick-translated M10 insert. MAG mRNA levels were found to be more abundant in the CNS than in the peripheral nervous system, and absent from all nonneuronal tissues examined, including liver, spleen, lung, and thymus (Fig. 4 *A*). These mRNA levels are in agreement with the tissue-specific expression of the MAG proteins, which have been reported to be four-fold more abundant in whole brain than in sciatic nerve, and could not be detected by radioimmunoassay in nonneuronal tissues (40).

Two size classes of MAG mRNAs were detected (Fig. 4 *A*). The predominant mRNA species is 2,500 nucleotides (nt) in length and is ~10-fold more abundant than the second species, which is ~3,000 nt in length. These messengers are likely to differ in their untranslated regions, since we have identified a coding region difference of 45 nt that accounts for the difference in the sizes of the two polypeptides (see below).

The levels of MAG mRNA detectable in rat brain during development were also determined (Fig. 4 *B*). Substantial mRNA levels are not present in rat brain until postnatal day 14. Peak mRNA levels are present between 20 and 27 d postnatally, coincident with the period of rapid myelination (2), and then decline substantially in the adult. At all stages both the 2,500-nt and 3,000-nt mRNAs are present.

Nucleotide and Deduced Amino Acid Sequences of the MAG Proteins

The sequencing strategy used is illustrated in Fig. 5 and the complete nucleotide sequence and deduced amino acid sequence for both M10D and M10E are shown in Fig. 6. M10D is 2348 nt long and contains an open reading frame of 1878 nt that begins with an ATG 126 nt downstream from the 5′ end of the clone and 24 nt downstream from an in-frame stop codon. This open reading frame is followed by a TGA (position 2004–2006) and 342 bp of 3′ untranslated sequence. M10E is 1083 nt long. It is identical to the 3′ half of M10D except for an additional internal sequence of 45 nt (that begins after nt 1841 of M10D). This segment introduces a stretch of 10 amino acids followed by an in-frame termination codon that shortens the open reading frame by 135 nt (Fig. 6). Both cDNA inserts contain the Poly A acceptor sequence (AATAAA) (36) and M10E ends in a Poly A tract.

M10D encodes a polypeptide of 626 amino acids with a calculated molecular mass of 69.3 kD. This value agrees well with the molecular masses estimated by SDS PAGE for the translation product of the M10D transcript (73 kD) and for the deglycosylated L-MAG protein (72 kD). M10E encodes a MAG polypeptide that is calculated to be smaller at the carboxy terminus by 5.1 kD. Because the native MAG polypeptides in myelin are known to differ by 5 kD, it is likely that M10D is a full-length L-MAG cDNA and M10E is an incomplete S-MAG cDNA.

A hydrophobicity analysis (22) of L-MAG revealed two extended hydrophobic segments. The first, which occurs at the amino terminus of L-MAG, is a stretch of ~20 nonpolar amino acids that may be a cleavable signal peptide similar to those typically present on virtually all secretory and many transmembrane proteins with an extracellularly disposed amino terminus (44). The NH_2-terminal amino acid of native MAG has not been identified. However, based on an analysis of the amino acids found near known signal sequence cleavage sites (58, 59), the predicted site of cleavage is between the glycines at positions 19 and 20. The glycine at position 20 is therefore a potential candidate for the NH_2-terminal amino acid of the mature protein.

The second hydrophobic region is a segment of 23 nonpolar amino acids (amino acids 514 to 536), which is long enough to traverse the bilayer and likely to be membrane embedded. This segment is followed immediately by an extremely basic sequence (amino acids 537 to 540), a feature of the cytoplasmic domain of many membrane proteins (44) that suggests that the ensuing portion of the polypeptide re-

