

# The Myelin-associated Glycoproteins: Membrane Disposition, Evidence of a Novel Disulfide Linkage between Immunoglobulin-like Domains, and Posttranslational Palmitylation

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**Abstract.** The myelin-associated glycoproteins (MAG) are members of the immunoglobulin gene superfamily that function in the cell interactions of myelinating glial cells with axons. In this paper, we have characterized the structural features of these proteins. The disposition of MAG in the bilayer as a type 1 integral membrane protein (with an extracellularly disposed amino terminus, single transmembrane segment, and cytoplasmic carboxy terminus) was demonstrated in protease protection studies of MAG cotranslationally inserted into microsomes in vitro and in immunofluorescent studies with site specific antibodies. A genetically engineered MAG cDNA, which lacks the putative membrane spanning segment, was constructed and shown to encode a secreted protein. These results confirm the identity of this hydrophobic

sequence as the transmembrane segment. Sequencing of the secreted protein demonstrated the presence of a cleaved signal sequence and the site of signal peptidase cleavage. To characterize the disulfide linkage pattern of the ectodomain, we cleaved MAG with cyanogen bromide and used a panel of antibodies to coprecipitate specific fragments under nonreducing conditions. These studies provide support for a novel disulfide linkage between two of the immunoglobulin domains of the extracellular segment. Finally, we report that MAG is posttranslationally palmitylated via an intramembranous thioester linkage. Based on these studies, we propose a model for the conformation of MAG, including its RGD sequence, which is considered with regard to its function as a cell adhesion molecule.

THE myelin sheath is formed by Schwann cells in the peripheral nervous system and the oligodendrocytes in the central nervous system, as a concentric, multilamellar spiral around an axon. Myelin forms in close apposition to, and its formation is regulated by contact of the glial cell with the surface of the axon (44). Cell adhesion molecules present on the extending glial process, are thought to mediate the interactions of the glial cell with the axon (36). The myelin-associated glycoproteins (MAGs)<sup>1</sup> are considered likely candidates for mediating these interactions (31). This suggested role is consistent with their localization to the periaxonal membrane (23, 50) and their ability to bind to large caliber axons that are destined to become myelinated (35). Two isoforms of MAG, designated large MAG (L-MAG) and small MAG (S-MAG), have been identified (19, 37). Both are heavily glycosylated integral membrane proteins (~30% by weight) that comigrate on SDS-PAGE as a broad band of 100 kD. Distinct functions for each isoform is suggested by their differential expression during development: L-MAG is synthesized during the rapid phase of myelination

and S-MAG is synthesized predominately in the adult (8, 19). These isoforms are also differentially phosphorylated in vivo (6) and in vitro (1).

cDNAs for both MAG isoforms have been isolated and characterized (19, 37). They are encoded by a single gene whose RNA transcript is alternatively spliced. An analysis of their deduced amino acid sequences, has suggested potential structure-function correlates (37). Both proteins are predicted to have an identical, large extracellular disposed amino-terminal segment and a single transmembrane segment (e.g., they are type 1 integral membrane proteins). They differ in their carboxy-terminal, presumptive cytoplasmic sequences. The extracellular segment, which is expected to mediate binding to the axon, contains five immunoglobulin homology units. The MAG proteins are therefore members of the immunoglobulin gene superfamily (IgSF) (4, 20, 37). They are homologous to a subset of the IgSF that includes several well-characterized cell adhesion molecules, including the neural cell adhesion molecule (NCAM), L1, and I-CAM 1 (52). Also present in the ectodomain of MAG is the tripeptide recognition sequence Arg-Gly-Asp (RGD). RGD sequences are frequently an important determinant of the binding site of cell recognition and extracellular matrix

1. *Abbreviations used in this paper:* IgSF, immunoglobulin family; MAG, myelin-associated glycoproteins.

molecules that bind to a family of heterodimeric surface receptors, the integrins (11, 33). Determination of whether the RGD sequence of MAG is an important functional element therefore, has clear implications as to whether its axonal receptor is an integrin or whether, like most IgSF members, its axonal receptor is immunoglobulin related.

While the disposition of MAG within the lipid bilayer has been predicted to be conventional, several features of its sequence are atypical for IgSF members. MAG has 14 cysteines in its ectodomain distributed amongst its five immunoglobulin domains. This suggests that either MAG contains free cysteines or that it has more than the single intradomain disulfide linkage that is typical of immunoglobulin domains. There is an additional cysteine within the predicted membrane segment, that may also be covalently linked.

In this paper, we have characterized the structural features of MAG. We have confirmed its disposition as a type 1 integral membrane protein and identified the site of signal peptide cleavage. In addition, we have investigated the covalent linkages of the cysteines present in the ectodomain and demonstrated the existence of a novel disulfide linkage between immunoglobulin domains of MAG. Finally, we have shown that a cysteine within the lipid bilayer is posttranslationally palmitylated. Based on these studies, we propose a model for the conformation of MAG, including the location of its RGD sequence. This model is considered with regard to the function of MAG as a cell adhesion molecule.

## Materials and Methods

### *In Vitro Transcription and Translation*

An L-MAG cDNA was subcloned into the plasmid, pGEM4 (Promega Biotech, Madison, WI). Complementary RNA was transcribed using SP6 polymerase in the presence of the RNA cap analogue m<sup>7</sup>G(5')ppp(5')G (Pharmacia Fine Chemicals, Piscataway, NJ) according to the directions of the manufacturer. 1  $\mu$ l of the transcription reaction was used to program a rabbit reticulocyte translation system (New England Nuclear, Boston, MA) containing [<sup>35</sup>S]methionine (New England Nuclear) for 30 min at 37°C with or without canine pancreatic microsomes. For protease protection studies, translation mixtures were divided into equal aliquots: one was treated on ice for 45 min with proteinase K (Boehringer-Mannheim Biochemicals) at 0.5 mg/ml final concentration and the other served as a control. A 10-fold excess of ice-cold methanol was then added to precipitate proteins and pellets were collected at 13,000 g. Immunoprecipitation procedures are described below.

### *Generation of anti-MAG Antibodies*

Rabbit polyclonals were prepared against two MAG segments: a 20-mer corresponding to amino acids 109–128 of the published amino acid sequence of MAG(37), which includes the RGD sequence (peptide 1) and the carboxy terminal 20 amino acids of L-MAG (peptide 2). Both peptides were synthesized with carboxy terminal cysteines and were coupled, by cross-linking with *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce Chemical Co., Rockford, IL) after the manufacturer's instructions, to keyhole limpet hemocyanin (KLH). Rabbits were inoculated with the coupled peptides as previously described (37).

### *Expression of MAG Isoforms in a Schwann Cell Line*

Construction of a recombinant retrovirus encoding L-MAG has been previously described (27). Construction of an S-MAG encoding retrovirus was performed in a similar manner. Briefly, Hind III linkers were added to the 2087 bp Apa 1 cDNA fragment of S-MAG, corresponding to the complete coding sequence and this cDNA was subcloned into the Hind III site of the retroviral vector, pMV-7(16). This construct was introduced into the ecotropic packaging cell Psi2 by calcium phosphate-mediated DNA trans-

fection (51). Viral stocks shed by the transfected psi 2 cells were used to infect an immortalized Schwann cell line (29) as described (16). Individual clones that were resistant to the neomycin analogue, G418 (Gibco Laboratories, Grand Island, NY) at a concentration of 800  $\mu$ g/ml, were selected and checked for their expression of MAG by immunofluorescent staining.

