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COVALENT LINKAGE OF PHOSPHOINOSITIDES TO MYELIN BASIC PROTEIN: IN VIVO OCCURRENCE AND IN VITRO STUDIES WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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SUMMARY: We have previously reported the covalent attachment of phosphoinositides to myelin basic protein (MBP) in vitro. In this study, in vivo phosphoinositidation of MBP was observed to occur after intracerebral injection with $[^{32}P]HPO_4^{2-}$. In the in vitro study of experimental allergic encephalomyelitis, a four-fold increase in phosphoinositidation of MBP was observed in the myelin from the guinea pigs sacrificed during the development of motor dysfunction, as compared to the control group. A decrease (40%) was observed among most of the animals sacrificed before the onset of motor dysfunction.

The covalent linkage between phosphoinositides and membrane proteins has been reported to occur (1,2,3). We have identified the covalent attachment of phosphoinositides to myelin basic protein (MBP) in vitro (4,5). After proteolysis, [^{32}P] labelled MBP yielded two "lipopeptides" (4), which contained phosphoinositides covalently attached to a tetrapeptide at serine-54 (5).

In this communication, phosphoinositidation of MBP was observed to occur in vivo. In addition both in vitro [^{32}P] incorporation and phosphoinositidation of MBP were studied in the guinea pigs with experimental allergic encephalomyelitis (EAE).

phosphorylation of MBP is an active process that occurs both in vivo and in vitro, and may not only partially contribute to the microheterogeneity of MBP (6) but may also alter the immunogenicity of MBP (7). It is possible that

Abbreviations: MBP, myelin basic protein; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; SDS, sodium dodecyl sulfate; PIPES, 1.4-peperazine-bis-(ethanesulfonic acid); HPLC, high performance liquid chromatography; TLC, thin layer chromatography; CSF, cerebrospinal fluid.

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changes in the state of phosphorylation might be involved in the pathogenesis of demyelinating diseases (8).

MATERIALS AND METHODS

Materials: New Zealand white rabbits, 15 days old, and adult albino guinea pigs, weighing 450-550g, were obtained from our vivarium; carrier free $[^{32}P]H_3PO_4$ was from ICN; Spectra/Pro 3 dialysis membrane was from Spectrum Medical Industries, Inc. And others were the same as we described previously (4,5).

In Vivo Study: Developing female rabbits were used for in vivo labelling experiment. Animals were lightly anesthetized with ether, injected intracerebrally with 1 mCi of $[^{32}P]HPO_4^{2-}$, and sacrificed by decapitation 2 hours later (9). Age-, sex-, and weight-matched animals without injection were sacrificed in the same manner for in vitro study as the reference to in vivo study. Brains were taken for myelin isolation (10). The average body and brain weights were 241±33g (S.D.) and 5.05±0.27g, respectively, and the yield of dry myelin was 43.67±4.92 mg/brain.

EAE Experiment: Adult female guinea pigs were housed for two weeks before use. EAE was induced according to the protocol described by Westall and Thompson (11). Animals were assigned at random for sacrifice by decapitation between the 7th and 15th days postinoculation despite their clinical status, and brains were taken for myelin isolation (10). Guinea pigs were assigned at random to either the control or EAE group and the animals of the control group were housed, fed, and sacrificed concurrently in the same manner as EAE group but were not injected. The clinical signs of disease included weight loss, fecal incontinence, hind leg weakness or paralysis and tremors. All animals in the control group gained weight (52.4±39.3g), while those in the experimental group lost weight (73.9±43.2g).

Assay for $[^{32}P]$ incorporation: Reaction mixture contained 70-100 µg myelin protein, 50 mM TrisHCl pH 7.4, 10 mM $MgCl_2$, 0.2% Triton X-100 and 100 µM [gamma-^{32}P]ATP (300-500 cpm/pmole) in a total volume of 100 µl. Reactions were incubated at 37°C for 15 minutes, and terminated by addition of 25 µl "SDS stop solution" containing 0.5 M TrisHCl pH 6.8, 10% SDS, 10% 2-mercaptoethanol, and 50% glycerol. Aliquots (50 µl) were analyzed by SDS-polyacrylamide slab gel electrophoresis (12). The gel slices containing MBP (18.5K), determined by staining, were cut out and counted in a liquid scintillation counter (13). The amount of protein was determined by the modified Lowry method (14).