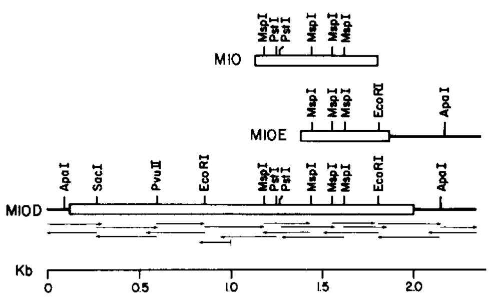

Figure 5. Restriction maps of MAG cDNA clones and sequencing strategy. Restriction enzyme sites in the three MAG cDNA clones were deduced by conventional procedures (25). All restriction sites were subsequently verified by nt sequence analysis. Restriction fragments were directionally subcloned into compatible sites of M13 before sequencing. The direction and extent of the sequence determination is shown by the arrows. In one case, a synthetic primer was used and is indicated by a vertical line at the tail of an arrow. The scale is calibrated in kilobases and shows the 5′ to 3′ orientation of the cDNAs relative to MAG mRNA. Coding regions are represented by the open bars and 3′ and 5′ untranslated regions by heavy lines. The 5′→3′ orientation of the M10D insert was deduced by sequence analysis and confirmed by the in vitro transcription/translation studies described in the text.

mains on the cytoplasmic side of the bilayer. Taken together our data would place the amino terminus and the major portion of the polypeptide on the extracellular side of the bilayer, a single segment within the bilayer, and a relatively short carboxy-terminal portion of the protein (90 amino acids of L-MAG, 45 amino acids of S-MAG) in the cytoplasmic compartment. Studies to establish the precise disposition of the MAG polypeptides within the phospholipid bilayer are currently in progress.

There are eight potential N-glycosylation sites, Asn-X-Ser/Thr (51), located in the putative extracellular domain. The MAG proteins are heavily glycosylated in vivo (30% carbohydrate by weight) and have been estimated to contain up to nine oligosaccharide chains (10). However, because the precise number of oligosaccharide chains has not been determined, we do not know how many of these sites are actually glycosylated in vivo.

Discussion

The formation of the vertebrate myelin sheath requires an intimate and specific interaction of a myelinating glial cell with a closely apposed axonal process (48). The MAG proteins have been considered to be likely candidates for mediating

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   1  CGCGGAGCAGAGCGTGCAGAAGCCAGACCATCCAAGTTGACTGGCCACTTGGAGCGGAATCAGGAGACATTCCCAACTCAGGGAGACTGAGGTGAGGGCCCTAGCTCGCCCACTTGCTGGACAAG

   1  Met Ile Phe Leu Thr Thr Leu Pro Leu Phe Trp Ile Met Ile Ser Ala Ser Arg Gly Gly His Trp Gly Ala Trp Met Pro Ser Ser Ile Ser Ala
 126  ATG ATA TTC CTT ACC ACC CTG CCT CTG TTT TGG ATA ATG ATT TCA GCT TCT CGA GGG GGG CAC TGG GGT GCC TGG ATG CCC TCG TCC ATC TCA GCC

  33  Phe Glu Gly Thr Cys Val Ser Ile Pro Cys Arg Phe Asp Phe Pro Asp Glu Leu Arg Pro Ala Val Val His Gly Val Trp Tyr Phe Asn Ser Pro
 222  TTC GAG GGC ACG TGT GTC TCC ATC CCC TGC CGT TTC GAC TTC CCG GAT GAG CTC AGA CCG GCT GTG GTA CAT GGC GTC TGG TAT TTC AAC AGT CCC

  65  Tyr Pro Lys Asn Tyr Pro Pro Val Val Phe Lys Ser Arg Thr Gln Val Val His Glu Ser Phe Gln Gly Arg Ser Arg Leu Leu Gly Asp Leu Gly
 318  TAC CCC AAG AAC TAC CCG CCA GTG GTC TTC AAG TCC CGC ACA CAA GTG GTC CAC GAG AGC TTC CAG GGC CGT AGC CGC CTG TTG GGA GAC CTG GGC