### *Indirect Immunofluorescence*

Permanently transfected L-MAG Schwann cells, plated onto glass coverslips, were washed twice with PBS, fixed for 10 min with 4% formaldehyde and, in some cases, were then permeabilized with 0.05% Triton X-100 in PBS for 5 min. Cells were washed twice with PBS and then incubated for 30 min in PBS/5% normal goat serum to block nonspecific binding. The primary antibodies used were the polyclonal antibodies to peptide 1 and peptide 2, used at a 1:100 dilution in PBS/5% normal goat serum, and an anti-MAG mAb (28), (a gift of Dr. M. Schachner, ETH, Zurich) used as undiluted hybridoma culture supernatant. Cells were incubated for 1 h at room temperature then washed, and a secondary antibody, fluoresceinated goat anti-rabbit or antimouse (Cappel Laboratories, Malvern, PA), was added at 1:100 in PBS/5% normal goat serum. Coverslips were washed three times in PBS, mounted in a PBS-gelatin solution, and viewed by epifluorescence microscopy.

### *Construction of a Recombinant cDNA Corresponding to the Ectodomain of MAG*

To generate a secreted form of MAG, we introduced a translation stop codon amino terminal to the predicted membrane spanning segment of an L-MAG cDNA (after nucleotide position 1,665 of the published MAG sequence [37]). A sense oligonucleotide and a complementary antisense oligonucleotide were synthesized (model #380A DNA Synthesizer; Applied Biosystems, Inc., Foster City, CA), gel purified, and annealed together. The resulting double stranded DNA fragment shown below contained an Xho I-compatible overhang 5' on the sense (top) strand, the coding sequences of the 15 amino acids proximal to the predicted membrane spanning segment, a translation stop codon (TGA), and an Eco RI-compatible overhang (3' on the sense strand) to facilitate subcloning into an expression vector.

5'-TCGAGCTGCCTTTCCAGGGAGCACACCGACTGATGTGGGCCAAATGAG-3'  
3'-CGACGGAAAGGTCCCTCGTGTGGCTGACTACACCGGTTTACTCTTAA-5'

This double stranded fragment was then ligated to the 1,619-bp Eco RI-XhoI fragment that encodes the major portion of the ectodomain of MAG. The resultant cDNA, which encoded the entire extracellular domain of MAG, was subcloned into the Eco RI site of the expression vector pMT2 (15) to create pMT2-ECD. A similar strategy was also used recently to generate a soluble form of MAG in transfected cells (13).

### *COS Cell Expression and Purification of Soluble MAG*

COS-7 cells were transfected with pMT2-ECD by the DEAE-dextran (22). 72 h after transfection, culture media was harvested. In most experiments, cells were grown under low serum conditions (DMEM with 0.5% Serum Plus; Gibco Laboratories), which greatly simplified subsequent purification of the secreted MAG protein because of the reduced level of proteins in the media without adversely affecting the level of MAG secretion. The media was then concentrated by ultrafiltration over an Amicon Filter (Amicon Corp., Danvers, MA) (mol mass cutoff of 30 kD), and the soluble MAG was purified by lentil lectin chromatography, elution with 0.5 M methyl mannoside, followed by fast protein liquid chromatography on a Mono Q anion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). The secreted MAG eluted in phosphate buffered saline using a gradient of NaCl, at a concentration of 0.2 M.

### *Peptide Sequencing*

Purified soluble MAG was sequenced by automated gas-phase protein sequencing (model 470; Applied Biosystems, Inc.).

### *Immunoprecipitation and Immunoblotting*

Total cell lysates were prepared by scraping up 10<sup>6</sup> transfected cells and boiling in 2% SDS. Samples were diluted fourfold with PBS containing Triton-X-100 (0.5%) and 1 ml samples were precleared with protein A coupled to Sepharose CL4B (Sigma Chemical Co.) by incubating with 10  $\mu$ l of beads for 2 h and briefly centrifuging at 13,000 g. The supernatants were

incubated overnight at 4°C with 30  $\mu$ l of the anti-peptide antibodies. Immunoprecipitates were collected with protein A/Sepharose, released by boiling for 2 min in SDS-PAGE sample buffer, and fractionated by electrophoresis on 7.5–15% polyacrylamide gradient gel (18). The gels were fixed in 30% methanol, 10% acetic acid, 60% water, treated with EN<sup>3</sup>HANCE following the manufacturer's instructions (New England Nuclear), and fluorographed. Western blotting of samples was carried out by described procedures (47) and antibodies were visualized with <sup>125</sup>I-Protein A (ICN Biochemicals, Irvine, CA).

### Alkylation of Cysteines

Purified soluble MAG or immunoprecipitated L-MAG (from transfected Schwann cells) were alkylated with neutralized <sup>14</sup>C iodoacetic acid (5  $\mu$ Ci/ml) (ICN Biochemicals) at room temperature in the dark in a solution consisting of 0.2 M Tris-HCl (pH 8.1), 2 mM EDTA, and 6 M guanidine hydrochloride. Samples were either alkylated directly or alkylated after prereduction with a 100-fold molar excess of DTT for 1 h at 37°C. After 60 min, the alkylation was stopped by quenching with an excess of  $\beta$ -mercaptoethanol. The samples were dialyzed against 0.1 M Tris, pH 7.4 and analyzed by SDS-PAGE.

### Metabolic Labeling of Cells

Confluent T-75 flasks of transfected Schwann cells were labeled with either [<sup>35</sup>S]cysteine, [<sup>35</sup>S]methionine, or [<sup>3</sup>H]palmitate. To label MAG with [<sup>35</sup>S]cysteine, cells were preincubated in cysteine-free DME for 30 min, and then labeled with 100  $\mu$ Ci/ml of [<sup>35</sup>S]cysteine in cysteine-free DME (Gibco Laboratories) for 2 h at 37°C, followed by an additional 2-h chase in DME supplemented with 10% FCS. For the palmitoylation studies, the transfected Schwann cells were labeled with 400  $\mu$ Ci/ml of 9-10-[<sup>3</sup>H]palmitic acid (30 mCi/mmol; New England Nuclear) in DME with 5 mM Na pyruvate plus 1% FCS for 5 h at 37°C. In all cases, at the end of the labeling period the cells were washed twice with PBS, lysed in 2% SDS, scraped into a conical tube, sonicated for 60 s and boiled for 2 min before immunoprecipitation with anti-MAG antibodies.

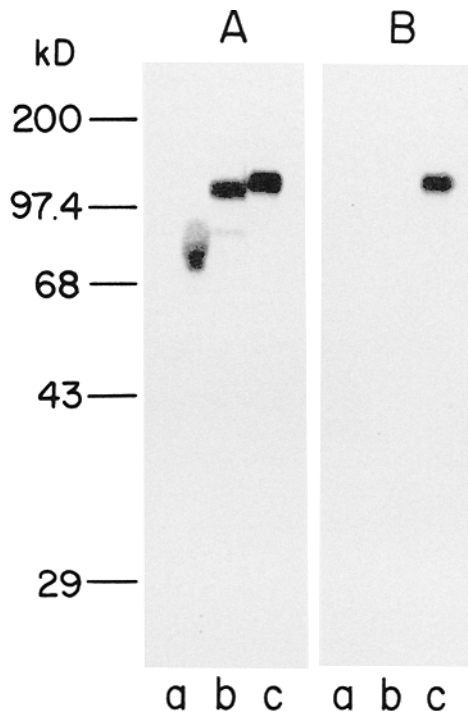
### Cyanogen Bromide Cleavage

5  $\mu$ l of a cyanogen bromide (CNBr) solution (2 g/ml in DMSO) were added to [<sup>35</sup>S]Cysteine-labeled L-MAG immunoprecipitates resuspended in 0.5 ml of 70% formic acid. The reaction mixture was incubated in the dark overnight at room temperature. To control for partial cleavage of Asp-Pro bonds resulting from incubation in the acidic medium, additional samples were incubated in 70% formic acid without CNBr. The reaction mixture was then diluted 10-fold with water and lyophilized. The lyophilized samples were solubilized in sample buffer (without  $\beta$ -mercaptoethanol), sonicated for 30 s, and boiled for 2 min. Samples were then immunoprecipitated with antiMAG antibodies and analyzed by SDS-PAGE with or without  $\beta$ -mercaptoethanol present.