Lipopeptide Isolation: $[^{32}P]$ labelled myelin, containing 0.7-1.0 mg myelin protein, was delipidated twice with ether:ethanol (3:2, V/V, once with ether, and with acetone. The delipidated precipitate was homogenized in a 1 ml "sample buffer" and then applied onto a preparative SDS-polyacrylamide slab gel (12). The gel containing $[^{32}P]$ labelled MBP (18.5K), determined by autoradiography, was cut out and embedded in a tube gel containing the same as stacking gel (12). $[^{32}P]$ labelled MBP was eluted into a Spectra/Pro 3 dialysis membrane, dialyzed against 10 mM TrisHCl pH 7.4 for 24 hours, precipitated with 4 volumes of ice cold acetone, and then hydrolyzed by trypsin in 2 ml of 15 mM $CaCl_2$ and 10 mM TrisHCl pH 7.8 at 37°C for 4 hours. After extraction with 10 ml methanol once and 10 ml chloroform:ethanol (2:1, V/V) once, the supernatant was dried with rotary evaporation and resuspended in 2 ml of 10 mM PIPES pH 6.8 for hydrolysis by carboxypeptidase Y at 37°C for 14 hours. At the end of proteolysis, 40 ml of ether:ethanol (3:2, V/V) were added. The supernatant was dried with rotary evaporation and resuspended in 200 µl of chloroform: methanol (2:1, V/V) for purification by reverse phase

HPLC. A μBondapak C_{18} column (3.9 mm I.D. x 30 cm, 10μ), held at room temperature with flow rate 0.5 ml/min, was first eluted with 50% methanol and then with 100% methanol at fraction 20. Fractions of 50 drops were collected and counted in a liquid scintillation counter.

Thin Layer Chromatography (TLC): Silica gel 60 was developed in chloroform: methanol:15 N NH_4OH:water (90:90:7:22, V/V/V/V).

RESULTS

Based on reverse phase HPLC (Figure 1) and TLC (Figure 2), lipopeptides isolated from in vivo [^{32}P] labelled MBP were indistinguishable from lipopeptides isolated from in vitro experiments. After treatment of [^{32}P] labelled

