Determination of a Native Proteolytic Site in Myelin-Associated Glycoprotein

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ABSTRACT: Myelin-associated glycoprotein (MAG) is a transmembrane structural protein that is thought to be involved in the formation and/or maintenance of the myelin sheath. MAG is proteolyzed at a discrete location near its transmembrane domain by a calcium activated myelin-associated cysteine protease in the central nervous system. The soluble proteolysis product, dMAG, can be found in the cerebrospinal fluid. The proteolysis of MAG may be involved in the molecular mechanism of demyelination, as the proteolytic degradation of myelin proteins has been observed in disease states. The site for the proteolysis of MAG to dMAG was identified. This was accomplished by developing a protocol for the purification of soluble dMAG and by protein sequencing of short peptides containing the carboxy-terminus of dMAG. The results from these experiments indicated that the native proteolytic site in MAG was located extracellularly and occurred between residues 512 (Ala) and 513 (Lys), with a large hydrophobic residue at the P_2 position (Trp-511). This finding in turn indicated that the protease for which MAG was a substrate had cathepsin L-like activity. Cathepsin L-like activity in myelin was confirmed by peptidolysis experiments using known cathepsin L substrates. Additional experiments are in progress to determine the identity of this protease.

Myelin-associated glycoprotein (MAG)[1] is a transmembrane protein located in the periaxonal myelin [for a review, see Quarles et al. (1992)]. MAG is a unique, quantitatively minor, component of myelin sheaths, which does not occur in other membranes. Primary structural analysis places MAG in the Ig superfamily, the members of which often are involved in cell−cell interactions. More specifically, MAG is predicted to have five extracellular Ig-like domains (D1−D5),[2] a transmembrane domain (D6), and two cytoplasmic domains (D7−D8). MAG is a highly conserved protein. MAG is thought, based on its location and structure, to be important in glial cell−axon contact which is involved in the formation and/or maintenance of the myelin sheath.

MAG (M_r = 100 000), in the CNS, is cleaved proteolytically by a myelin-associated cysteine protease (Sato et al., 1982) to a large derivative, dMAG (M_r = 90 000). This large fragment is soluble and quite stable. It contains all five extracellular Ig-like domains and all potential glycosylation sites. dMAG is present in the CSF of normal human subjects (Yanagisawa et al., 1985). dMAG apparently has lost some, if not all, of the transmembrane properties of MAG through proteolysis, as evidenced by its solubility in the extracellular medium. The enzyme responsible for proteolysis of MAG is thought to be a member of the ubiquitous calpain family, as calcium is reported to increase the apparent MAG to dMAG conversion rate *in vitro* (Sato et al., 1984) in purified myelin membranes. Further, calpains are cysteine proteases and the highly specific cysteine protease inhibitor E-64 inhibits the formation of dMAG *in vitro* (Sato et al., 1984). However, the specificity and identity of this protease are not known with certainty.

Immunocytochemical (Gendelman et al., 1985; Itoyama et al., 1980; Prineas et al., 1984;) and biochemical (Johnson et al., 1986; Möller et al., 1987) analyses of MS lesions demonstrate a preferential loss of MAG at the periphery of some, but not all, plaques. In addition, homogenates of white matter from MS plaque areas show high levels of dMAG (Möller et al., 1987). There also is an increase in the proteolytic activity (Quarles et al., 1992) in CNS tissue from MS patients as compared to the values obtained from age matched control samples. Recently, a species differences has been demonstrated in the proteolysis of MAG (Möller, 1996). Specifically, *in vitro* experiments with purified myelin have shown that the MAG to dMAG conversion rate is significantly faster in human and nonhuman primate myelin as compared to the rate in other mammalian myelin membranes. Taken together, the above observations suggest that proteolysis of MAG may be relevant to human disease. The native proteolytic site for the conversion of MAG to dMAG was determined in order to further characterize MAG proteolysis. Preliminary data were presented (Stebbins et al., 1996).