### Characterization of the Palmitoyl Moiety

To analyze the sensitivity of the MAG-palmitoyl bond to neutral hydroxylamine, MAG labeled with [<sup>3</sup>H]palmitate was separated by SDS-PAGE and the gels were soaked for 4 h at room temperature in at least 10 vol of 1 M Tris (pH 7.0) or 1 M hydroxylamine (pH 7.0). The gels were then washed for 1 h in water and prepared for fluorography. Quantitation of fluorographs was performed by optical densitometry.

To identify the nature of the acyl group, the immunoprecipitates of the MAG labeled with [<sup>3</sup>H]palmitic acid were washed in deionized water and then in methanol. The immunoprecipitates were incubated for 4 h in 1 ml 0.1 M KOH in methanol. The supernatant was removed and extracted with 2 vol of chloroform/water (1:1); the upper (aqueous) phase was reextracted with 1 vol of chloroform. The organic phases were combined and washed three times with chloroform/methanol/water (1:10:10). The final organic phase was dried under a nitrogen stream, redissolved in methanol, and analysed by reverse-phase HPLC on a C18 column (Supelcosil LC-318, 4.6 mm  $\times$  25 cm). The mobile phase was acetonitrile/water (85:15, vol/vol); with a flow rate of 1 ml/min. Standards consisting of myristic acid, palmitic acid, methyl myristate, and methyl palmitate (Sigma Chemical Co.) were injected with the radioactive sample. Fractions were collected at 1 min intervals while monitoring at 210 nm by UV absorption, and radioactivity in individual fractions was determined by scintillation counting. Data analysis and graphics were done on a Macintosh II computer.

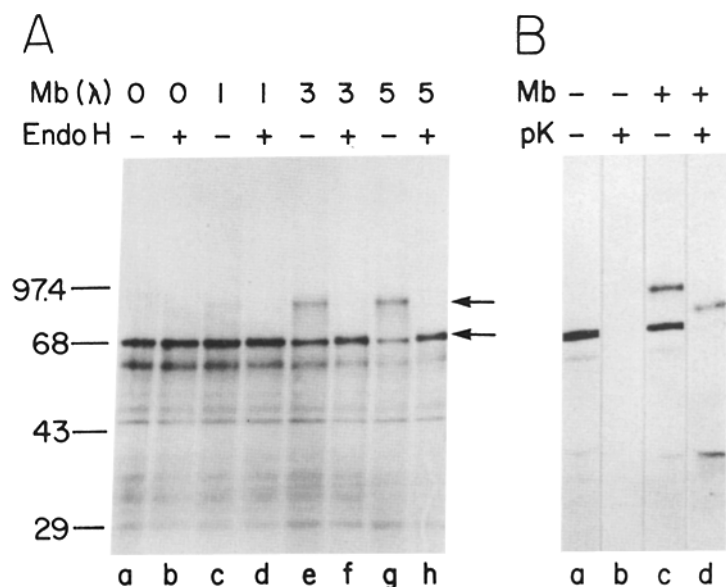


**Figure 1.** Characterization of anti-peptide antibodies. MAG proteins, expressed by transfected cells, were fractionated by SDS PAGE and blotted as described (47). Samples include a recombinant soluble form of the ectodomain of MAG (*rs* MAG) secreted by transfected COS cells (lane *a*), and total cell lysates of S-MAG (lane *b*) and L-MAG (lane *c*) transfected Schwann cells. Primary antibodies were used at a 1:1,000 dilution and consisted of the anti-peptide 1 antibody directed against the RGD and surrounding sequences (*A*) and the anti-peptide 2 antibody specific to the carboxy terminus of L-MAG (*B*). The blots were incubated with <sup>125</sup>I-protein A and exposed for autoradiography.

## Results

### Characterization of the Anti-peptide Antibodies

Site-specific antibodies were generated to the RGD sequence of MAG (peptide 1) and to the carboxy-terminal 20 amino acids of L-MAG (peptide 2). The specificity of each antibody was confirmed by Western blotting. Samples of culture supernatant containing the soluble extracellular domain of MAG (*rs*MAG) (lane *a*), or cell lysates of transfected S-MAG (lane *b*), and L-MAG (lane *c*) cells were fractionated by SDS PAGE and blotted (Fig. 1). Primary antibodies used were directed against anti-peptide 1 in Fig. 1 *A* and anti-peptide 2 in Fig. 1 *B*. The anti-peptide 1 antibody (which recognizes the RGD segment of MAG) reacted with all three forms of MAG whereas the anti-peptide 2 antibody (which recognizes the carboxy terminus of L-MAG) only reacted with L-MAG. These results confirm the specificity of these antibodies. Their use in the analysis of the disposition of MAG and the arrangement of the disulfide linkages of MAG is described below. It should be noted that the relative molecular mass of both isoforms of MAG expressed by fibroblasts (Fig. 1) and Schwann cells (Fig. 6 and 7) is somewhat larger than that present in myelin (L-MAG has a *M<sub>r</sub>* of 120 kD and S-MAG has a *M<sub>r</sub>* of 115 kD), reflecting the more extensive glycosy-



**Figure 2.** Disposition of MAG in the bilayer. An mRNA for L-MAG was translated in a reticulocyte lysate system in the presence of increasing amounts of dog pancreas microsomal membranes (*mb*). The primary translation product, is indicated by the lower arrow (*A*), and the core glycosylated polypeptide, synthesized in the presence of microsomal membranes (lanes *c*, *e*, and *g*), is indicated by the upper arrow. Samples treated with endoglycosidase H are shown in lanes *d*, *f*, and *h*. (*B*) Protease K treatment of MAG co-translationally inserted into microsomes. MAG peptides were immunoprecipitated with an antibody directed against the RGD segment after in vitro translation without (lanes *a* and *b*) or with (lanes *c* and *d*) microsomal membranes. Samples treated with proteinase K are in lanes *b* and *d*. The cotranslationally inserted polypeptide that remains after proteinase K treatment is indicated (arrow, lane *d*).

lation of MAG previously observed (12) in some transfected cells.

### Disposition of MAG in the Membrane

Previous analysis (37) of its amino acid sequence, suggested that MAG, like most immunoglobulin gene superfamily members, is a typical type I (34) integral membrane protein, e.g., it has an extracellularly disposed amino terminus, a single membrane spanning segment, and a cytoplasmic carboxy terminus. We have confirmed this disposition using an in vitro transcription-translation system to direct the synthesis and cotranslational insertion of L-MAG into microsomes and then analyzed its disposition within the microsome in protease protection studies. An mRNA for L-MAG was translated in a reticulocyte lysate with or without purified dog pancreas microsomes. When synthesized in the presence of microsomal membranes, the translated L-MAG shifts from an  $M_r$  of 69 to an  $M_r$  of 94 kD (Fig. 2 *A*), corresponding to the core glycosylated polypeptide. This glycosylated polypeptide is cleaved to an  $M_r$  of 70 kD by endoglycosidase H. Although predicted to have a cleaved signal sequence, the molecular weight of the processed protein after endo H treatment was not decreased compared to that of the primary translation product. This is likely to reflect N-acetyl glucosamine residues that remain after enzymatic deglycosylation.

