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Identification of a mitogen-activated protein kinase site in human myelin basic protein in situ

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Abstract

Ultrastructural localization of a specific phosphorylated isomer of myelin basic protein (MBP) has been achieved with a monoclonal antibody specific for human MBP sequence, 89–105, in which Thr^{98} was phosphorylated. Cryosections of human brain white matter revealed that gold particles were found localized almost exclusively to the major dense line demonstrating that threonine 98 in the sequence Thr-Pro-Arg-Thr-Pro-Pro-Pro, a mitogen-activated protein kinase-specific site, was phosphorylated in vivo. In two cases of multiple sclerosis, the density of gold particles in myelin was reduced by about 30%, in one case by 42%, and by 80% in a fourth case. However, gold labelling was seen in areas of demyelination suggesting that the phosphorylated threonyl peptide was protected from degradation.

Keywords: Mitogen-activated protein kinase; Phosphorylation; Myelin basic protein; Multiple sclerosis

1. Introduction

Myelin basic protein (MBP) represents a family of proteins coded for by a single gene. Through the process of differential splicing of the message, a number of isoforms are generated. In rodents, at least four isoforms in the 14–21-kDa range are known; the 14-kDa variant represents the principle isoform. In the human the pattern of isoforms is less complex; the 18-kDa isoform represents the principal species. To add to the complexity, each isoform shows considerable microheterogeneity, which has been studied extensively in the bovine and human 18-kDa isoforms. This microheterogeneity arises from a number of post translational modifications which include phosphorylation (Chou et al., 1976; Ulmer, 1988 (review)), deamidation (Chou et al., 1976); deimination (Wood and Moscarello, 1989); GTP-binding (Chan et al., 1988); ADP-ribosylation (Boulias and Moscarello, 1994) and heterogeneous N-terminal acylation (Moscarello et al., 1992). Of these modifications, phosphorylation has been studied extensively (Deibler et al., 1975; Ulmer, 1988).

Phosphorylation was first studied in 1971 when a cyclic-AMP-dependent protein kinase was reported to phosphorylate MBP in vitro (Johnson et al., 1971). Since this time, calcium-phospholipid-dependent (Turner et al., 1984), calcium-calmodulin-dependent and mitogen-activated protein kinases (MAP kinase) (Erickson et al., 1990) have been reported to phosphorylate MBP in addition to the cyclic-AMP-dependent kinase. Of 14 potential sites of phosphorylation, five sites have been identified after [^{32}P]phosphate labelling following intracranial injection (Ulmer, 1988). Other sites were identified by in vitro labelling by specific protein kinases.

In the majority of the studies reporting post translational modifications of MBP, the microheterogeneity was observed in isolated MBP. One study, using an antibody which reacts only with citrullinyl residues, reported the localization of the citrullinated form of MBP to the intraperiod line of myelin predominantly but not exclusively, suggesting that this modification of MBP occurred in vivo and may be important in targeting the MBP to specific

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sites in the myelin sheath (McLaurin et al., 1993), maintaining a sophisticated level of organization.

The report by Erickson et al. (1990) demonstrated that bovine MBP contained a MAP kinase site, Thr-Pro-Arg-Thr^{97}-Pro-Pro-Pro which was phosphorylated in vitro at Thr^{97} of the bovine sequence (Thr^{98} in the human sequence) by a purified MAP kinase. Accordingly, a synthetic peptide was prepared (Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr^{98}-Pro-Pro-Pro-Ser-Gln-Gly-Lys: human sequence) and phosphorylated at Thr^{98}. A monoclonal antibody was raised which recognized only the phosphorylated form of the peptide. It did not react with MBP phosphorylated at other sites (Yon et al., 1994, 1995). Using a similar immuno-gold method on cryosections of human white matter as employed with the citrulline-specific antibody we were able to demonstrate labelling of myelin in situ. With these data we now have demonstrated that both the citrullinated form of MBP and the Thr^{98}-phosphorylated form occur naturally in the myelin sheath.

