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PHOSPHORYLATION OF MYELIN BASIC PROTEIN AND PEPTIDES BY GANGLIOSIDE-STIMULATED PROTEIN KINASE

Kai-Foon Jesse Chan

Laboratory of Experimental Neuropathology, NINDS, NIH, Bethesda, MD 20892

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Summary: Rabbit myelin basic protein (MBP) was phosphorylated by a ganglioside-stimulated protein kinase to a stoichiometry of 1.4 and 2.1 mol phosphate/mol MBP in the presence and absence of G_{T1b}, respectively. Two-dimensional peptide mapping analyses revealed that two of the sites of phosphorylation were distinct from those catalyzed by cAMP-dependent protein kinase or protein kinase C. Phosphorylation of one of these sites by ganglioside-stimulated protein kinase was inhibited by G_{T1b}, suggesting that the inhibitory effect of gangliosides on MBP phosphorylation may be substrate-directed. Although ganglioside-stimulated protein kinase did not phosphorylate MBP at a domain containing residues 82-117, a synthetic peptide Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys corresponding to residues 111-120 was phosphorylated by the kinase in a ganglioside-stimulated manner. These findings suggest that the conformation of MBP may be important in determining its phosphorylatability.

Myelin basic protein (MBP) comprises approximately one-third of the total protein in central nervous system myelin (1,2). It is located on the cytoplasmic side of the major dense line (3-7) and may act as an adhesion molecule for the formation, compaction, and maintenance of myelin's multilamellar structure. MBP can undergo post-translational modification through phosphorylation and dephosphorylation mechanisms. In vivo phosphorylation of MBP is a dynamic event (8). Both depolarization of axons and impulse conduction have been shown to enhance this covalent modification process (9-10). Phosphorylation of MBP under in vitro conditions can be catalyzed by Ca^{2+}-dependent and Ca^{2+}-independent protein phosphotransferases (11-19). These include protein kinase C, a Ca^{2+}/phospholipid-dependent protein kinase, and cAMP-dependent protein kinase, a Ca^{2+}-independent protein kinase (11-15, 17-18). Although these two kinases can phosphorylate MBP at several seryl and threonyl residues (14), they still cannot account for all

Abbreviations: MBP, myelin basic protein; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; cAMP, adenosine-3':5'-monophosphate. Designation of ganglio-series gangliosides is based on the Svennerholm system (23).

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the endogenous phosphorylation sites found in purified rabbit MBP (20). Recent investigations in this laboratory revealed that gangliosides have profound effects on the phosphorylation of MBP and several other myelin proteins (17). A novel ganglioside-stimulated protein kinase has been identified and partially purified from guinea pig brain (21). To investigate whether this ganglioside-dependent enzyme, a Ca^{2+}-independent protein kinase, also may play a role in modulating myelin function, its ability to phosphorylate MBP was studied. In this report, it is shown for the first time that MBP can be phosphorylated by ganglioside-stimulated protein kinase in the presence or absence of G_{T1b}. Two of the phosphorylation sites are distinct from those catalyzed by protein kinase C and by cAMP-dependent protein kinase. These findings suggest that this protein phosphorylation reaction may be important in the modification of MBP.

MATERIALS AND METHODS

Rabbit myelin basic protein was obtained from Calbiochem. The purity of the protein was monitored by using high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis techniques. A ganglioside-stimulated protein kinase was partially purified from guinea pig brains as previously described (21). A membrane-associated protein kinase C and the catalytic subunit of cAMP-dependent protein kinase were purified to apparent homogeneity according to previously reported procedures with minor modifications (18, 22). G_{T1b} was obtained from Bachem, Inc. Synthetic peptides including an encephomyelitogenic peptide Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg, MBP residues 68-84 (guinea pig), and Leu-Arg-Arg-Ala-Ser-Leu-Gly were purchased from Peninsula. A peptide with the sequence of Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys was custom synthesized and purified to homogeneity by using high-performance liquid chromatography.