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[1] Abbreviations: MAG, myelin-associated glycoprotein; Ig, immunoglobulin; CNS, central nervous system; M_r, relative molecular mass; dMAG, a proteolytically derived form of myelin-associated glycoprotein; CSF, cerebrospinal fluid; MBP, myelin basic protein; MS, multiple sclerosis; DTT, dithiothreitol; RP-HPLC, reversed phase high-performance liquid chromatography; HP-SCX, high-performance strong cation exchange chromatography; PCEB, 0.1 M phosphate, 2 mM cysteine, 1 mM EDTA, 0.2% Brij; E-64, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-Leu-4-aminobutylamide; (Z)-Arg-MBNA, benzoyl-Arg-4-methoxy-β-napthylamide; (Z)-Phe-Arg-MBNA, benzoyl-Phe-Arg-4-methoxy-β-napthylamide; (Z)-Val-Val-Arg-AMC, benzoyl-Val-Val-Arg-7-amido-4-methylcoumarin; DMSO, dimethylsulfoxide; MBNA, 4-methoxy-β-napthylamide; PTH, phenylthiohydantoin.

[2] The eight domains of MAG are designated D1 through D8.

[3] The amino-terminal residue of MAG peptides 1, 6, and 7 were found to correspond to positions 464, 498, and 509 of the MAG consensus sequence (Figure 5). The residues of peptides 1, 6, and 7 that are shown in plain text are identical to the MAG consensus sequence, and the residues shown in bold vary from the consensus sequence.

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MATERIALS AND METHODS

Purification of Myelin. Frozen bovine brain, which immediately was frozen on dry ice after slaughter, was obtained from Pel Freeze. Bovine CNS tissue was homogenized and myelin membranes were purified according to an adapted protocol of Norton and Poduslo (1973). Purified myelin was used for both dMAG purification and enzyme assays. Enzyme assays were performed with highly purified myelin membranes, which had gone through three successive cycles of sucrose gradients and osmotic shocks. Protein concentrations were determined by Biorad's version of the Bradford dye binding assay (Bradford, 1976) with bovine serum albumin as a standard.

Purification of dMAG. Purified myelin membranes were incubated in 0.5 M Tris-HCl, pH 7.5, 0.05 M $CaCl_2$ for 30 min at 37 °C, in order to release dMAG from the membranes. The membranes were removed by centrifugation and soluble proteins salted out from the supernatant by the addition of $(NH_4)_2SO_4$ to 60% saturation at 4 °C. Following centrifugation, the $(NH_4)_2SO_4$ pellet was resuspended in Ultrogel ACA-34 (BioSepra) buffer [0.1 M Tris-HCl, pH 7.5, 0.1M $CaCl_2$, 0.1% betaine, 1 mM dithiothreitol (DTT)] and centrifuged again. The resulting supernatant was fractionated and reprecipitated with a 30% and a 60% $(NH_4)_2SO_4$ saturation step, respectively. The final pellet from the 60% step was resuspended in Ultrogel ACA-34 buffer in preparation for size exclusion chromatography and vacuum concentrated to a volume equal to 1/100 of the column used in the following step. Size exclusion chromatography then was accomplished by an Ultrogel ACA-34 column (1.5 × 100 cm) which was run under gravity flow conditions at 4 °C. Fractions containing the dMAG eluted at approximately 40% of the total column volume and the fractions greater than 90% homogenous, as judged on silver stained 10% SDS−PAGE, were pooled. dMAG pools were concentrated and twice diafiltrated with dH_2O in a Microcon 3 (Amicon) or Filtron 3K (Filtron) filtration unit. Finally, the dMAG was vacuum dried in a Speed Vac (Savant).

Enzyme Assays to Test for Cathepsin L-like Activity. Enzymatic activity of highly purified myelin membranes was determined in PCEB (0.1 M phosphate, 2 mM cysteine, 1 mM EDTA, 0.2% Brij) buffer at either pH 6.0 or 7.5. Highly purified myelin membranes (20 μL) were preincubated at 37 °C in 70 μL of PCEB buffer with or without E-64 (Calbiochem) followed by the addition of concentrated stock solutions of (*Z*)-Arg-MBNA (Bachem), (*Z*)-Phe-Arg-MBNA (Bachem), or (*Z*)-Val-Val-Arg-AMC (Bachem). Stock solutions of substrates were made in 100% DMSO, and the final assay volumes were 100 μL containing 1.5 mM peptide and 10% DMSO. In the case of (*Z*)-Arg-MBNA and (*Z*)-Phe-Arg-MBNA, the product was coupled to Fast Garnet GBC dye (Sigma) and extracted with 100% *n*-butanol according to the method of Barrett (1972). The molar absorbance of the dye coupled product was determined at 515 nm with a Ceres 900 microplate workstation (Bio-Tek) using MBNA (Sigma) as a standard. In the case of (*Z*)-Val-Val-Arg-AMC, the molar fluorescence (365 nm excitation and 460 nm emission) of the product was determined with a TKO 100 minifluorometer (Hoefer) using AMC (7-amido-4-methylcoumarin, Sigma) as a standard according to the method of Kirschke and Wiederanders (1994).