                  ◆
  97  Leu Arg Asn Cys Thr Leu Leu Leu Ser Thr Leu Ser Pro Glu Leu Gly Gly Lys Tyr Tyr Phe[Arg Gly Asp]Leu Gly Gly Tyr Asn Gln Tyr Thr
 414  CTA CGA AAC TGC ACC CTG CTT CTC AGC ACG CTG AGC CCT GAG CTG GGA GGG AAA TAC TAT TTC[CGA GGT GAC]CTG GGC GGC TAC AAC CAG TAC ACC

 129  Phe Ser Glu His Ser Val Leu Asp Ile Ile Asn Thr Pro Asn Ile Val Val Pro Pro Glu Val Val Ala Gly Thr Glu Val Glu Val Ser Cys Met
 510  TTC TCG GAG CAC AGC GTC CTG GAC ATC ATC AAC ACC CCC AAC ATC GTG GTG CCC CCA GAA GTG GTG GCA GGA ACG GAA GTA GAG GTC AGC TGC ATG

 161  Val Pro Asp Asn Cys Pro Glu Leu Arg Pro Glu Leu Ser Trp Leu Gly His Glu Gly Leu Gly Glu Pro Thr Val Leu Gly Arg Leu Arg Glu Asp
 606  GTG CCG GAC AAC TGC CCA GAG CTG CGC CCT GAG CTG AGC TGG CTG GGC CAC GAG GGG CTA GGG GAG CCC ACT GTT CTG GGT CGG CTG CGG GAG GAT

                                                                                                                          ◆
 193  Glu Gly Thr Trp Val Gln Val Ser Leu Leu His Phe Val Pro Thr Arg Glu Ala Asn Gly His Arg Leu Gly Cys Gln Ala Ala Phe Pro Asn Thr
 702  GAA GGC ACC TGG GTG CAG GTG TCA CTG CTA CAC TTC GTG CCT ACT AGA GAG GCC AAC GGC CAC CGT CTG GGC TGT CAG GCT GCC TTC CCC AAC ACC

                                                                                      ◆
 225  Thr Leu Gln Phe Glu Gly Tyr Ala Ser Leu Asp Val Lys Tyr Pro Pro Val Ile Val Glu Met Asn Ser Ser Val Glu Ala Ile Glu Gly Ser His
 798  ACC TTG CAG TTC GAG GGT TAC GCC AGT CTG GAC GTC AAG TAC CCC CCG GTG ATT GTG GAG ATG AAT TCC TCT GTG GAG GCC ATT GAG GGC TCC CAC

 257  Val Ser Leu Leu Cys Gly Ala Asp Ser Asn Pro Pro Pro Leu Leu Thr Trp Met Arg Asp Gly Met Val Leu Arg Glu Ala Val Ala Glu Ser Leu
 894  GTC AGC CTG CTC TGT GGG GCT GAC AGC AAC CCG CCA CCG CTG CTG ACT TGG ATG CGG GAT GGG ATG GTG TTG AGG GAG GCA GTT GCT GAG AGC CTG

                                                                                                      ◆
 289  Tyr Leu Asp Leu Glu Glu Val Thr Pro Ala Glu Asp Gly Ile Tyr Ala Cys Leu Ala Glu Asn Ala Tyr Gly Gln Asp Asn Arg Thr Val Glu Leu
 990  TAC CTG GAT CTG GAG GAG GTG ACC CCA GCA GAG GAC GGC ATC TAT GCT TGC CTG GCA GAG AAT GCC TAT GGC CAG GAC AAC CGC ACG GTG GAG CTG

                                              ◆
 321  Ser Val Met Tyr Ala Pro Trp Lys Pro Thr Val Asn Gly Thr Val Val Ala Val Glu Gly Glu Thr Val Ser Ile Leu Cys Ser Thr Gln Ser Asn
1086  AGC GTC ATG TAT GCA CCT TGG AAG CCC ACA GTG AAT GGG ACG GTG GTG GCG GTA GAG GGG GAG ACA GTC TCC ATC CTG TGT TCC ACA CAG AGC AAC