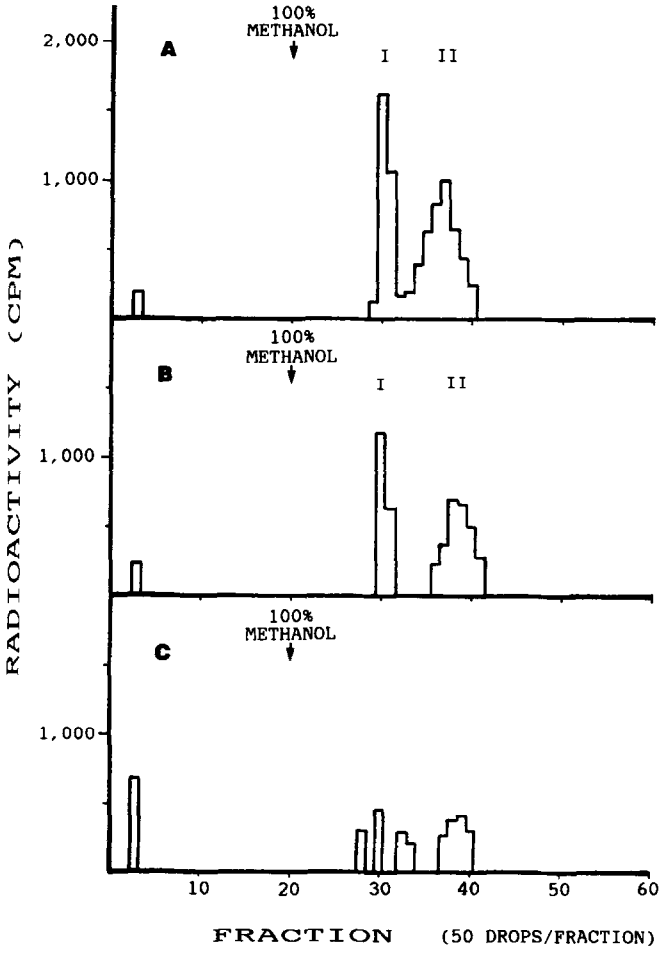

FIGURE 1: HPLC ANALYSIS OF LIPOPEPTIDES FROM MYELIN BASIC PROTEIN

Lipopeptides I and II were isolated from (A) rabbit MBP in the in vitro study, (B) rabbit MBP in the in vivo study, and (C) rabbit MBP in the in vivo study after treatment with phospholipase C in 10 mM Tris-HCl pH 7.3 at 37°C for 6 hours. HPLC system was described in the methods section.

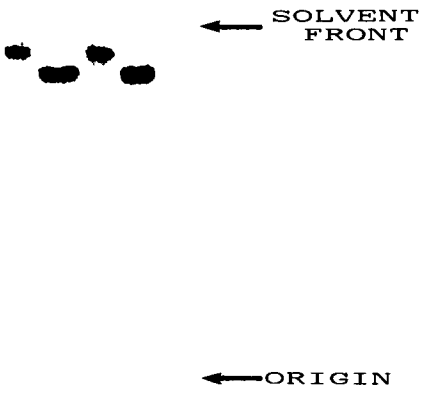

FIGURE 2: THIN LAYER CHROMATOGRAPHY OF LIPOPEPTIDES FROM MYELIN BASIC PROTEIN

Lipopeptides I and II were isolated from (A) rabbit MBP in the in vitro study and (B) rabbit MBP in the in vivo study. TLC system was described in the methods section and the radioactive spots were detected by autoradiography.

MBP obtained from in vivo experiment with phospholipase C, it yielded 50% less of lipopeptides as compared to untreated [^{32}P] labelled MBP (Figure 1). Those peaks not corresponding to lipopeptide I or II were not identified.

In the in vitro study of EAE myelin, the extent of [^{32}P] incorporation and phosphoinositidation of MBP from EAE guinea pigs were very different from those of the control animals (Figure 3). The extent of [^{32}P] incorporation and phosphoinositidation were apparently related to the clinical status of disease. As compared to the control group, all six guinea pigs sacrificed during the development of hind leg weakness or paralysis showed a marked increase in [^{32}P] incorporation (285%) and in phosphoinositidation (446%) of MBP and five out of seven animals sacrificed before the development of motor dysfunction showed a decrease in [^{32}P] incorporation (50%) and in phosphoinositidation (40%) of MBP. However, it should be noted that two animals sacrificed before the development of motor dysfunction did show an increase in [^{32}P] incorporation and phosphoinositidation. The lipopeptides I and II, isolated from the control and EAE groups, were indistinguishable by reverse