Comparative Peptide Mapping, with and without Carboxypeptidase Y Pretreatment, of dMAG. Purified dMAG in 0.05 M sodium citrate-HCl, pH 6.0 (1−2 μg/μL) was divided into two equal halves, which were kept separate throughout the following protocol according to the method of Isobe et al. (1986). Carboxypeptidase Y (1 μg, sequencing grade; Boehringer Mannheim) was added to one half, while the other half was used as a nontreated control. The samples were incubated at 37 °C for 2 h and then dried in a Speed Vac (Savant). The dried samples were dissolved in 8 M urea/0.4 M NH_4HCO_3 (1.25−2.5 μg of protein/μL) and heated at 100 °C for 3 min. Each sample was reduced and alkylated by the addition of DTT to 4.5 mM for 15 min at 50 °C followed by the addition of iodoacetamide to 10 mM for 15 min at ambient temperature (Stone & Williams, 1993). Following reduction and alkylation, each sample was diluted with dH_2O so that the urea and NH_4HCO_3 were 1 and 0.05 M, respectively. Endoproteinase Glu-C (1 μg, sequencing grade; Boehringer Mannheim) was added to both. The samples were analyzed by RP-HPLC using a 218TP52 column (Vydac) following overnight incubation at ambient temperature.

Anhydrotrypsin Affinity Chromatography. Endoproteinase Lys-C treatment: Purified dMAG in 8 M urea/0.4 M NH_4HCO_3 (1−2 μg/μL) was reduced and alkylated by the addition of DTT to 4.5 mM for 15 min at 50 °C followed by the addition of iodoacetamide to 10 mM for 15 min at ambient temperature (Stone & Williams, 1993). The sample was then diluted with dH_2O (0.25−0.5 μg of protein/μL). Endoproteinase Lys-C (1 μg, sequencing grade; Boehringer Mannheim) was added and the sample was incubated overnight at 37 °C. Clostripain treatment: Purified dMAG in 8 M urea/0.4 M NH_4HCO_3 (2−4 μg/μL) was reduced and alkylated by the addition of DTT to 9 mM for 15 min at 50 °C followed by the addition of iodoacetamide to 20 mM for 15 min at ambient temperature (Stone & Williams, 1993). Additional DTT was added to 20 mM, following reduction and alkylation, in order to scavenge any remaining iodoacetamide, and the sample was diluted with dH_2O (0.5−1 μg of protein/μL). Clostripain (2 μg, sequencing grade; Promega) was added, and the sample was incubated overnight at 37 °C.

The enzyme treated samples were adjusted to pH 5.0 by addition of glacial HOAc and diluted with dH_2O to a final volume of 300 μL, after the overnight incubations. An equal volume of 0.1 M NaOAc and 0.04 M $CaCl_2$, pH 5.0, was added, and the sample was applied to an anhydrotrypsin agarose column (Takara Biochemical), which previously was equilibrated with a buffer (0.05 M NaOAc and 0.02 M $CaCl_2$, pH 5.0). The column was washed with buffer after sample application. Unbound peptides were collected in the effluent, and bound peptides were eluted with 0.1 M HOAc. The flow-through was concentrated in a Speed Vac (Savant) and separated by RP-HPLC using a 218TP52 column (Vydac).

Preparation of Peptides Containing the Carboxy-Terminus of dMAG. The endoproteinase Lys-C treatment of dMAG (see above) resulted in a large carboxy-terminus containing peptide. Therefore several incubations and purifications were needed to collect enough material for complete protein sequencing and mass spectral analysis. The fractions coming off the 218TP52 column (Vydac), containing the carboxy-terminal peptide fragment, were pooled and concentrated in a Speed Vac (Savant). This sample was further purified by

RP-HPLC using an ODS AQ column (YMC). The clostripain treatment of dMAG (see above) resulted in a very short peptide containing the carboxy-terminus of dMAG. No extra steps were needed to prepare this sample for protein sequencing.