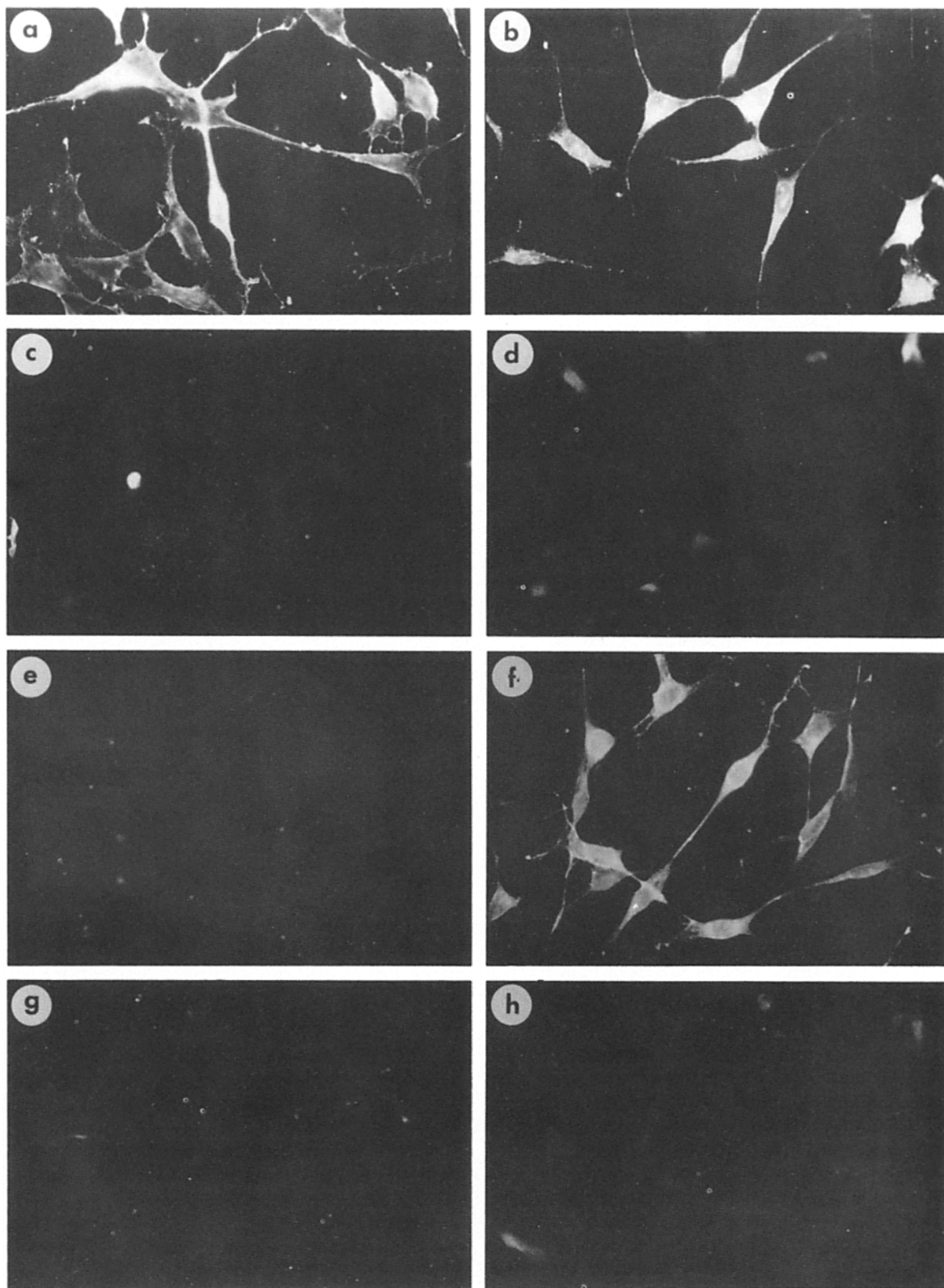
To determine the orientation of the cotranslationally inserted polypeptide within the microsome membrane, the total translation mixture was treated with proteinase K. After protease digestion, a fragment of  $M_r$  of 85 kD remains (Fig. 2 *B*, arrow). This protected fragment corresponds to the intraluminal segment (the future ectodomain) and the membrane-spanning segment. This fragment was specifically immunoprecipitated by the anti-peptide 1 antibody directed against the RGD and surrounding sequences but not by the anti-peptide 2 antibody directed against the carboxy terminus of L-MAG (data not shown). These results indicate that the major portion of the amino terminus of MAG is extracellularly disposed (e.g., intraluminal), that MAG crosses the

membrane only once (since only one fragment results from the protease treatment), and that there is a short (10 kD) carboxy-terminal segment that is cytoplasmic (extraluminal and therefore degraded by proteinase K).

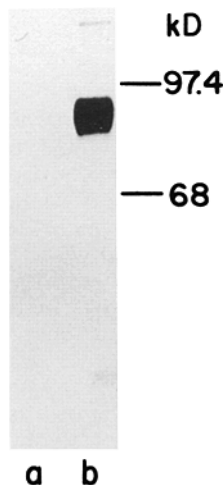
This disposition was confirmed by immunofluorescent studies of the transfected cells expressing L-MAG or S-MAG with the anti-peptide antibodies (Fig. 3). The surface expression of L-MAG is apparent in both nonpermeabilized (*a*) and permeabilized cells (*b*) when stained with MA513, an mAb that recognizes the native conformation of MAG(28). Surprisingly, essentially no staining was visible with the anti-peptide 1 antibodies that recognize the RGD sequence of MAG either before (Fig. 3 *c*) or after (Fig. 3 *d*) permeabilization with 0.05% Triton X-100. These results suggest that this region of MAG is conformationally inaccessible unless MAG is first denatured, and are consistent with other studies described below. By contrast, excellent staining was seen with the antibody specific for the carboxy terminus of L-MAG, but only after cells were permeabilized, consistent with the intracellular localization of this epitope (compare Fig. 3, *e* and *f*). The specificity of the anti-peptide 2 antibody in immunofluorescence was confirmed using S-MAG expressing cells; no staining of these cells was apparent before or after permeabilization (Fig. 3, *g* and *h*).

### MAG Has a Cleaved Signal Sequence and a Single Transmembrane Segment

The myelin associated glycoproteins have two extended hydrophobic segments. The first consists of ~20 amino acids at the amino terminus that is predicted to be a cleaved signal sequence (10). The second, consisting of 23 nonpolar amino acids (514–536 of the published MAG sequence [37]), is likely to be the single membrane spanning segment of MAG demonstrated in the in vitro translation studies described above. To confirm the function of these two hydrophobic segments directly, we inserted a translation termination codon in the cDNA of L-MAG immediately 5' to the predicted membrane-spanning segment (after amino acid 513), and subcloned this recombinant cDNA construct into the eu-



**Figure 3.** Accessibility of MAG epitopes: reactivity with a MAG monoclonal and anti-peptide antibodies. Immunofluorescent staining of cells expressing L-MAG (a–f) or S-MAG (g and h) is demonstrated. Cells on the right side of the figure (b, d, f, and h) were permeabilized with 0.05% Triton before incubation with antibodies. Antibodies used included a MAG monoclonal (MA513) that recognizes the native conformation of MAG(28) (a and b), and antibodies against the RGD (c and d) or carboxy-terminal segments (e–h) of L-MAG.