Phosphorylation of MBP (0.4 mg/ml) or synthetic peptides by different protein kinases was carried out at 30 °C in reaction mixtures containing 50 mM HEPES, pH 7.4, 10 mM magnesium acetate, and 167 μM [γ-^{32}P]ATP. The reaction catalyzed by the partially purified ganglioside-stimulated protein kinase contained 266 μg/ml G_{T1b} and 1.0 mM EGTA; the reaction catalyzed by the catalytic subunit of cAMP-dependent protein kinase (2.3 μg/ml) contained 1.0 mM EGTA; and the reaction catalyzed by protein kinase C contained 0.4 mM calcium chloride and 40 μg/ml phosphatidylserine. At timed intervals, aliquots were taken and ^{32}P incorporation into MBP was monitored by using phosphocellulose P-81 paper assays (17, 21). In other experiments, the reactions were terminated with 20% (w/v) ice-cold trichloroacetic acid (TCA). The precipitated MBP was centrifuged and washed with 5% TCA (3 times) and anhydrous ethyl ether (3 times). The sites of phosphorylation in MBP were analyzed by using two-dimensional peptide mapping techniques after digestion with trypsin (17, 21) or by using reversed-phase HPLC after digestion with Staphylococcus aureus V8 protease as described previously (18).

RESULTS AND DISCUSSION

A ganglioside-stimulated protein kinase partially purified from guinea pig brain could undergo autophosphorylation and phosphorylation of exogenous substrates such as Leu-Arg-Arg-Ala-Ser-Leu-Gly in the presence of G_{T1b} (21). This protein kinase also phosphorylated MBP isolated from rabbit brain (Fig.

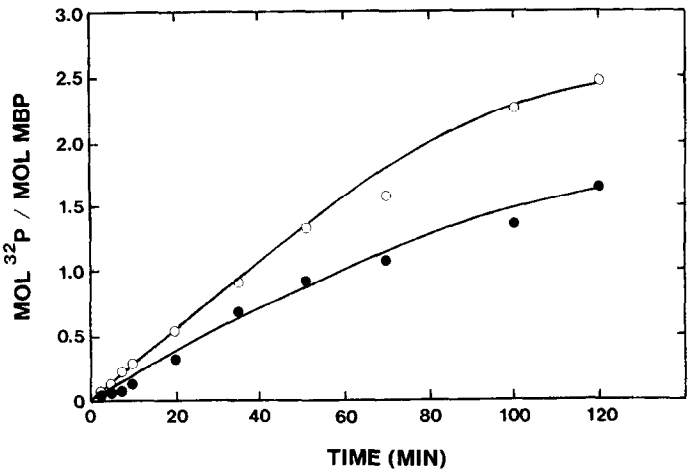

Fig. 1. Phosphorylation of rabbit MBP by ganglioside-stimulated protein kinase. Rabbit MBP (0.4 mg/ml) was phosphorylated by a partially purified ganglioside-stimulated protein kinase in the presence (●) or absence (○) of G_{T1b} (266 μg/ml) under conditions similar to those described under Materials and Methods. At timed intervals, ^{32}P incorporation into MBP was monitored by using phosphocellulose P81 paper assays. The experiments were repeated three times and the results of one representative experiment are shown.

1). However, phosphorylation of MBP was observed even when G_{T1b} was absent. The stoichiometry of phosphorylation in the presence and absence of G_{T1b} was 1.4 and 2.1 mol ^{32}P/mol MBP, respectively (Table I), indicating that the phosphorylation reaction was attenuated by G_{T1b}. Thus, gangliosides can exert seemingly opposite effects on the enyzmic activity of ganglioside-stimulated protein kinase.

Phosphorylation of MBP by the catalytic subunit of cAMP-dependent protein kinase also was inhibited by G_{T1b} (Table I). However, gangliosides had no inhibitory effects on protein kinase C-catalyzed phosphorylation of MBP. Approximately 4.3 and 4.0 mol phosphate were incorporated into MBP in the presence and absence of G_{T1b}, respectively (Table I).

Table I. Effects of gangliosides on the stoichiometry of phosphorylation of rabbit myelin basic protein by various protein kinases

Protein kinases	Stoichiometry (mol ^{32}P/mol MBP)	
	$-G_{T1b}$	$+G_{T1b}$
PKJ	2.12 ± 0.38	1.39 ± 0.24
PKA	1.93 ± 0.37	1.44 ± 0.50
PKC	4.00 ± 0.51	4.34 ± 0.56

Rabbit MBP (0.4 mg/ml) was phosphorylated by different protein kinases with or without the presence of 266 μg/ml G_{T1b} under conditions similar to those described under Materials and Methods. The stoichiometry of phosphorylation was determined after 100 min of reaction as described in Fig. 1. The protein kinases were: ganglioside-stimulated protein kinase (PKJ); the catalytic subunit of cAMP-dependent protein kinase (PKA); and protein kinase C (PKC).