RP-HPLC. RP-HPLC was accomplished on a System Gold HPLC equipped with a Model 507 autosampler, Model 126 programmable solvent module, and Model 168 diode array detector (Beckman). A guard column (Separations Group) and either a narrow bore (2.1 × 250 mm) 218TP52 column (Vydac) or a narrow bore (2.0 × 250 mm) ODS AQ column (YMC) at 35 °C were used. Samples were eluted at 0.25 mL/min according to a gradient described by Fernandez et al. (1992). Column effluent was monitored at 215 and 280 nm, and fractions were collected at 30 s intervals and stored at −70 °C.

Protein Sequencing. Fractions containing peptides were applied to a Biobrene-treated glass fiber filter (Applied Biosystems) and dried prior to amino acid sequencing on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer (Applied Biosystems) using methods and cycles supplied by the manufacturer. Data were collected and analyzed on a Model 610A data analysis system (Applied Biosystems). The presence of cysteine at a particular cycle was confirmed by the presence of the PTH of its iodoacetamide derivative which eluted 0.2 min after the PTH of glutamate on our system. A special proline cycle, according to the method of Vensel and Kasarda (1991), was utilized when a proline was anticipated in a particular cycle, in order to minimize the incomplete Edman degradation usually observed with this residue. The synthetic peptide LMWA (QCB, Hopkinton, MA) was used as a control.

Mass Spectral Analysis of dMAG Peptides. Mass spectra were obtained with a Model TSQ-700 spectrometer (Finnigan) operating in the Finnigan electrospray mode. Lyophilized peptides were dissolved in 20 μL of 0.5% HOAc in 1/1 v/v aqueous methanol and admitted at a flow rate of 1 μL/min. The spray needle was held at 5.3 kV and the spectrometer optimized and tuned with myoglobin/ MRFA as described in the instruction manual. The capillary temperature was 200 °C, and spectra were acquired in the profile mode and averaged for 1−2 min. MALDI spectra were obtained on a Krotos Kompact Maldi III. Samples were prepared by adding a water solution of the peptide to a deposit of α-cyano-4-hydroxycinnamic acid on the sample holder.

RESULTS

Purification of dMAG. Purified myelin from bovine CNS tissue was incubated at 37 °C for 30 min in a buffer containing calcium, which activated an endogenous myelin-associated protease, in order to form dMAG *in vitro*. A rapid and efficient protocol for the purification of the soluble dMAG, which accumulated as a result of this incubation, was developed. In short, soluble dMAG was removed from the insoluble myelin membranes, fractionated by antichaotropic agents, and separated by size exclusion chromatography. Typical preparations started with 10 g of bovine CNS tissue and yielded 100−200 μg of dMAG. dMAG was purified to approximately 90% homogeneity, as seen in Figure 1.

Comparative Peptide Mapping, with and without carboxypeptidase Y pretreatment, of dMAG. Comparative RP-HPLC peptide maps of the endoproteinase Glu-C digests of dMAG, with and without pretreatment with carboxypeptidase Y, showed a peak eluting at 74.8 min in the carboxypeptidase Y pretreated digest, which was decreased slightly relative to its adjacent peaks at 74.0 and 75.4 min as compared to the nonpretreated digest (data not shown). Automated Edman degradation of a fraction eluting between 74.5 and 75.0 min containing this peak resulted in sequence data consistent with a mixture of five MAG peptides. The partial sequence of peptide 1 indicated that it was generated from the region of interest in MAG (Figure 5) and probably included the carboxy-terminus of dMAG:

RSGLLLTSILTLRGQAQAPPRVICTS**H**NLYGT**K**-
SLELPFQGAH..... (peptide 1)[3]

The sequences of peptides 2−5 (data not shown) corresponded to regions that were more proximal to the amino-terminus of MAG. The complete sequence of peptide 1 could not be determined, due to its length (>43 residues) and its contamination with four other peptides. Attempts to isolate peptide 1 from the mixture by other RP-HPLC and HP-SCX steps were unsuccessful. Attempts to obtain molecular weight information and sequence data on peptide 1 by electrospray mass spectrometry of the mixture or of partially purified peptide 1 resulting from other RP-HPLC and HP-SCX steps also were unsuccessful.