**Figure 4.** Absence of free cysteines in the ectodomain of MAG. A recombinant protein (*rsMAG*) corresponding to the ectodomain of MAG, was treated with the alkylating reagent,  $^{14}\text{C}$ -iodoacetamide, and the resultant material was separated by SDS PAGE. In lane *a*, *rsMAG* was treated directly with iodoacetamide. In lane *b*, *rsMAG* was first reduced with DTT before treatment with iodoacetamide. Minor contaminants in the *rsMAG* preparation (lane *b*) are faintly visible at 160 and 25 kD.

karyotic expression vector, pMT2 (15). This construct was then used to transiently transfect COS cells. In contrast to the full length L-MAG protein, which is targeted appropriately to the plasma membrane, the truncated protein, which lacks the second hydrophobic segment, is no longer retained at the cell surface but is instead secreted into the culture media (Fig. 1). This recombinant, soluble MAG protein (*rsMAG*), migrates on SDS-PAGE with a  $M_r$  of 85 kD, compared to native MAG present in myelin that migrates as a broad band of 100 kD. The shift in molecular mass of  $\sim 15$  kD is consistent with the deletion, in *rsMAG*, of the membrane and cytoplasmic segments. After enzymatic deglycosylation, the  $M_r$  shifts to 57 kD, reflecting removal of the extensive N-linked carbohydrate chains (data not shown).

Next, we confirmed the role of the amino-terminal hydrophobic segment as a cleaved signal segment by direct amino acid sequencing of *rsMAG*. Although predicted to have a cleaved signal peptide, the site of signal peptidase cleavage was unknown, as MAG purified by published methods (32) has been reported to have a blocked amino terminus. We purified the secreted MAG protein by a combination of ultrafiltration, lentil lectin chromatography, and FPLC, to obtain a preparation of *rsMAG* that was  $>90\%$  pure by SDS-PAGE. We determined the amino terminal sequence by subjecting this material to 15 cycles of Edman degradation. The directly determined amino acid sequence begins with gly-his-trp-gly-etc. and corresponds to a sequence beginning at amino acid 20 of the published MAG sequence (37). These results confirm our previous prediction that MAG is cleaved during its cotranslational insertion into the microsomal membrane and that the site of cleavage is after amino acid 19 of the propeptide (37).

The results of these studies indicate that the two isoforms of MAG correspond to polypeptides of 607 amino acids for L-MAG and 563 amino acids for S-MAG with molecular masses of 67.1 and 62.1 kD, respectively. Both proteins have identical ectodomains of 494 amino acids and membrane-spanning segments of 23 amino acids, but differ in the extent and sequences of their cytoplasmic segments (90 amino acids for L-MAG and 46 amino acids for S-MAG).

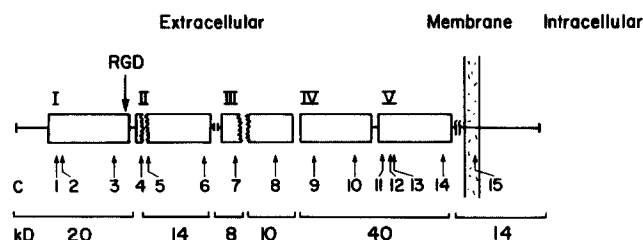
### A Novel Disulfide Linkage Present in the Ectodomain

To further characterize the structure of MAG, we have deter-

mined its pattern of disulfide bonds. Immunoglobulin domains typically contain a single intradomain disulfide linkage whose function may be to stabilize the conformation of the Ig fold and confer stability against extracellular proteases (52). The ectodomain of MAG has five immunoglobulin domains, containing a total of 14 cysteines. The distribution of these cysteines consists of three in each of the first two Ig domains, two each in the third and fourth domains (the most conventional Ig-like domains of MAG), and four in the fifth (membrane proximal) domain (see Fig. 5).

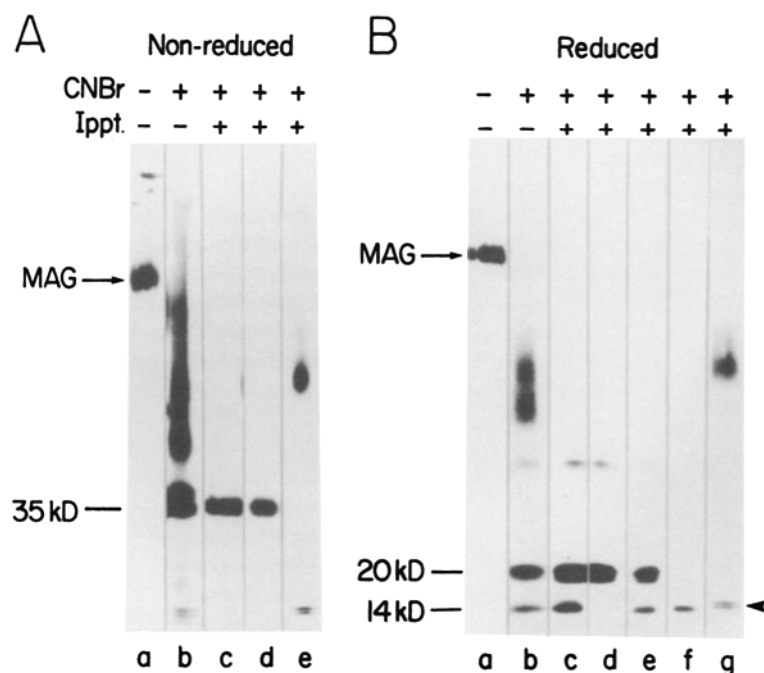
We first determined whether any of the cysteines of the ectodomain of MAG are unconjugated, or whether they all form disulfide bonds.  $^{14}\text{C}$ -labeled iodoacetamide, an alkylating reagent, was used to label free sulfhydryl groups. None of the cysteines of *rsMAG* (Fig. 4) or L-MAG (data not shown) were labeled by this reagent, unless they were first reduced with DTT. These results suggest that all of the cysteines in the ectodomain are disulfide linked, a finding that is further supported by a comparison of the electrophoretic mobility of L-MAG and *rsMAG* under reducing and non-reducing conditions. Both L-MAG and *rsMAG* migrate more rapidly under nonreducing conditions than under reducing conditions; for example L-MAG migrates with an  $M_r$   $\sim 90$  vs.  $\sim 120$  kD, respectively (lanes *a*, Fig. 6 *A* vs. Fig. 6 *B*). This migration pattern is consistent with the presence of extensive disulfide linkages in the ectodomain that stabilize a compact structure during SDS-PAGE under nonreducing conditions.

Next, we determined how the additional cysteines present in the first, second, and fifth domains are conjugated. One possibility was that MAG might be covalently linked to itself to form a homodimer, or to another protein to form a heteromer, similar to the covalently linked heterodimers formed by immunoglobulin heavy and light chains. The migration of MAG as a monomer under nonreducing conditions on SDS PAGE (Fig. 6 *A*, lane *a*) excludes these possibilities, however. Therefore, the disulfide linkages of MAG must all be intrachain. Two other models were thus considered likely: either that the additional cysteines present in domains one and two are linked to each other (linking domains one and two), or alternatively that these cysteines are disulfide linked



**Figure 5.** Cyanogen bromide cleavage pattern of MAG. In this diagram, the five Ig domains of MAG are shown as boxes. The predicted sites of CNBr cleavage (corresponding to the location of the methionine residues) are indicated by breaks in the diagrammatic model of MAG. The location of the 15 cysteines (*C*) are numbered sequentially. The size in kilodaltons of the CNBr fragments predicted for complete cleavage, including 4 kD for each potential N-linked glycosylation site, is given at the bottom. The location of the RGD peptide (against which an antibody was raised) is shown at the end of domain one. Modified from reference 37.