 353  Pro Asp Pro Ile Leu Thr Ile Phe Lys Glu Lys Gln Ile Leu Ala Thr Val Ile Tyr Glu Ser Gln Leu Gln Leu Glu Leu Pro Ala Val Thr Pro
1182  CCG GAC CCT ATT CTC ACC ATC TTC AAG GAG AAG CAG ATC CTG GCC ACG GTC ATC TAT GAG AGT CAG CTG CAG CTG GAA CTC CCT GCA GTG ACG CCC

                                                                                      ◆
 385  Glu Asp Asp Gly Glu Tyr Trp Cys Val Ala Glu Asn Gln Tyr Gly Gln Arg Ala Thr Ala Phe Asn Leu Ser Val Glu Phe Ala Pro Ile Ile Leu
1278  GAG GAC GAT GGG GAG TAC TGG TGT GTA GCT GAG AAC CAG TAT GGC CAG AGA GCC ACC GCC TTC AAC CTG TCT GTG GAG TTT GCT CCC ATA ATC CTT

 417  Leu Glu Ser His Cys Ala Ala Ala Arg Asp Thr Val Gln Cys Leu Cys Val Val Lys Ser Asn Pro Glu Pro Ser Val Ala Phe Glu Leu Pro Ser
1374  CTG GAA TCG CAC TGT GCA GCG GCC AGA GAC ACC GTG CAG TGC CTG TGT GTG GTA AAA TCC AAC CCG GAA CCC TCC GTG GCC TTT GAG CTG CCT TCC

          ◆           ◆
 449  Arg Asn Val Thr Val Asn Glu Thr Glu Arg Glu Phe Val Tyr Ser Glu Arg Ser Gly Leu Leu Leu Thr Ser Ile Leu Thr Leu Arg Gly Gln Ala
1470  CGC AAC GTG ACT GTG AAC GAG ACA GAG AGG GAG TTT GTG TAC TCA GAG CGC AGC GGC CTC CTG CTC ACC AGC ATC CTC ACG CTC CGG GGT CAG GCC

 481  Gln Ala Pro Pro Arg Val Ile Cys Thr Ser Arg Asn Leu Tyr Gly Thr Gln Ser Leu Glu Leu Pro Phe Gln Gly Ala His Arg Leu Met Trp Ala
1566  CAA GCC CCA CCC CGC GTC ATT TGT ACC TCC AGG AAC CTC TAC GGC ACC CAG AGC CTC GAG CTG CCT TTC CAG GGA GCA CAC CGA CTG ATG TGG GCC

          ___________________________________________________________________________
 513  Lys Ile Gly Pro Val Gly Ala Val Val Ala Phe Ala Ile Leu Ile Ala Ile Val Cys Tyr Ile Thr Gln Thr Arg Arg Lys Lys Asn Val Thr Glu
1662  AAA ATC GGC CCT GTG GGT GCT GTG GTC GCC TTT GCC ATC CTG ATT GCC ATT GTC TGC TAC ATC ACC CAG ACA AGA AGA AAA AAG AAC GTC ACA GAG

 545  Ser Pro Ser Phe Ser Ala Gly Asp Asn Pro His Val Leu Tyr Ser Pro Glu Phe Arg Ile Ser Gly Ala Pro Asp Lys Tyr Glu[Ser Arg Glu Val
1758  AGC CCC AGC TTC TCA GCG GGA GAC AAC CCT CAT GTC CTG TAC AGC CCC GAA TTC CGA ATC TCT GGA GCA CCT GAT AAG TAT GAG[TCC AGA GAG GTC

 573  Ser Thr Arg Asp Cys His xxx            ]Ser Glu Lys Arg Leu Gly Ser Glu Arg Arg Leu Leu Gly Leu Arg Gly Glu Pro Pro Glu Leu
1842  TCT ACC CGG GAT TGT CAC TGA GAG CCC CAG GAG]AGT GAG AAG CGC CTG GGG TCC GAG AGG AGG CTG CTG GGC CTT AGG GGG GAA CCC CCA GAA CTG