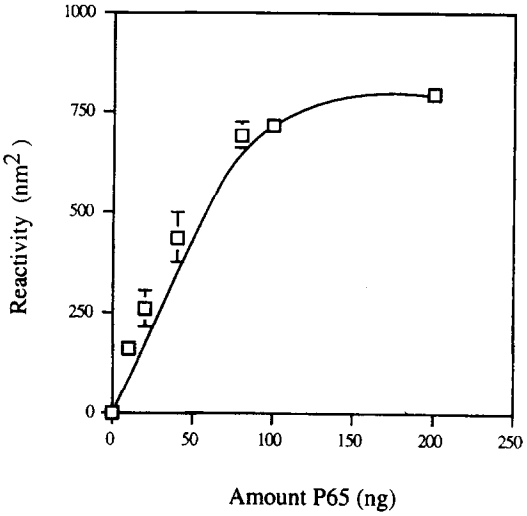

Fig. 1. Standard curve for slot blot determination of the amount of MBP phosphorylated at Thr 98. To prepare the curve, the phosphorylated synthetic peptide (P65) FFKNIVTPRT^{98}PPPSQEK was used at different concentrations. All amounts of MBP phosphorylated at Thr 98 were extrapolated from the linear part of the curve.

2. Materials and methods

2.1. Isolation of myelin basic protein

Myelin basic protein was isolated from the paraventricular regions of normal brain white matter and from normal appearing white matter from victims of multiple sclerosis essentially as described by Deibler et al. (1972).

2.2. SDS-PAGE and Western blots

SDS-gels were run by the method of Laemmli (1970), and Western blots by the method of Towbin et al. (1979). Briefly, after electrophoresis of 2 μg protein per lane in a 10–20% gradient tricine gel, the proteins were transferred to nitrocellulose and the blots incubated for 1 h with anti-bovine IgG (polyclonal) at room temperature. To detect the Thr^{98}-phosphorylated MBP, the monoclonal antibody P12 was used (Yon et al., 1995). In this case, the blots were rinsed three times, 5 min each time with 0.15% glycine containing 0.5% bovine serum albumin, then 2% blotto for 1 h followed by incubation with antibody (1:2000) for 1 h. The secondary antibody conjugated to horse radish peroxidase (1:5000) was added to all blots for 1 h.

2.3. Quantitation of MBP by slot blot

A standard curve was constructed using 0–200 ng of peptide 89–105 (Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-Pro-Pro-Ser-Gln-Lys) phosphorylated at Thr^{98} (P65). The blots were incubated with P12 at 4°C overnight, washed three times with Tris-buffered saline (pH 7.5) and reacted with goat anti-mouse IgG conjugated to horse radish peroxidase (Biorad). The absorbance of each slot was obtained by scanning in a densitometer at 633 nm. To measure the amount of MBP in each sample, several concentrations were used. The amount of P12 reactive MBP was obtained by extrapolating from the standard curve (Fig. 1).

2.4. Amino acid analysis

Quantitation of the amount of peptide P65 and of MBP was done by amino acid analyses. Each sample was hydrolysed in 6 N HCl at 110°C in vapour phase for 24 h. For each sample triplicate analyses were done. Variation among analyses was not greater than 10%.

2.5. Immunogold electron microscopy

Biopsies of human white matter were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 for 2–4 h then rinsed 3–5 min in phosphate-buffered saline (PBS) and stored in PBS containing 20 mM sodium azide. 12 h prior to cutting, the samples were infused with 2.3 M sucrose for cryoprotection. Sections were cut in a cryo-ultramicrotome at −95°C. Immunogold labelling was done as described by McLaurin et al. (1993). Anti-bovine MBP was used to detect IgG followed by gold-labelled (3 nm particles) goat anti-rabbit IgG. When the P12 antibody was used, goat anti-mouse IgG complexed to 3-nm gold particles was used. The number of particles/μm^2 of intact myelin was obtained by image analysis (Weibel et al., 1966).

Several control experiments were included. Adsorption of P12 with synthetic peptide FFKNIVTRRTPP had no effect on the labelling whereas the phosphorylated peptide (Thr 98) completely abolished the labelling. Adsorption

with MBP component 1 (C-1) had no effect on the labelling.

3. Results

3.1. Immunoreactivity of MBP from normal and MS white matter

MBP was isolated from normal and MS white matter as described in Materials and methods. The Coomassie brilliant blue-stained gel of MBP from one MS sample and an age-matched control is shown in Fig. 2, lanes 2 and 3. The immunoblot using anti-MBP antibodies is shown in lanes 4–7. The reactivity of MBP from normal white matter (lane 4) was similar to MBP from two victims of MS (lanes 5 and 6). Component 1 (C-1) prepared by CM52 column chromatography of MBP isolated from normal white matter is shown in lane 7. The reactivity of MBP from normal white matter and one of the four MS samples with the P12 antibody is shown in lanes 8 and 9. Despite the fact that the same amounts of material were loaded in each well, the MS sample did not react as well as the normal, suggesting that less of the Thr^{98} phosphorylated MBP was present in this sample.