**Figure 6.** Evidence that domain one and two of MAG are disulfide linked. [ $^{35}$ S]Cysteine-labeled MAG from transfected Schwann cells was immunoprecipitated and cleaved with cyanogen bromide. This material was run on SDS-PAGE under non-reducing conditions (A) or reducing conditions (B). In A, the uncleaved initial immunoprecipitate of MAG is shown in lane a and the total material after cyanogen bromide cleavage in lane b. Fragments were specifically immunoprecipitated with antibodies to the RGD sequence of domain one (lane c), the antiMAG monoclonal GEN S3 (lane d), or an antibody to the carboxy-terminal 20 amino acids of L-MAG (lane e). In B, the uncleaved immunoprecipitate of MAG is shown in lane a and the total material after cyanogen bromide cleavage in lane b. Fragments that precipitated with the antiRGD antibody before reduction (lane c) or after reduction (lane d) or with GEN S3 before (lane e) or after (lane f) reduction are shown. The arrowhead indicates the 14-kD doublet precipitated by the antibody specific for the carboxy terminus of L-MAG (lane g). Molecular weights shown are estimated from the position of standards.

to two of the four cysteines present in domain five (thereby linking domains one, two, and five).

To distinguish these possibilities, we first cleaved [ $^{35}$ S]-cysteine-labeled L-MAG with cyanogen bromide (CNBr) to generate specific fragments of MAG. (The position of the methionines in the L-MAG molecules, and the size of the expected fragments after CNBr cleavage are shown in Fig. 5). We then determined which domains remain covalently linked after immunoprecipitation under non-reducing conditions. We found that after cyanogen bromide treatment, antibodies against peptide 1 (the RGD sequence of domain 1) immunoprecipitate a fragment of  $M_r \sim 35$  kD (Fig. 6 A, lane c). After reduction, this fragment resolves into two fragments of  $M_r$  20 and 14 kD (Fig. 6 B, lane c). The larger 20-kD fragment was demonstrated to correspond to domain one as it is the only fragment immunoprecipitated by the anti-RGD antibody under reducing conditions (Fig. 6 B, lane d). The 14-kD fragment with which it coprecipitates corresponds to the size expected for the fragment of domain two resulting from CNBr cleavage. In addition, the intensity of labeling of these two fragments is proportionate to their expected content of cysteines (four in the 20-kD fragment and two in the 14-kD fragment; see Fig. 4). Domain five (which is contained within a larger fragment of 40 kD that also includes domain four), does not coprecipitate with domain one and two under nonreducing conditions. These results indicate that only domain one and two are disulfide linked and that domain five, which contains four cysteines, must have two intradomain disulfide linkages.

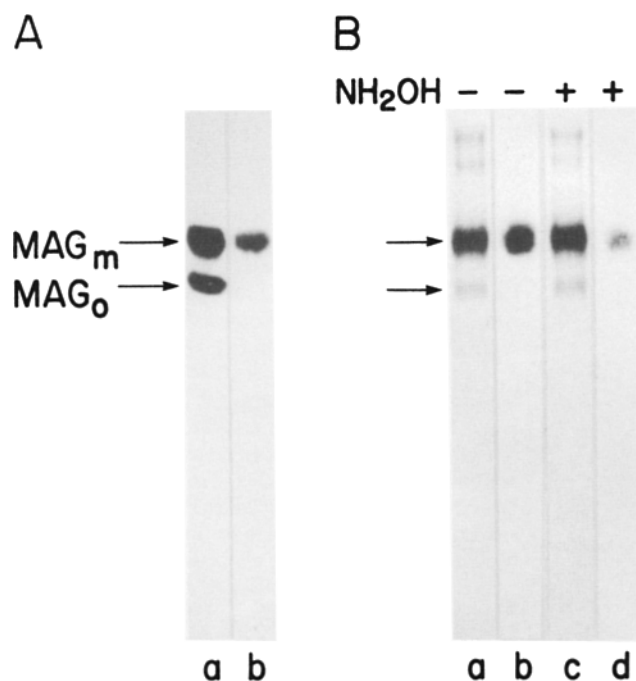
To demonstrate the specificity of the immunoprecipitation of domain one with the antiRGD antibody, parallel studies were performed with two other antibodies. A monoclonal anti-MAG antibody, GEN S3 (25), whose epitope was previously unknown, specifically immunoprecipitates the domain two fragment (Fig. 6 B, lane f) under reducing conditions. Like the antiRGD antibody, under nonreducing conditions the monoclonal antibody recognizes a fragment of  $M_r = 35$

kD (Fig. 6 A, lane d), which resolves into the 20- (domain one) and 14-kD (domain two) fragments when analyzed by reducing SDS PAGE (Fig. 6 B, lane e), confirming the linkage of domain one with two. By contrast, the antipeptide 2 antibody specific for the carboxy terminus of L-MAG, immunoprecipitates a predicted fragment of 14 kD as well as a larger, partially cleaved fragment of MAG (Fig. 6 A, lane e and Fig. 6 B, lane g). The electrophoretic mobility of the 14-kD carboxy-terminal fragment is identical under reducing and nonreducing conditions, consistent with the absence of a disulfide linkage in this segment. This carboxy-terminal fragment typically runs as a doublet, possibly reflecting heterogeneity in the extent of its phosphorylation (1) or palmitylation (see below).

#### **MAG Is Posttranslationally Palmitylated via a Thioester Linkage**

There is an additional cysteine in the membrane spanning segment of MAG. When L-MAG was treated with [ $^{14}$ C]iodoacetamide, this cysteine remained unlabeled suggesting it also participates in a covalent linkage (data not shown). As cysteines present within or near the cytoplasmic side of the bilayer in other integral membrane proteins have been demonstrated to be posttranslationally palmitylated via a thioester linkage (48), we examined whether MAG might be similarly palmitylated.

Transfected Schwann cells expressing MAG, were pulse-labeled with either [ $^{35}$ S]methionine or [ $^3$ H]palmitate and the label associated with MAG was characterized. Under conditions of continuous labeling with [ $^{35}$ S]methionine, two forms of L-MAG are detected (Fig. 7 A, lane a). These correspond to  $M_r$ 's of 94 and 120 kD. The lower molecular weight form corresponds to the core glycosylated, ER form of MAG as evidenced by pulse-chase studies in which this form appears first in short labeling periods (15 min). After a 60-min chase, the mature form (the upper band) first ap-



**Figure 7.** (A) MAG is posttranslationally palmitylated. MAG was immunoprecipitated from transfected Schwann cells expressing L-MAG that were continuously labeled for 5 h with [ $^{35}$ S]methionine (lane a) or [ $^3$ H]palmitate (lane b), and then fractionated by SDS-PAGE (7.5–15% acrylamide gradient). Two forms of MAG are evident in lane a, the core glycosylated form (MAG<sub>o</sub>) and the mature, fully glycosylated form (MAG<sub>m</sub>). [ $^3$ H]Palmitate is specifically only incorporated into the mature form of MAG (lane b). (B) Treatment with hydroxylamine releases  $^3$ H-palmitate. Transfected Schwann cells were pulse labeled with [ $^{35}$ S]methionine for 2 h followed by a chase for an additional 2 h (lanes a and c) or with [ $^3$ H]palmitate continuously for 5 h (lanes b and d). The majority of the MAG evident in lanes a and c, is the mature, fully glycosylated form of MAG. After electrophoresis, the gel was divided in two and incubated for 1 h at room temperature in 1 M Tris, pH 7.0 (lanes a and b) or 1 M hydroxylamine, pH 7.0 (lanes c and d). After rinsing with water, the gels were processed for fluorography.