 594  Asp Leu Ser Tyr Ser His Ser Asp Leu Gly Lys Arg Pro Thr Lys Asp Ser Tyr Thr Leu Thr Glu Glu Leu Ala Glu Tyr Ala Glu Ile Arg Val
1905  GAC CTC AGT TAT TCC CAC TCA GAC CTG GGG AAA CGA CCC ACC AAG GAC AGC TAC ACC CTG ACA GAG GAG CTG GCT GAG TAC GCA GAA ATC CGA GTC

 626  Lys xxx
2001  AAG TGAGGAAGCTGGGGGCTGGCCCTGTGGCTCACCCCCCATCAGGACCCTCGCTTGGCCCCCACTGGCCGTGGGCTCCCTTTCTCTTGAGAGTGGTAGGGGTGGGGGCGGGAAGGGGCGGGGCAG

2126  GAAACAGTGAGGTCTTAGGGGCCCGGCCTCCCCTCCTTCCCGGCTGCTCCTCTCTGCCAACATCCTGCACCTATGTTACAGCTCCCTCTCCCCTCCTTTTAACCTCAGCTGTTGAGAGGGGTGCTCT

2254  GTCTGTCCATGTTATTTATTGTTATCCTGGTCTCCTGTCCCCTTACCCGGCCCCAGGACCTGTACAAAAGGGACATGAAATAAATGTCCTAATGA
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Figure 6. nt sequences and deduced amino acid sequences of the MAG polypeptide. nt and amino acid residue positions for clone M10D are numbered on the left. Clone M10E begins at residue No. 1379 of M10D and is completely identical at every position except for the 45-bp insert shown enclosed by the box. There are eight potential N-linked glycosylation sites in the predicted extracellular portion of MAG which are indicated by ◆ over Asn. The Arg-Gly-Asp segment (position 118–120), described in the text, is bracketed from above and below. The predicted 23 residue membrane spanning segment is indicated above by the heavy bar. Both 5′ and 3′ untranslated segments are indicated as stretches of continuous nt. A potential Poly A acceptor site in the 3′ untranslated sequence is bracketed.

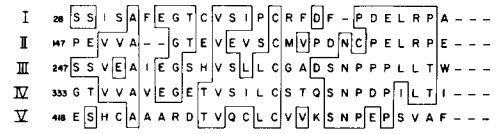

Figure 7. Alignment of internal homologies present in L-MAG. Portions of the primary sequence from Fig. 6 are shown in the single letter code for amino acids. The five segments of internal homology were visually aligned to maximize identities between segments. A break introduced into the sequence is indicated by a dashed line. The position of the initial amino acid of each of the five homology segments (I–V) is indicated to the left of the figure. Additional, but less striking, homologies were found to exist outside of the sequence shown and are described in the text.

this process (40). The analysis of their amino acid sequences and structural features, discussed below, may aid in identifying mechanisms underlying these interactions.

Of particular interest in the MAG amino acid sequence is the segment RGD (residues 118 to 120). This tripeptide sequence has been demonstrated to be a crucial element in the interaction of a growing number of cell surface receptors with extracellular proteins (42, 34). For example, the cell receptors for fibronectin (37, 53, 65), vitronectin (38), and osteopontin (33) have been shown to interact with RGD sequences on these extracellular matrix proteins. The RGD sequence of L-MAG is found near the potentially extracellularly disposed amino terminus of the protein (Fig. 6) and is therefore in a position that would allow it to interact with a ligand of the closely apposed axonal surface. Whether the RGD sequence present on the MAG polypeptides actually mediates an interaction between the glial cell and the axon will need to be determined directly.