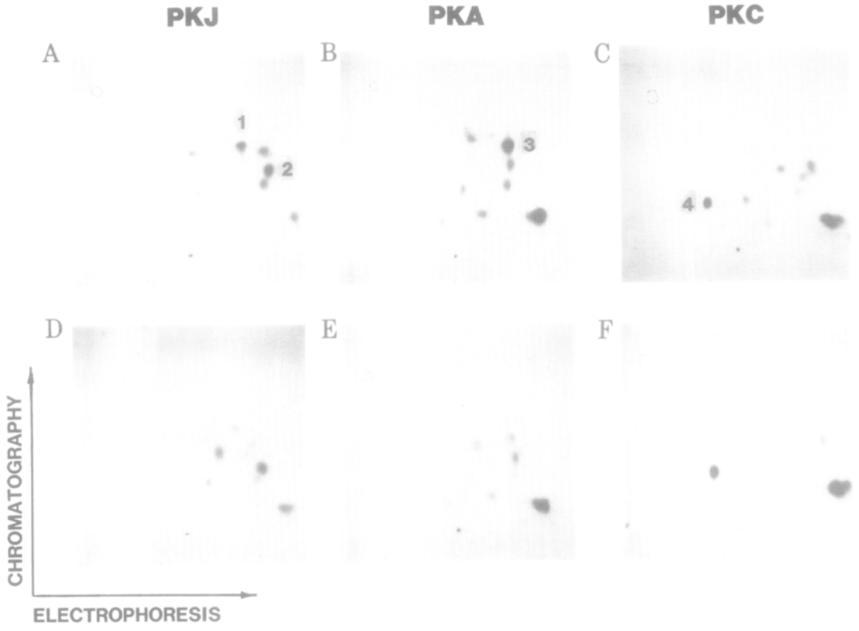

Fig. 2. Two-dimensional peptide mapping of ^{32}P-labeled rabbit MBP phosphorylated by ganglioside-stimulated protein kinase, cAMP-dependent protein kinase or protein kinase C. Rabbit MBP was phosphorylated by ganglioside-stimulated protein kinase (A and D), the catalytic subunit of cAMP-dependent protein kinase (B and E), or protein kinase C (C and F) in the absence (A to C) or presence (D to F) of G_{T1b} (266 μg/ml) as described in the legend to Table I. The ^{32}P-labeled proteins were digested with trypsin and analyzed by using two-dimensional peptide mapping technique. High-voltage electrophoresis was carried out in a buffer containing pyridine/acetic acid/water (1:10:189; v/v) and ascending chromatography was carried out in a buffer comprised of 1-butanol/acetic acid/water/pyridine (12:15:3:10; v/v). The tracking dye was phenol red. The radioactive phosphopeptides were located by using autoradiography.

Analyses of the sites of phosphorylation in MBP by using two-dimensional peptide mapping techniques revealed that the substrate specificity of ganglioside-stimulated protein kinase was distinct from cAMP-dependent protein kinase and protein kinase C (Fig. 2). Two ^{32}P-labeled tryptic peptides (sites 1 and 2) were preferentially phosphorylated by ganglioside-stimulated protein kinase (Fig. 2A). In contrast, phosphorylation of site 3 was preferred by cAMP-dependent protein kinase (Fig. 2B) whereas site 4 were specifically phosphorylated by protein kinase C (Fig. 2C). Addition of G_{T1b} to the reaction mixtures had no apparent effects on MBP phosphorylation catalyzed by protein kinase C (Fig. 2F). However, phosphorylation of both site 1 and site 3 was inhibited by gangliosides (Figs. 2D and 2E). These results indicate that modulation of MBP phosphorylation by G_{T1b} may be substrate-directed, perhaps due to binding of gangliosides at or near the phosphorylation sites 1 and 3 of MBP.

To gain more information on the location of the sites of phosphorylation catalyzed by ganglioside-stimulated protein kinase, phosphorylated MBP was trisected at glutamic acid residues into three domains (designated A, B, and

C, respectively) by digestion with Staphylococcus aureus V8 protease at pH 4.0 as previously described (18). The ^{32}P-labeled peptides were separated into three major polypeptide peaks (I-III) by using reversed-phase HPLC (Fig. 3A). Only peak I showed the highest absorbance at 280 nm. This unique characteristics is due to the presence of a single Trp residue (position 114) in MBP and suggests that peak I represents residues 82-117 (domain B). Peaks II and III may correspond to domain A (residues 1-81) and domain C (residues 118-168) of MBP, respectively (18). Analyses of the chromatographic fractions revealed that only peaks II and III contained radioactivity (Fig. 3B). Other studies indicated that phosphorylation of peak II was increased in the absence of G_{T1b} (results not shown). No phosphorylation of peak I was observed, regardless of the presence or absence of gangliosides (Fig. 3B). Thus, although domain B contains four potential phosphorylation sites (13-15, 18-20) including a seryl residue located within an experimental allergic encephalitogenic determinant (residues 112-120), ganglioside-stimulated protein kinase does not seem to be able to phosphorylate isolated MBP at this highly conserved region.