Anhydrotrypsin Affinity Chromatography of Endoproteinase Lys-C Treated dMAG. RP-HPLC analysis of endoproteinase Lys-C treated dMAG before (Figure 2A) and after (Figure 2B) anhydrotrypsin affinity chromatography is shown in Figure 2. The major peptide peak eluting at 73.7 min in the flow-through sample (Figure 2B) also was seen in the control (Figure 2A) endoproteinase Lys-C digest of dMAG, which was not subjected to anhydrotrypsin affinity chroma-

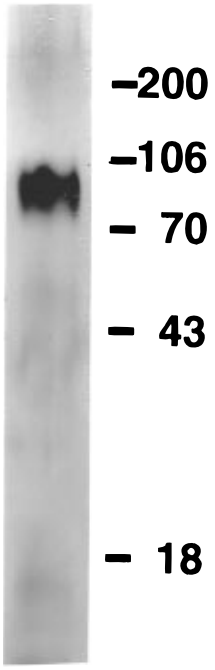

FIGURE 1: Purified dMAG on a silver stained SDS−PAGE. dMAG (M_r = 90 000) was released from purified myelin by activation of an endogenous myelin protease. dMAG was purified biochemically following release from the membrane. The M_r × 1000 and electrophoretic migration of the molecular weight markers are indicated.

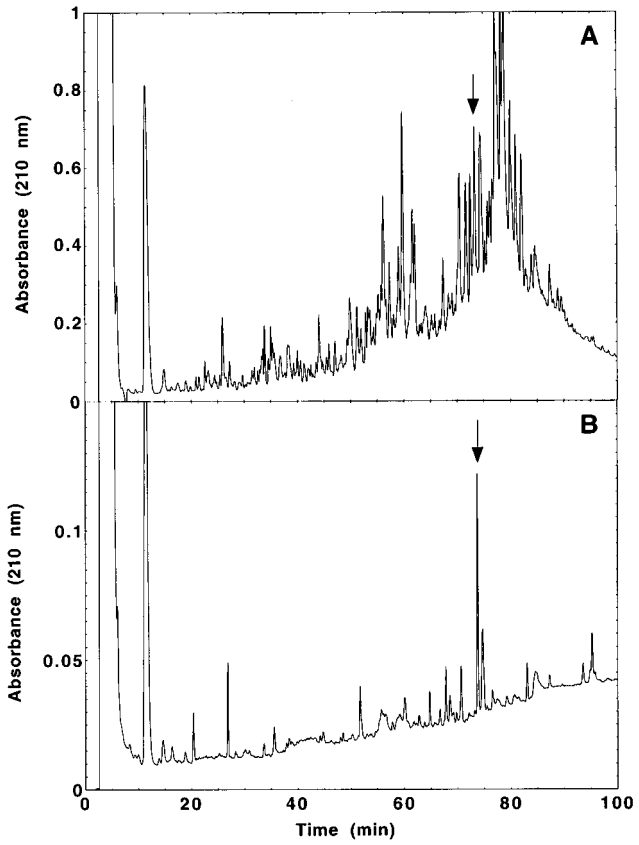

FIGURE 2: Profiles of an endoproteinase Lys-C digest of dMAG. Panel A: the control digest not subjected to anhydrotrypsin affinity chromatography. Panel B: the flow-through (nonretained) fraction after anhydrotrypsin affinity chromatography (the full-scale absorbance is 0.15). A peptide eluting at 73.7 min (indicated by the arrow in panels A and B) was identified as containing the carboxy-terminus of dMAG.

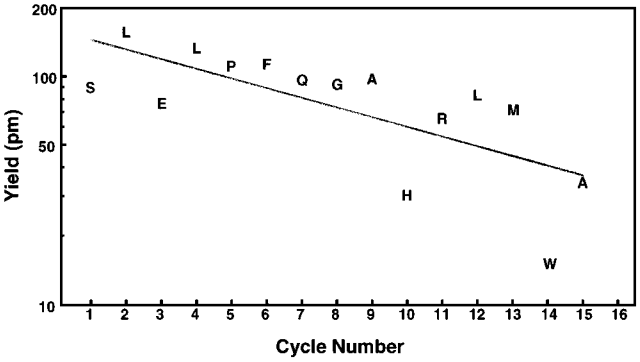

FIGURE 3: Sequence analysis of peptide 6 from an endoproteinase Lys-C digest of dMAG which was not retained on an anhydrotrypsin column. The nonretained peptide fragment was purified by RP-HPLC and then subjected to automated Edman degradation. The amino acid identity and lag corrected yields after each cycle are indicated by their location and letter.

tography. The 73.7 min peak was pooled from several control digests and purified by an additional RP-HPLC step prior to analysis by automated Edman degradation and mass spectrometry. Automated Edman degradation of this peptide (Figure 3) revealed the following sequence (which overlapped with the carboxy-terminus of peptide 1):