Another striking feature of the amino acid sequence of L-MAG is the presence of five segments (I–V) of internal homology, each of which contain sequences that resemble those of immunoglobulin domains. They are sequentially arranged in the extracellular portion of L-MAG. These segments are most closely related to each other over the sequences shown in Fig. 7; lesser degrees of homology also exist outside the illustrated sequences. In particular, segments III and IV share 38 identical amino acids and 24 conservative replacements over the 83 amino acid stretch shown in Fig. 8. Furthermore, both segments III and IV contain sequences that closely resemble the consensus sequence (E-D-x-G-x-Y-x-C) present in the variable region of several immunoglobulins (63) (e.g., positions 299–305 and 385–392).

A computer search of known sequences in the protein data

*Table I. Proteins Homologous to MAG**

	Identity	MAG region	Z value
	%		
N-CAM (169–367)	27.8	224–408	14.3
HLA class II, DR (53–167)	19.7	181–294	8.91
HLA class II, DC-1 (50–223)	19.3	177–346	8.81
H-2 class II, E_α (107–167)	25.8	235–294	7.14
Ig heavy chain V–III region (72–104)	30.3	279–311	8.02
Poly-Ig receptor (364–478)	19.0	317–432	7.29
Alpha 1β-glycoprotein (333–448)	23.9	283–392	6.15

* A search of the National Biomedical Research Foundation protein data base with the amino acid sequence of L-MAG by the FASTP program (24) revealed significant homology to a number of immunoglobulin-related proteins. These proteins, which are listed above, include chicken N-CAM (14), human HLA class II antigens DR (5) and DC-1 (1), the murine H-2 antigen E_α (3) and immunoglobulin heavy chain V–III (60), the rabbit poly-Ig receptor (29), and human alpha 1β-glycoprotein (19). Numbers in parentheses correspond to the sequences of each protein most homologous to the indicated sequences of MAG. The significance of the homology of each protein to MAG is given by its Z value (24), which corresponds to the number of standard deviations by which the score of an optimally aligned segment of MAG differs from the mean score of randomly permutated MAG sequences aligned with the same protein. It has been suggested (24), that Z scores >6 are probably significant, and those >10 are definitely significant.

base revealed significant homologies between MAG and a number of immunoglobulin-related proteins (Table I). These include the human plasma alpha 1 β-glycoprotein (19), the rabbit poly-Ig receptor (29), several histocompatability class II antigens (1, 3, 5), and the variable region of several immunoglobin heavy chains (41, 60). By far the strongest homology encountered, however, was to N-CAM, a well characterized cell adhesion molecule in the vertebrate nervous system which is believed to mediate, via homophilic binding, neuron–neuron interactions (9, 43). The partial amino acid sequence reported for chicken N-CAM (14) contains four segments of internal homology that are described as variable-like in sequence characteristics (14) and constant-like in structural characteristics (17). The two homology units of MAG that most closely resemble variable regions in sequence characteristics (e.g., III and IV) have a strong homology (e.g., 31% identity over 168 amino acids) to a similar stretch of N-CAM containing two internal repeats. These segments of MAG and N-CAM are shown, optimally aligned, in Fig. 8. Significant but less striking homology also exists between the other internal domains of MAG to each of those of N-CAM (data not shown). It is particularly striking that these two molecules, which are believed to mediate

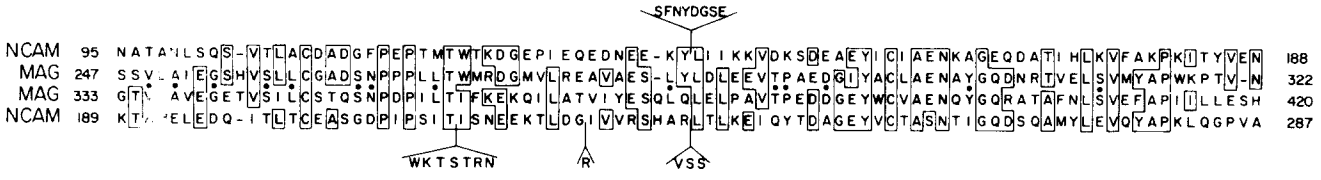

Figure 8. Homology between MAG and N-CAM. Portions of the rat L-MAG and chicken N-CAM (14) primary sequence are shown in the single letter code for amino acids. The segments shown correspond to MAG homology units III and IV (lines 2 and 3) and the two internal repeats of N-CAM that they most closely resemble. Identities between segments were optimized by pairwise alignment with the ALIGN program (49). Only those amino acids that are identical on both a MAG and an N-CAM segment are boxed. Additional identities shared between the MAG homology units are indicated by dots between the second and third lines. Amino acid positions are indicated on the left and right margins.