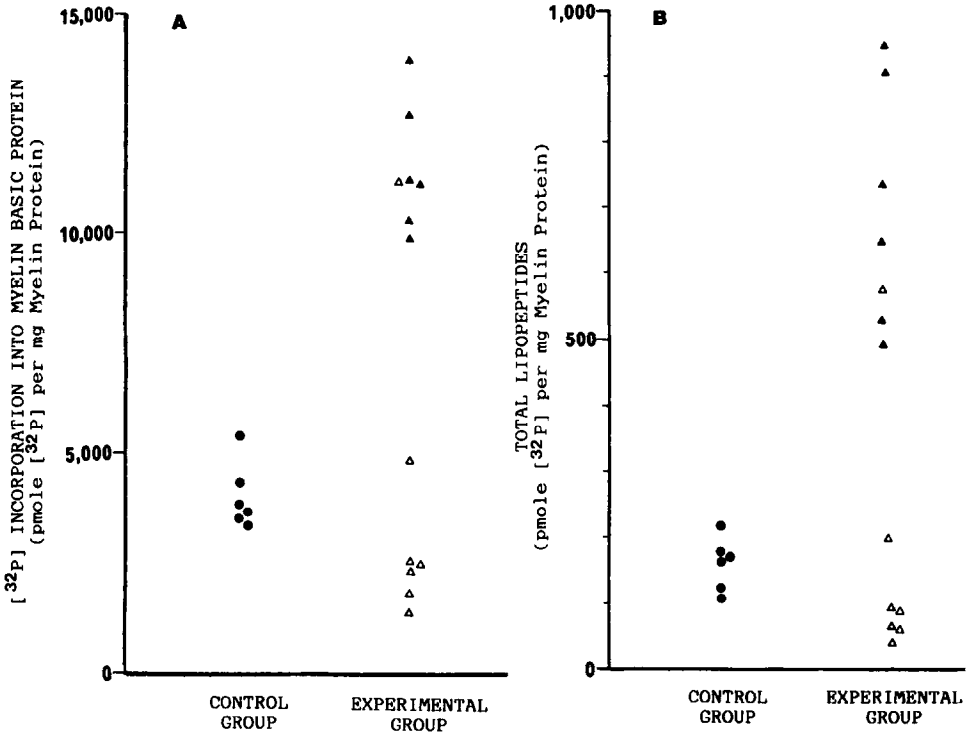

FIGURE 3: THE EFFECTS OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS ON IN VITRO [^{32}P] INCORPORATION INTO MYELIN BASIC PROTEIN AND ON IN VITRO PHOSPHOINOSITIDATION OF MYELIN BASIC PROTEIN FROM [γ-^{32}P]ATP

(A) Amount of [^{32}P] was incorporated into the guinea pig MBP and (B) amount of total lipopeptides was isolated from [^{32}P] labelled guinea pig MBP. There were 6 guinea pigs in the control group (●). In the experimental group, 6 guinea pigs (▲) were sacrificed during the development of hind leg weakness or paralysis and 7 guinea pigs (△) were sacrificed before the onset of motor dysfunction. Each marker represents the average result of n assays performed on a single animal (n=16 in [^{32}P] incorporation and n=3 in phosphoinositidation). See methods section for details.

phase HPLC and by TLC as described before. However, the quantity of lipopeptides was significantly different between the control and EAE groups.

DISCUSSIONS

The suggestion that the covalent linkage between phosphoinositides and membrane proteins is not only important in maintaining the integrity of membranes (15) but is also important in the specific release of proteins from membranes (16) is consistent with our observation that this covalent linkage

in the myelin membrane, which indeed occurs in vivo, may play an important role in the diseases of myelin.

Myelin is the primary vulnerable target in multiple sclerosis (MS) (17). Electron microscopic studies revealed that demyelination is a result of phagocytosis of myelin by macrophages and demyelination does not occur before the appearance of monocytes (18). Each of the protein antigens in the myelin sheath is capable of eliciting an immune response in a heterologous species, but only MBP is encephalitogenic (19). MBP, bound to the cytoplasmic side of the myelin membrane (20), is normally inaccessible for immune attacks directed against myelin (21). Immunocytochemically a good reaction of MBP in the normal myelin with antibody is only achieved after partial delipidation (22). However, MBP is much more exposed in myelin obtained during the acute form of MS (23). Under these conditions, MBP must somehow be made accessible to the immunocytes and/or antibodies before demyelination.

The observation, by Alvord et al. (24), of the release of MBP into the cerebrospinal fluid (CSF), which has shown no essential differences between EAE and MS, could be the result of change of phosphoinositidation of MBP. The release of MBP into CSF resulted from the decreased phosphoinositidation of MBP and the subsequent increase of phosphoinositidation of MBP resulted in the rapid drop of MBP concentration in CSF. In this case, that the decrease in phosphoinositidation should be the result of peripheral sensitization to MBP is also consistent with our observation in which it occurred only after intradermal injection of MBP.

Our observations indicate that the covalent linkage between phosphoinositide and MBP, which indeed occurs in vivo, may play a major role in maintaining the integrity of myelin sheath and keeping MBP in the cytoplasmic apposition of myelin sheath away from the immune system, which may be involved in the pathogenesis of demyelinating diseases.

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