SLELPFQGAHRLMWA (peptide 6)

Electrospray and MALDI mass spectrometric analysis of this peptide determined the masses to be 1755.4 and 1757.9 *m/z*,

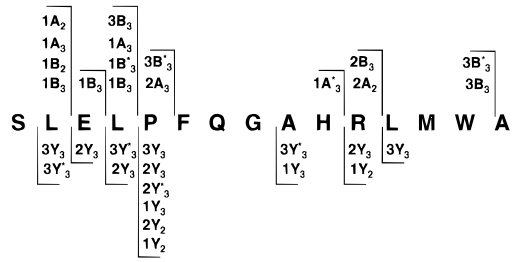

FIGURE 4: Tandem electrospray mass spectroscopic analysis of peptide 6. The 33 ions which were obtained are consistent with the fragmentation pattern shown [e.g., $1A_2$ is a singly charged ion of a type A fragmentation (RCO^+) from a doubly charged parent ion].

A

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460 VYSERSGLLL TSILTLRGQA QAPPRVICTS RNLYGTQSLE  consensus
    ....RSGLLL TSILTLRGQA QAPPRVICTS HNLYGTKSLE  bovine

501 LPFQGAHRLM WAKIGPVGAV VAFAILIAIV CYITQTRRKK  consensus
    LPFQGAHRLM WA....                            bovine
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B

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508  -R-L-M-W-A*K-I-G-P-V-
     P4 P3 P2 P1 P1' P2' P3' P4'
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FIGURE 5: The primary structure of MAG in the area of interest, the region of the transmembrane domain. Panel A: the consensus sequence of MAG derived from a human (Sato et al., 1989); mouse (Fujita et al., 1989) and rat (Lai et al., 1987) MAG sequence. The amino acid residues in plain text are identical and the ones in bold vary between the three sequences. The underlined residues are predicted to be in the MAG transmembrane domain. The letters below this consensus sequence indicate the bovine MAG primary structure that was determined from the sequencing of dMAG peptides. The residues in bold of bovine MAG were found to be different from the consensus sequence. The arrow indicates the location of the carboxy-terminus of bovine dMAG. Panel B: the expanded region of primary structure including the native proteolysis site in MAG. The residues of the specificity site are designated P_4 to P_4' (Berger & Schechter, 1970), and the star indicates the location of the scissile bond.

respectively. These results were in good agreement with the calculated mass of 1756.0 *m/z*. Further, tandem electrospray mass spectrometry (Figure 4) confirmed much of the sequence of this peptide.

Anhydrotrypsin Affinity Chromatography of Clostripain Treated dMAG Anhydrotrypsin affinity chromatography also was performed on clostripain treated dMAG (data not shown). This experiment yielded a major peptide peak eluting at 51.7 min in the column flow-through. Automated Edman degradation of this peptide (data not shown) revealed the following sequence (which overlaped with the carboxy-terminus of peptide 6):

LMWA (peptide 7)

A synthetic peptide of the same sequence exhibited an identical retention time and UV spectrum.

Enzyme Assays to Test for Cathepsin L-like Activity in Myelin. As seen in Table 1, both the known cathepsin L substrates, (*Z*)-Phe-Arg-MBNA, and (*Z*)-Val-Val-Arg-AMC (Barrett & Kirschke, 1981; Kirschke & Wiederanders, 1994) were hydrolyzed by highly purified myelin membranes in PCEB buffer pH 6.0. The hydrolysis of (*Z*)-Phe-Arg-MBNA was inhibited almost completely by E-64 at a concentration of 5 μM (Table 1). No significant hydrolysis was detected of the (*Z*)-Arg-MBNA substrate at pH 6.0 or of the (*Z*)-Val-

Table 1: Peptidolysis Activity of Myelin Membranes[a]

peptide substrate	sensitive[b] to Cathepsin L Proteases	specific activity[c]
(*Z*)-Arg-MBNA	No	1.7 ± 0.7
(*Z*)-Phe-Arg-MBNA	Yes	72.8 ± 12.0
(*Z*)-Phe-Arg-MBNA + E-64	Yes	3.0 ± 0.4
(*Z*)-Val-Val-Arg-AMC	Yes	103.5 ± 22.0
(*Z*)-Val-Val-Arg-AMC (pH 7.5)	No	0.7 ± 1.2

[a] Various synthetic peptides were used to assay highly purified myelin membranes in PCEB buffer at pH 6.0 (unless indicated otherwise). [b] Peptide substrates were classified according to known cathepsin L activities (Kirschke & Barrett, 1985; Kirschke & Wiederanders, 1994). [c] The specific activity was expressed as picomoles per hour per milligram of protein. The values shown are averages (±sd) of three determinations using highly purified myelin from three separate myelin isolations.