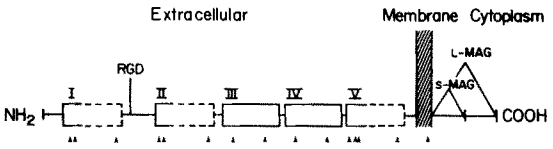

Figure 9. The major structural features of the MAG polypeptides. This diagram summarizes the major structural features of the MAG polypeptides discussed in the text. Both S-MAG and L-MAG are thought to have an identical large extracellular domain and membrane spanning segment, but differ in their cytoplasmic domains. The extracellular domain contains five segments of immunoglobulin-like internal homology that are shown as boxes; regions of strong homology are shown by continuous lines, and less precise sequence homologies are dotted. Arrowheads indicate the position of cysteines. Note that each homology unit contains at least two cysteines spaced an average of 51 amino acids apart. The position of the RGD sequence that may participate in cell adhesion is also indicated.

cell–cell interactions in the nervous system, share such close homology. It may be that other cell adhesion molecules in the nervous system will be found to share sequence and structural homologies with N-CAM and MAG as well.

MAG may therefore be considered a member of the immunoglobulin gene superfamily (15, 17, 62, 63). This is a family of proteins that share a common extracellular subunit structure termed the immunoglobulin homology unit (17). This unit is ~100 amino acids long and contains cysteine residues postulated to form an intrachain disulfide linkage that stabilizes a series of characteristically folded antiparallel beta sheets (62, 63).

At present nothing is known about how the extracellular portion of MAG is folded. It is of interest to note however, that each of the five homology units contains at least two cysteine residues that are spaced, on average, 51 amino acids apart. Furthermore, the sequences surrounding these cysteines are highly conserved in each unit, suggesting that these cysteines and their flanking sequences are structurally important. In view of the homologous sequences and similarly spaced cysteine residues shared by the MAG and immunoglobulin domains, it is reasonable to suggest that these protein domains are structurally similar as well, and that two cysteines within each MAG homology unit are disulfide linked. Although MAG contains sequences resembling those of variable domains, the cysteine spacing (as well as a preliminary analysis of the secondary structure of the extracellular segments) is more closely related to that described for the constant domains of immunoglobulins (62, 63).

Our data therefore predicts that MAG contains five extracellularly disposed, disulfide-linked homologous domains (summarized in Fig. 9). In this proposed structure it would resemble the alpha 1 β-glycoprotein (19), and the poly-Ig receptor (29). As has been proposed for these proteins and other members of the immunoglobulin gene superfamily, MAG may have evolved by gene duplication from an ancestral immunoglobulin-like gene involved in cell recognition phenomena (19, 29, 17).

A most unexpected finding of the homology search was that a cDNA with an identical amino acid and nucleotide sequence to the 3′ end of L-MAG had been previously isolated. This cDNA, clone 1B236 (52), corresponds to the 3′ half of M10D except that it contains an additional 90 nt in the 3′ untranslated region, including an alternate Poly A acceptor site, and ends in a Poly A tract. 1B236 had been considered to be a neuron-specific cDNA. This conclusion was based on immunocytochemical studies in which antibodies, raised against chemically synthesized peptides (based on the deduced amino acid sequence of the cDNA), were reported to show staining of specific neuron groups in the CNS (4, 23). We cannot reconcile these observations with the results of the previously discussed immunocytochemical studies that localized MAG to myelinating cells (50, 55, 27). If a localization to neurons is confirmed, however, it would suggest that MAG may have a more general role in cell–cell interactions in the nervous system than previously appreciated.