Quantitation of the amount of Thr^{98} phosphorylated MBP in the normal and MS samples was done by the slot blot method described in Materials and methods. The data are shown in Table 1. Whereas the proportion of Thr^{98}-phosphorylated MBP was 43% of the total MBP, two of the MS samples were 32 and 31%, one was 25% and one

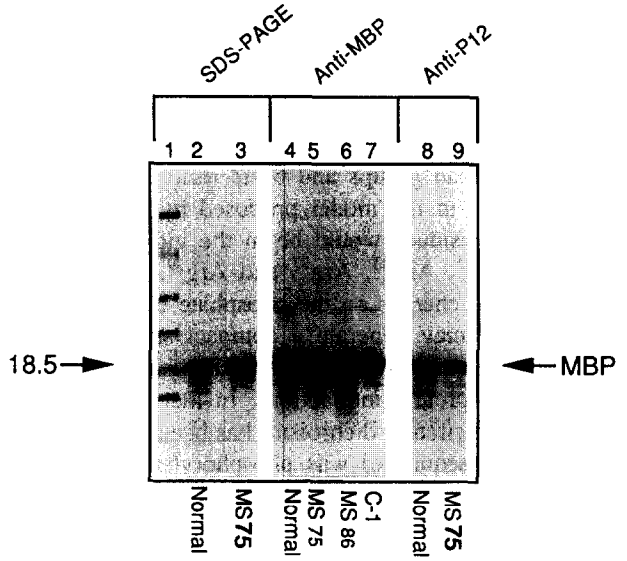

Fig. 2. SDS-PAGE of MBP isolated from normal human white matter and from white matter of a victim of MS. Coomassie brilliant blue stained, lanes 2, 3, respectively. Western blot with anti-bovine MBP antibody, lane 4, normal; lane 5, MS 75; lane 6, MS 86; and lane 7, component 1 (C-1) of MBP; lane 1, molecular mass standards. Western blot reacted with mAb P12. Lane 8, normal; lane 9, MS 75.

Table 1
Amount of Thr 98 phosphorylated MBP in MBP isolated from normal and multiple sclerosis white matter

	MBP (ng) [a]		Proportion of phosphorylated MBP	Phosphorylated MBP relative to normal
	Added	Measured [b]		
Normal	200	86.5 ± 11.8	0.43 ± 0.06	1
MS 75	200	6.3 ± 8.2	0.32 ± 0.04	0.74
MS 86	200	62.4 ± 12.3	0.31 ± 0.06	0.72
MS 51	200	50.0 ± 6.0	0.25 ± 0.03	0.58
MS 27	200	19.0 ± 2.0	0.09 ± 0.02	0.21

[a] Amount MBP was determined by amino acid analysis.
[b] The immunoassay results are the means of eight determinations.

9%. Since MS 27 was obtained from a fulminating case of 5 weeks duration in a young woman, these data reflect the severity of the disease process.

3.2. Localization of Thr^{98} phosphorylated MBP in myelin

Cryosections of biopsy samples were prepared from normal and MS white matter (Fig. 3). The myelin sheaths from both normal and MS samples were labelled with Fab-conjugated, 3-nm gold particles. In both samples (Fig. 3a and c, respectively), the gold particles are arranged in linear arrays along the major dense line. Particles were rarely observed at the intraperiod line. When the distribution of gold particles was determined by morphometric analysis 82.7 ± 5.9% of the gold particles in the normal and 79.8% ± 6.9% in the MS case were localized to the major dense line.

When we compared the density of gold particles in cryosections of two normal and two cases of MS (MS 75, MS 86), the number of particles in the MS samples were decreased by 24 and 30%, respectively (Table 2). In the cryosection of the MS case shown in Fig. 3b, gold particles were found in plaques suggesting that the peptide containing Thr^{98} in phosphorylated form was resistant to proteolysis. This result is consistent with an earlier report (Deibler et al., 1990) in which bovine MBP, monophosphorylated at Thr^{97}, was resistant to thrombin digestion. When a different antibody was used (mAb 26), which recognizes residues 69–74 (Tyr-Gly-Ser-Leu-Pro-Gln) of MBP, no labelling was found in the plaque areas.