Val-Arg-AMC substrate at pH 7.5 even with extended incubation times (>16 h). The lack of hydrolysis of (*Z*)-Arg-MBNA at pH 6.0 can be used as an internal control for lysosomal contamination of purified myelin membranes, as this substrate is sensitive to both cathepsin B and H proteases (Barrett & Kirschke, 1981; Kirschke & Barrett, 1985). The lack of hydrolysis of (*Z*)-Val-Val-Arg-AMC at pH 7.5 indicates that cathepsin S is not present in highly purified myelin (Kirschke & Wiederanders, 1994). Cathepsin S has a specificity similar to cathepsin L. However under the conditions used at pH 7.5, the only cathepsin which still is active toward (*Z*)-Val-Val-Arg-AMC is cathepsin S (Kirschke & Wiederanders, 1994).

DISCUSSION

The location and sequence of the proteolytic site for the conversion of MAG to dMAG was identified in order to further characterize the proteolysis of MAG in intact membranes. This was accomplished by determining the carboxy-terminus of dMAG. For these experiments, an endogenous protease was used to generate dMAG from CNS tissue *in vitro*. The soluble dMAG then was purified biochemically (Figure 1) and subjected to either differential peptide mapping (data not shown) or anhydrotrypsin affinity chromatography (Figure 2).

Differential peptide mapping was accomplished by comparing a RP-HPLC proteolytic peptide map of carboxypeptidase Y pretreated dMAG to a RP-HPLC proteolytic peptide map of untreated dMAG. The exopeptidase treatment prior to peptide mapping altered the retention time of the carboxy-terminal containing fragment. Endoproteinase Glu-C was selected to create the proteolytic peptide map of dMAG, in order to yield peptide fragments that resulted from the hydrolysis of the bonds following Glu-464 and/or Glu-500 (Figure 5), since it was expected that MAG would be proteolyzed near its transmembrane domain by the endogenous enzyme. These experiments demonstrated a slight decrease of the relative peptide yield at 74.8 min in the map generated from pretreated dMAG, as compared to the relative peptide yield at 74.8 min in the map generated from untreated dMAG (data not shown). These results suggested that the 74.8 min fraction included a peptide fragment containing the carboxy-terminus of dMAG. Automated Edman degradation of the fraction eluting between 74.5 and 75.0 min generated sequence data that was consistent with a mixture of five peptides (1−5).

Peptide 1 was assumed to contain the desired carboxy-terminus based on its proximity to the transmembrane region of MAG and the lack of other endoproteinase Glu-C specificity sites, with the exception of the bond following Glu-500 which did not hydrolyze under the conditions used. Unfortunately, the exact carboxy-terminal residue of peptide 1 could not be determined unequivocally by protein sequencing, because of its size (>43 residues) and the presence of other comigrating peptides, in particular peptide 2 (49 residues). Attempts to purify peptide 1 also were unsuccessful. Nevertheless, these experiments were consistent with the hypothesis that the native proteolytic site in MAG was located near the transmembrane domain. These experiments also revealed a histidine and a lysine residue at positions 491 and 497, respectively, of the bovine MAG sequence, whereas arginine and glutamine occur at these positions, respectively, in the MAG consensus sequence (Figure 5). The lysine at position 497 was useful in the following differential peptide chromatography experiments.

The second strategy used to identify the carboxy-terminus of dMAG involved the use of anhydrotrypsin affinity chromatography. dMAG first was subjected, in preparation for anhydrotrypsin affinity chromatography, to proteolytic digestion with two highly specific proteases, endoproteinase Lys-C and clostripain (endoproteinase Arg-C), which yielded fragments with a lysine or arginine, respectively, at the carboxy-terminus. Peptide fragments of this type, with a terminal lysine or arginine, were anticipated to be retained by the anhydrotrypsin resin, as this resin selectively binds the carboxy-terminal products of tryptic digests. Thus, a peptide containing the dMAG carboxy-terminus, which did not end in a lysine or arginine, was expected to be found in the nonretained flow-through of the column. Indeed, a peptide was isolated from the flow-through fraction with each enzyme used (peptides 6 and 7). The two peptides contained identical carboxy-termini.