We have demonstrated that the two MAG proteins encoded by M10D and M10E differ in the extent of their intracytoplasmic domains (Fig. 6). This is likely to be the only difference between these proteins, since the calculated molecular mass difference (5 kD) of their carboxy terminal regions is in close agreement with the directly determined molecular mass difference between the native polypeptides (Fig. 1). Furthermore, protease V8 peptide maps of the two MAG polypeptides also suggest that they differ by a single peptide fragment unique to L-MAG (10). Until a full-length cDNA for S-MAG has been characterized, other amino acid differences cannot be ruled out. It is of interest that N-CAM, whose similarities to MAG in structure and function have already been noted, also exists in multiple forms that differ in the extent of their cytoplasmic domains (14). These proteins, termed large domain and small domain N-CAM (14), contain cytoplasmic segments of 362 and 101 amino acids, respectively, and, like MAG, are expressed differentially during development (11).

The data presented in this paper suggest that the MAG proteins are products of a single gene whose primary transcript may be alternatively spliced to yield the two MAG mRNAs. This is consistent with the identity of M10D and M10E at all nt positions, with the exception of the internal 45-nt segment present only in M10E (Fig. 6). Furthermore, preliminary Southern blot studies of rat genomic DNA are also consistent with the presence of a single MAG gene (data not shown). Finally, we have also detected a much larger difference in the size of MAG mRNAs by RNA blot analysis (Fig. 4), e.g., mRNAs of 2,500 and 3,000 nt. This is almost certainly due to sequence differences in the untranslated regions, possibly an alternate polyadenylation site.

The functional significance of the two different intracytoplasmic domains is not known. One possibility is that the cytoplasmic segments may have important and perhaps distinct interactions with intracytoplasmic constituents, particularly with cytoskeletal elements. In this regard it is noteworthy that the putative cytoplasmic segment of MAG is homologous to a similarly disposed cytoplasmic segment of integrin, a plasma membrane receptor that binds actin intracellularly (53). Specifically, the carboxy-terminal 21 amino acid segment of integrin shares eight identical and eight conserved amino acids with amino acids 551–573 in both L-MAG and S-MAG when two gaps are allowed (data not shown). It is also of interest that actin is known to have a similar periaxonal localization to that of MAG (64). Additional studies will be necessary to establish whether either or both MAG proteins directly interact with actin or other cytoskeletal elements.

It is also possible that the two cytoplasmic segments of

MAG are phosphorylated differently, as has been demonstrated for the two alternate cytoplasmic domains of N-CAM (46). Based on the amino acid sequences surrounding known phosphorylation sites of other proteins (30), several serines and threonines of the predicted cytoplasmic segments of the MAG proteins may be phosphorylated in vivo. These include potential phosphorylation sites for calcium/calmodulin-dependent protein kinase at amino acids 537–543 (RRKKNVT) and for protein kinase C at 575–582 (KRLGSERR) and 604–608 (KRPTK). In addition, a tyrosine of L-MAG (amino acid 620) lies in a sequence (TEELAEY) that closely resembles the tyrosine autophosphorylation site of the EGF receptor (TAENAEY) (8, 18). Phosphorylation of the EGF receptor at this tyrosine may be an important modulator of the activity of this receptor in vivo (18). Whether these sites on the MAG proteins are actually phosphorylated in vivo and their relevance for the roles of the two MAG proteins during development will require further investigation. It is intriguing to note that three of the potential sites described above are present only in L-MAG.

In summary, we have isolated cDNA clones that encode alternate forms of the MAG proteins. Sequence analysis of these clones revealed homologies to the immunoglobulin gene superfamily, particularly to N-CAM, and the presence of an RGD sequence that may be important for the postulated role of the MAG proteins in glial–axonal interactions. The availability of these clones will facilitate future studies directed at the precise role of the MAG proteins in cell–cell interactions and the significance of their distinct cytoplasmic domains.

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