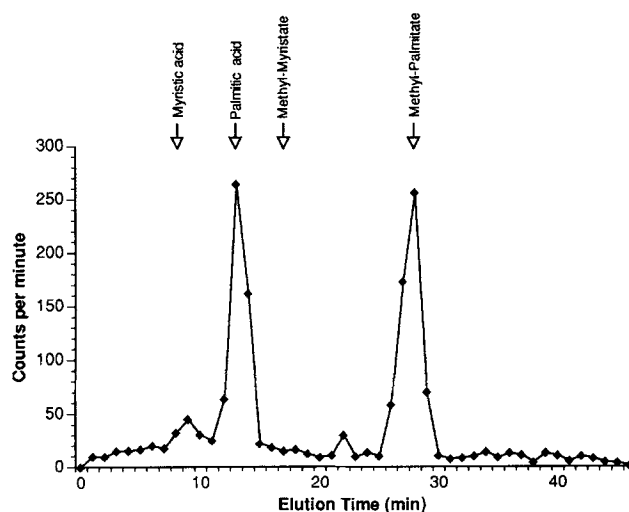
pears and after a 2-h chase, most of the labeled MAG present is the mature form (compare lanes a of Fig. 7, A and B). The higher molecular weight form of MAG is the only form present at the cell surface, based on external labeling (21) of Schwann cells (data not shown). When cells were labeled in parallel with [ $^3$ H]palmitic acid, only the mature, 120-kD form of MAG was labeled, while the core glycosylated, ER form of the protein was not (Fig. 7 A, lane b). In parallel studies, labeling by [ $^3$ H]palmitic acid of S-MAG was similarly restricted to just the mature form (data not shown). These results indicate that MAG is posttranslationally acylated. Further, these results suggest that minimal conversion of the [ $^3$ H]palmitic acid to an amino acid occurred during the period of labeling as the newly synthesized MAG (MAG<sub>o</sub>) was unlabeled.

To clarify the nature of the fatty acylation, and to exclude the possibility that the palmitate had been converted to myristate, we characterized the fatty acyl group and its covalent linkage. Palmitylation typically occurs via an alkaline labile thioester linkage, whereas myristylation typically occurs via a hydroxylamine stable amide linkage (48). First,

we incubated the gel displayed in Fig. 7 B in hydroxylamine for 4 h at room temperature under conditions expected to hydrolyze thioester but not amide linkages. Under these conditions, up to 80% of the [ $^3$ H]palmitic acid label in MAG was removed, while the  $^{35}$ S-methionine label in MAG was unaffected. This result indicates that the incorporated label represents lipid bound to this protein through a thioester linkage. The [ $^3$ H]palmitate-labeled MAG was also treated with KOH and methanol to release the acyl group. The fatty acid that was released by this treatment was then characterized by HPLC. As shown in Fig. 8, the  $^3$ H label migrates with the same mobility as methyl-palmitate and palmitate, consistent with its identification as a palmitoyl group.

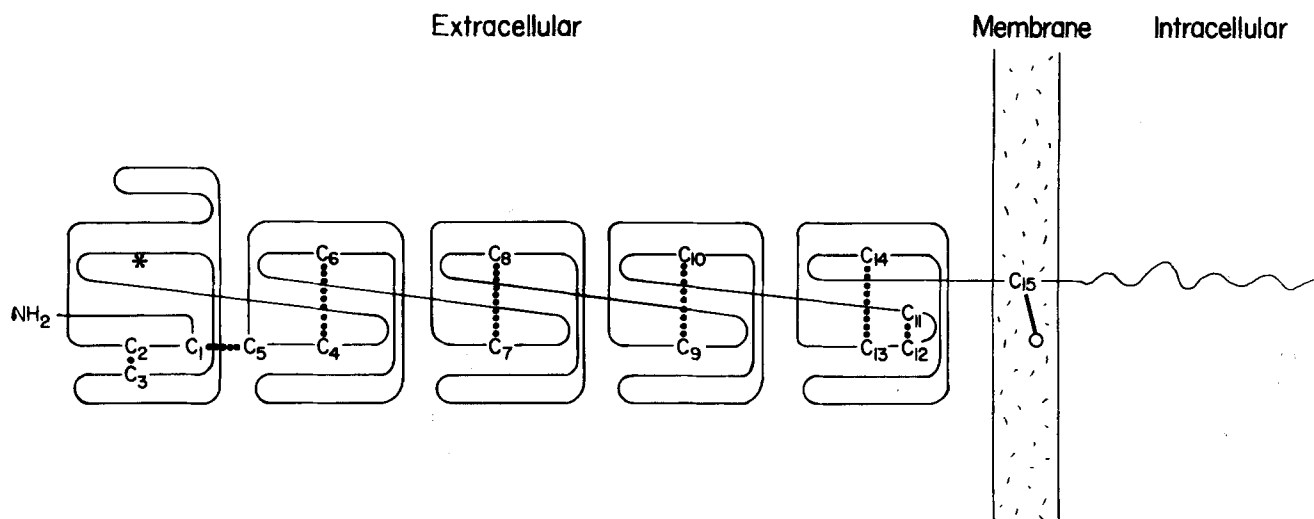
## Discussion

Although the role of MAG in myelination has not been established, evidence to date suggests that it mediates a complex set of interactions, that include binding to a receptor on a subset of axons (12, 27), binding to extracellular matrix components (7) and, possibly, promoting adhesion between the apposed membranes of the myelinating Schwann cell at specific sites (38, 49). These complex interactions in turn imply the existence of multiple functional sites that are perhaps reflected in the extraordinary degree of conservation displayed by all segments of MAG during evolution. Thus human (39, 43) and mouse (9) MAG show an overall 95 and 98% identity of amino acids respectively with rat MAG. The RGD sequence and the number and location of the cysteines have been conserved. As nothing was known about the conformation of MAG or its disposition within the membrane, we undertook the studies described in this paper to clarify the structure of these proteins as a prelude to identifying their discrete functional elements. These studies confirm the disposition of MAG within the bilayer as a type one integral



**Figure 8.** Characterization of the acyl group of MAG. Immunoprecipitates of MAG labeled with [ $^3$ H]palmitate were incubated for four hours in 0.1 M KOH in methanol. The released material was subjected to HPLC as described in Materials and Methods. Two peaks of radioactivity are evident. Fractions containing the standards myristic acid, palmitic acid, methyl myristate, and methyl palmitate are indicated.





**Figure 9.** Model for the conformation of MAG. Diagram showing the major structural features of MAG discussed in this paper. Shown are the five immunoglobulin domains of the extracellular segment, with the first domain folded as a variable domain and all other domains folded as constant (C2) domains. The predicted position of the cysteines and their disulfide linkages are also indicated including an interdomain disulfide linkage of C1 and C5, and the intrasheet disulfide linkages of C2 and C3, and C11 and C12. The palmitoyl group present on the intramembranous cysteines is indicated (O) and the predicted position of the RGD sequence (\*) of MAG within the first domain is shown.

membrane protein, suggest a unique arrangement of its immunoglobulin domains, and demonstrate the post-translational palmitoylation of its intramembranous cysteine.