To further investigate the site specificity of MBP phosphorylation, an oligopeptide Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys corresponding to residues 111-120 of rabbit MBP was synthesized and its phosphorylatability by different protein kinases tested. Analysis of this synthetic peptide by reversed-phase HPLC revealed that its retention time (35.07 min) (Fig. 3C) was similar to peak I (34.19 min) (Fig. 3A). This result supports the identification of peak I as domain B of MBP. The retention time for an experimental allergic encephalitogenic peptide Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg was 35.02 min (results not shown). Ganglioside-stimulated protein kinase could phosphorylate the synthetic peptide Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (Table II). The initial rate of phosphorylation was stimulated 6- to 10-fold by addition of G_{T1b} to the reaction mixture. These results suggest that Ser-113 in isolated rabbit MBP may not be accessible to ganglioside-stimulated protein kinase. No phosphorylation was observed using a peptide Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg as a substrate (Table II). Thus, the Arg residue located two positions to the amino-terminal side of the Ser residue may be important for substrate recognition by ganglioside-stimulated protein kinase. Of course, more detailed kinetics studies are required to ascertain this aspect.

Turner et al. (13) have suggested that Ser-115 in bovine MBP is phosphorylated by protein kinase C and this phosphorylation reaction may be important in determining the antigenicity of the basic protein to induce experimental allergic encephalomyelitis. Subsequent studies by Kishimoto et al. (14) revealed that phosphorylation of this site is preferred by cAMP-dependent protein kinase, rather than protein kinase C. As shown in Table