Sequence analysis of these peptides revealed that peptide 7 was contained within peptide 6 and that peptides 6 and 7 were generated by the enzymatic hydrolysis of the bonds following Lys-497 and Arg-508, respectively, in bovine dMAG (Figure 5). Confirmation of the primary structure of peptide 6 was accomplished by MALDI and electrospray tandem mass spectrometry (Figure 4). Confirmation of the primary structure of peptide 7 was accomplished by comparison of its RP-HPLC retention time to a synthetic peptide. The sequences of these two peptides resulting from enzymatic hydrolysis of dMAG by two different and highly specific proteolytic enzymes indicated that Ala-512 was the carboxy-terminal residue of dMAG. These results also were consistent with peptide 1 containing the carboxy-terminal residue of dMAG.

The above results indicated that the native scissile bond in MAG occurred between residues 512 and 513 (Figure 5A). Examination of this site revealed a large hydrophobic residue at the P_2 position (Figure 5B) and was consistent with cathepsin L-like activity (Barrett & Kirschke, 1981; Marks & Berg, 1987). The enzymatic hydrolysis of known cathepsin substrates by highly purified myelin membranes then was measured in order to confirm the presence of cathepsin L-like activity in myelin. These experiments demonstrated that cathepsin L-like activity was associated with the myelin membrane (Table 1). Specifically, the hydrolysis of (*Z*)-Phe-Arg-MBNA (pH 6.0) and lack of

hydrolysis of either (*Z*)-Arg-MBNA (pH 6.0) or (*Z*)-Val-Val-Arg-MCA (pH 7.5) were diagnostic for cathepsin L-like activity (Kirschke & Barrett, 1985; Kirschke & Wiederanders, 1994). The enzymatic hydrolysis of (*Z*)-Phe-Arg-MBNA also was inhibited by micromolar concentrations of E-64.

Summary and Conclusions. The determination of the carboxy-terminus of the proteolytic product dMAG indicates that the transmembrane protein MAG is the native substrate of an extracellular protease. This result is consistent with the solubility of dMAG, which loses the transmembrane characteristics of MAG and is present in CSF (Yanagisawa et al., 1985). Previous calcium activation experiments have suggested that a calpain was responsible for the hydrolysis of MAG in the CNS (Sato et al., 1984). This interpretation is not consistent with the location and sequence of the proteolytic site in MAG described in this manuscript, even though calpain is present in and can be isolated from myelin membranes [for a review, see Banik et al. (1992)]. Furthermore, calpains are thought not to be extracellular enzymes [for a review, see Goll et al. (1990)].

The presence of a large hydrophobic residue (Trp-511, Figure 5B) immediately preceding the carboxy-terminal residue of dMAG indicates that the protease, for which MAG is a substrate, has cathepsin L-like (Barrett & Kirschke, 1981), and not calpain-like (Sasaki et al., 1984), specificity. This result was supported by assaying highly purified myelin membranes for cathepsin L-like activity (Table 1). Cathepsin L is thought to play an extracellular role in normal tissue remodeling and pathologies (Dehrmann et al., 1995). This protease was shown to be active *in vitro* at physiological pH under high ionic strength conditions (Dehrmann et al., 1995). Sato et al. (1982) also described an increased rate of MAG to dMAG proteolysis with high ionic strength conditions. Further, an extracellular protease inhibitor also should be present in the CNS, if the extracellular proteolysis of MAG is a regulated process. This notion is supported by the observation that cystatin C is secreted into the CSF from the choroid plexus (Tu et al., 1992). However, the role of cystatin C in the regulation of MAG proteolysis, if any, has yet to be described.

The presence of dMAG in the CSF of healthy humans (Yanagisawa et al., 1985) suggests that the proteolysis of MAG is a naturally occurring process in CNS. During development, the removal of MAG from the myelin sheath may be required for the formation of compact myelin (Trapp, 1988), but the physiological significance of MAG proteolysis is not known. In addition, the degradation of the myelin sheath which is seen in disease states is thought to involve the proteolysis of myelin proteins [for a review, see Berlet (1992)]. Thus, the cathepsin L-like protease, for which MAG is a native substrate, and its natural inhibitor(s) also may play a role in the molecular mechanism of demyelination.

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