4. Discussion

Although MBP has been shown to be phosphorylated at several sites in vitro, phosphorylation at Thr^{97} of the bovine sequence by a MAP kinase isolated from 3T3 fibroblasts or EL4 thymoma cells stimulated with epidermal growth factors or phorbal esters has been demonstrated recently (Erickson et al., 1990). The Thr^{97} site in bovine MBP was not a substrate for cAMP-dependent kinase, protein kinase C or Ca^{2+}, calmodulin-dependent

kinase. MAP kinases are known to be important mediators of signal transduction coupling a variety of stimuli to specific physiological responses (Davis, 1994).

The data presented in this manuscript extend the in vitro studies by demonstrating that phosphorylated Thr^{98} of human MBP was detected in cryosections of human myelin. Since the myelin was in its natural in situ environment, we are confident that phosphorylated MBP (Thr^{98}) was present in vivo. Although the presence of substrate for an enzyme does not establish the presence of the enzyme at the same site, the correlation is certainly suggestive.

Fig. 3. Electron micrographs of cryosections of lightly fixed human white matter, immunogold labelled with mAb P12, and secondary antibody, 3 nm gold conjugated anti-murine Fab. All bars represent 0.1 μm. (A) Compact myelin from normal white matter (biopsy). Most of the gold particles are on the major dense line. (B) Partially degraded myelin in a plaque from victim of MS. Gold particles are seen in several areas. Arrows indicate plaque edge. (C) Compact myelin in white matter from a victim of multiple sclerosis. Majority of the particles are at the major dense line.

Table 2
Density of gold particles in cryosections of white matter from normal and multiple sclerosis brain

Sample	Number of gold particles per μm^2	Number of fields	Percent decrease [a] in MS
Normal (1)	784 ± 29	25	
Normal (2)	816 ± 44	25	
MS 75	596 ± 78	25	24
86	560 ± 73	25	30

Significance: $P < 0.005$.
[a] Percent decrease was obtained by comparing the gold particles in each MS case to the mean of the normals.

From earlier studies including the binding of GTP to MBP, (Chan et al., 1988), phosphorylation of MBP by protein kinase C (Ramwani and Moscarello, 1990; Cosentino et al., 1994), and ADP-ribosylation of MBP by cholera toxin (Boulias and Moscarello, 1994), we conclude that a signal transduction system must be part of the physiological machinery of myelin. The present data establishing the presence of MAP kinase site in MBP by detection of the Thr^{98} phosphorylated form of MBP in myelin in situ add strength to the above-mentioned in vitro studies. Since MAP kinase is a developmentally activated kinase a role for a signal transduction system funnelling through a MAP kinase appears reasonable. The decrease in the proportion of MBP phosphorylated at Thr^{98} in MS tissue is an interesting observation. Of the four cases reported here, three were chronic of at least 20 years duration, the one fulminating case (MS 27) was in a 27-year-old woman which terminated after 5 weeks.

The localization of the antibody P12, reactive with phosphorylated Thr^{98} to the major dense line was surprising since the presence of the phosphate group in this highly basic region of the molecule might be expected to decrease (or disrupt) protein–lipid interactions between the phospholipid head groups and basic residues on the protein. However, in the model proposed by Stoner (1984) several basic residues would be in the vicinity of Thr^{98}, including Lys^{105}, Arg^{33}, Arg^{97} and Arg^{99}. Neutralization of the negative charges on the phosphate by two of these basic residues may generate a compact structure in this area of the molecule with enough basic residues nearby to promote lipid–protein interactions. In fact an increase in β-structure (by circular dichroism) has been reported when Thr^{97} (bovine sequence) was phosphorylated (Deibler et al., 1990). Although the portion(s) of the molecule involved in secondary structure cannot be deduced from circular dichroism, these studies demonstrate that phosphorylation of a single threonyl residue induces structure.

Immunogold localization results have yielded important information concerning the disposition of certain post translationally modified forms of MBP. The citrullinated form of MBP was predominantly found at the intraperiod

line of myelin, while the Thr^{98} phosphorylated form was localized to the major dense line. In both cases, the deimination of arginine or the phosphorylation of Thr^{98} generate uncharged areas in the molecule. We previously reported that phosphorylation of Ser^{7} also generated a specific secondary structure with Arg^{5} and Arg^{9}, again neutralizing this portion of the molecule, generating a specific structure (Ramwani and Moscarello, 1990). Since all members of the MBP family are modified in some way the need to generate specific antisera for their localization is evident and should be highly rewarding.

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