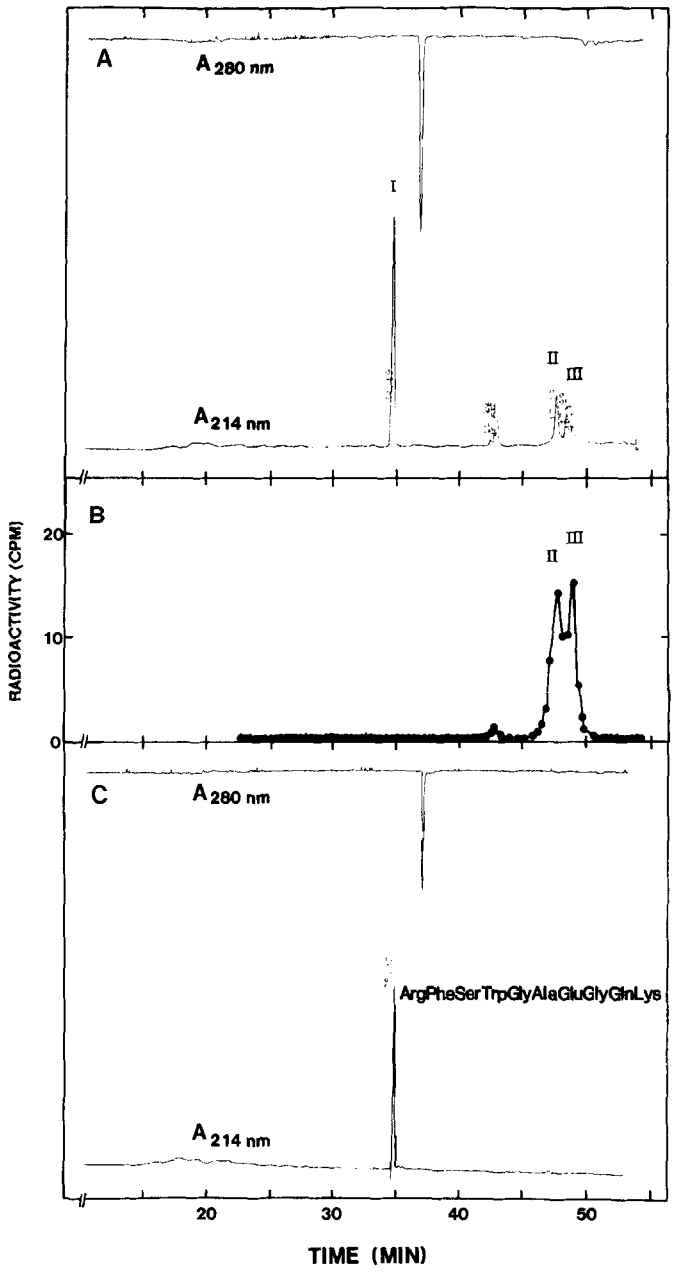

Fig. 3. Localization of the sites of phosphorylation in the three domains of MBP. MBP (5 μg) was phosphorylated by ganglioside-stimulated protein kinase to a stoichiometry of 1.9 mol ^{32}P/mol MBP in the absence of G_{Tib}. The radioactive MBP was digested by Staphylococcus aureus V8 protease at pH 4.0 and the ^{32}P-labeled peptides were analyzed by reversed-phase HPLC on a Vydac C18 column using a linear gradient of 0-60% (v/v) acetonitrile in 0.05% trifluoroacetic acid (v/v), commencing after 5 min of washing. The elution profiles were monitored at 214 nm (AUFS=0.5) and at 280 nm (AUFS=0.1) (**A**). Fractions of 0.4 ml were collected and the amounts of radioactivity were determined by liquid scintillation counting (**B**). The elution profile of a synthetic peptide Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys which corresponds to residues 111-120 in rabbit MBP is shown in **C**. Phosphorylation of this peptide (1.0 mg/ml) by ganglioside-stimulated protein kinase is summarized in Table II.

Table II. Comparison of substrate specificities of MBP and peptides for various protein kinases

Substrates	Activity (cpm incorporated per min)			
	PKJ		PKA	PKC
G_{Tub}:	-	+	-	-
Rabbit MBP	12,400	10,500	32,300	180,000
RFSWGAEGQK	1,400	13,400	3,900	60
LRRASLG	4,300	25,400	9,900	10,400
FSWGAEGQR	40	30	30	20
MBP #68-84 (G. pig)	10	80	20	50

Phosphorylation of rabbit MBP (0.4 mg/ml) and various synthetic peptides (1.0 mg/ml) by ganglioside-stimulated protein kinase (PKJ), cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) was carried out at 30 °C for 5 min in reaction mixtures similar to those described under Materials and Methods. The peptide substrates were: RFSWGAEGQK, Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys; LRRASLG, Leu-Arg-Arg-Ala-Ser-Leu-Gly; FSWGAEGQR, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg; and MBP #68-84 (guinea pig), His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro.

II, cAMP-dependent protein kinase could phosphorylate the synthetic peptide Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (1 mg/ml). In contrast, this peptide was a poor substrate for protein kinase C, especially when compared to MBP (0.4 mg/ml) or to a peptide with the sequence of Leu-Arg-Arg-Ala-Ser-Leu-Gly (1.0 mg/ml) (Table II). These observations support the results of Kishimoto et al. (14) which suggest that Ser-115 in bovine MBP may not be phosphorylated by protein kinase C.

The exact physiological significance of phosphorylation of MBP by ganglioside-stimulated protein kinase or other protein kinases still remains to be established. There are at least two possibilities: (i) phosphorylation of newly biosynthesized MBP may facilitate the protein to acquire a conformation that is necessary for insertion into specialized myelin membranes; and (ii) phosphorylation of MBP in different regions of the multilamellae may provide a mechanism for the modulation of the metabolic states or membrane stability of myelin. Because MBP can be phosphorylated at multiple sites, it is possible that phosphorylation of the different sites may depend on the various stages of myelin formation and the exact location or conformation of MBP within the multilamellae. Thus, although Ser-113 in isolated MBP is not significantly phosphorylated by ganglioside-stimulated protein kinase (Fig. 3), this site may become phosphorylatable if this region assumes a structure similar to the synthetic peptide corresponding to residues 111-120 (Table II). This conformational requirement also may explain why Thr-98 in human and bovine MBP is not phosphorylated by protein kinase C or cAMP-dependent protein kinase (14, 18) whereas a synthetic peptide corresponding to the sequence of 97-103 can serve as a substrate for

protein kinase C (15). The effects of gangliosides on MBP phosphorylation are complex (Figs. 1 and 2; Tables I and II). Further studies including the determination of the primary sequences of sites 1 and 2, the preferred sites of phosphorylation for ganglioside-stimulated protein kinase, and site 3 are required to better understand the structure-function relationship of myelin basic protein and the functional roles of gangliosides. Nevertheless, results presented in this report suggest that phosphorylation of MBP by ganglioside-stimulated protein kinase may be important in regulating some myelin functions.

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