MAG is a member of the C2 set of the immunoglobulin gene superfamily (53) and contains five Ig domains in its extracellular segment (19, 37). (C2 domains have features intermediate between variable [V] and constant [C] Ig domains in containing the cysteine spacing and seven  $\beta$  strands typical of C domains but sequences which are more homologous to V domains [53]). They are characteristic of multidomain IgSF members that mediate cell adhesion (36, 52). Domains three and four are the most conventional C2 domains of MAG. These domains show the highest degree of homology to other members of this subset of proteins, which includes NCAM and L1 in the nervous system, and I-CAM 1 in extraneural sites. Domains three and four each contain two cysteines that are presumed to form a typical intradomain disulfide linkage.

The other domains of MAG contain more than the two cysteines necessary to form an intradomain disulfide linkage. Domains one and two contain three cysteines each, whereas domain five contains four cysteines. As there are no free cysteines in the ectodomain of MAG (Fig. 4), the additional cysteines of domains one and two must either be disulfide bonded to each other (linking domains one and two) or to the extra cysteines of domain five (linking domains one, two, and five). A priori, the former possibility appeared more likely as a disulfide linkage of domains one and two with the clustered cysteines of domain five (three of which occur in a stretch of 12 amino acids), would seem to be precluded on steric grounds. Evidence presented here confirms this expectation, indicating that only domains one and two are disulfide linked to each other. Thus, these two domains were found to coprecipitate with each other stoichiometrically, but not with domain five, under nonreducing conditions (Fig. 6).

Several features of the primary sequence of the ectodo-

main are relevant to further predictions of the conformation of MAG. Because of the location of the cyanogen bromide cleavage site (shown in Fig. 5), the coprecipitation of the domain two fragment with domain one indicates that either cysteine five or six of this fragment must be disulfide linked to one of the first four cysteines of MAG. In view of the homology of their surrounding sequences to those present in other Ig domains, cysteines four and six would be expected to form the intradomain disulfide linkage of domain two, suggesting that cysteine five is covalently linked to a cysteine of the first domain. A. Williams and his colleagues have recently pointed out that the first domain of MAG has atypical features for a C2 domain (53). In particular, domain one shows the greatest homology to other IgSF proteins when it is folded as a V domain, with 9 beta strands, a model that also optimizes the conserved beta strand assignments for this domain (53). If the first domain of MAG is folded as a V domain, several additional consequences result. The second and third cysteines would be in predicted  $\beta$  strands b and e respectively of the immunoglobulin fold, and in a position to form an unusual intrasheet disulfide linkage with each other. This arrangement also places the first cysteine of domain one in a position to form a disulfide linkage with cysteine five of domain two.

The RGD sequence was originally proposed to be in the hinge region between domains one and two (37) and a potentially important functional determinant. However in the model discussed above, the RGD segment (shown as an asterisk in Fig. 9) now falls into the interior of the first domain, in  $\beta$  strand f. In this position, the accessibility and functional importance of the RGD sequence as a ligand binding site would likely be limited. A location buried within the first domain is consistent with the inability of the anti-RGD antibody used in these studies to recognize MAG in its native conformation (Fig. 3, c and d), although it reacts well with SDS denatured MAG (Fig. 1 A). Also consistent with a

limited functional role for this sequence are recent studies in which synthetic peptides containing the RGD sequence failed to block the adhesion of MAG, incorporated into liposomes, to axons (35). These more recent studies and this model of the conformation of MAG suggest that the axonal receptor MAG is more likely to be another member of the IgSF rather than an integrin.

The fifth domain of MAG is also atypical for C2 domains in containing four cysteines. As none of these cysteines are free, domain five must contain two disulfide linkages. C13 and C14 can be aligned to form a conventional intradomain disulfide linkage (between  $\beta$  strands b and f). The two additional cysteines, C11 and C12, are then predicted to be in  $\beta$  strands a and b, respectively, and in a position to form a second intrasheet disulfide linkage. Based on these arguments, we present a schematic model of MAG in Fig. 9 showing its orientation within the bilayer, its presumptive disulfide linkages, and the palmitoyl group on the intramembranous cysteine.

MAG is not the only IgSF member with additional cysteines present in the ectodomain. Of particular interest are the lymphoid differentiation antigen CD33(42) and the B cell adhesion molecule CD22 (45): integral membrane proteins with two and five extracellular immunoglobulin domains, respectively. Both proteins share an  $\sim 25\%$  amino acid identity with the first two domains of MAG. They each contain six cysteines in their amino-terminal two domains which are present in equivalent positions to their counterparts in MAG. These presumably form an interdomain disulfide linkage similar to that shown here for MAG. These findings further suggest that these two domains of MAG may represent a discrete functional element that may be evolutionarily related to these proteins.

Other IgSF members with an excess of cysteines per domain include c-Kit (30), ICAM 1 (46), and the poly Ig receptor (24). Whether these molecules will contain interdomain disulfide linkages remains to be determined, but the findings here suggest that an interdomain disulfide linkage may be fairly common among IgSF members and of potential importance in stabilizing these proteins in an optimal conformation.

One additional feature of the MAG proteins shown in these studies is the presence of a palmitoyl group on the cysteine within the membrane (seven amino acids in from the cytoplasm). Although more commonly located in the cytoplasm and near the bilayer, an intramembranous palmitoylated cysteine has previously been reported for the class II MHC antigen (14). Like many other proteins, MAG is palmitoylated only on the mature form of the protein, i.e., the palmitoyl moiety is added posttranslationally, possibly in a transition element of the ER or in the *cis*-Golgi (40, 41). The linkage of the palmitic acid moiety to the majority of the acyl proteins described to date has been identified as an oxyester or thioester bond. In the case of MAG, we have shown that the palmitic acid moiety is bound to the polypeptide through a thioester bond based on its sensitivity to alkaline methanolysis and hydroxylamine.

It is intriguing to note that fatty acylation of myelin proteins is a common theme. The major structural proteins of myelin, proteolipid protein in the central nervous system (5), and the PO protein in the peripheral nervous system (2), are both palmitoylated. The myelin protein cyclic nucleotide

phosphodiesterase has also recently been demonstrated to be palmitoylated (3). Evidence has been presented for the presence of a fatty acyl transferase activity within the myelin sheath (5), suggesting that all of these myelin specific proteins may be acylated by a common mechanism.

As is true with other proteins, the functional significance of this palmitoylation is not known. In the case of the structural proteins of myelin, PO and PLP, this fatty acylation may reflect their location and need to function within the highly hydrophobic environment of the compact myelin sheath. By contrast, MAG is present only in the noncompacted membranes of myelinating cells (50), suggesting that its palmitoylation serves a different role. One function of the fatty acylation of other integral membrane proteins, is to regulate the assembly of protein multimers. Thus, in the case of the major histocompatibility antigen, Ia, palmitoylation may prevent dimerization, by inhibiting the formation of disulfide bonds between two adjacent molecules (17). The inhibition of fatty acylation and dimerization in turn blocks post-translational maturation, in particular, the addition of sialic acid. There is also evidence that fatty acylation may be important for the assembly of the acetylcholine receptor complex and its surface expression (26). Whether palmitoylation may also modulate the intramembranous associations and lateral mobility of MAG or, alternatively whether it may regulate the binding activity of the ectodomain or of the cytoplasmic domains of MAG, is as yet unknown.

In sum, we have shown that MAG is a type one integral membrane protein with an unusual arrangement of its immunoglobulin domains, a sterically inaccessible RGD sequence, and a post-translational palmitoylation of its intramembranous cysteine. The availability of cDNA clones for MAG that can be modified and expressed in a myelinating tissue culture system (27), should facilitate our understanding of the functional significance of these structural features of MAG.

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