Colvalent Linkage of Phospholipid to Myelin Basic Protein: Identification of Phosphatidylinositol Bisphosphate as the Attached Phospholipid†

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ABSTRACT: Evidence presented demonstrates a covalent attachment of a phospholipid to bovine myelin basic protein. Partial characterization of the phospholipid moiety was performed on myelin basic protein obtained from ^{32}P-phosphorylated whole mylein that was first delipidated by two ether/ethanol (3:2 v/v) extractions, ether extraction, and acetone extraction and then purified by preparative sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The myelin basic protein was precipitated with aqueous acetone and treated with proteases. Treatment with carboxypeptidase Y or trypsin for several hours released a lipophilic fragment, which was purified by reverse-phase high-performance liquid chromatography to yield two "lipopeptides". Such lipopeptides were obtained from both the major and minor myelin basic proteins of rat and bovine brain. Treatment with either mild base or phospholipase C removes the lipophilic character of the peptide fragment. The lipophilic fragment is a substrate for phospholipase D, but it does not comigrate on thin-layer chromatography with any ^{32}P-labeled lipid obtained from myelin incubated with [γ-^{32}P]ATP. Polyphosphoinositides were shown to be released by mild acid treatment of myelin basic protein that had been extracted with organic solvent and then purified by SDS–polyacrylamide gel electrophoresis. Along with the fact that inositol monophosphate was identified in the partial acid hydrolysate of the lipopeptide, we have concluded that polyphosphoinositide (phosphatidylinositol 4-phosphate and/or phosphatidylinositol 4,5-bisphosphate) was the original phospholipid portion of the lipopeptide.

Proteins are known to be covalently modified in vivo by such processes as phosphorylation, methylation, acetylation, glycosylation, and adenylation [for review, see Wold (1981)]. Though there is precedent in bacterial systems for the presence of a covalent phospholipid protein linkage, the existence of a covalent linkage of phospholipid to proteins of eukaryotic systems has not been previously demonstrated. Proteolipids containing esterified fatty acids are well-known and have been reviewed recently (Schlesinger, 1981). To date, the only protein containing a covalently attached phospholipid is the penicillinase of *Bacillus licheniformis* (Yamamoto & Lampen, 1976a). Its N-terminal serine is linked via a phosphodiester bond to phosphatidic acid (Yamamoto & Lampen, 1976b). There is one report that suggests there is phospholipid associated with plasma lipoproteins (Fisher & Gurin, 1964a,b). Upon enzymatic digestion of the protein and partial purification of the resulting peptides, fatty acids were found to be associated with a peptide fraction that also contained organic phosphate. The phosphate was not liberated by bacterial alkaline phosphatase treatment of the lipopeptide.

There are numerous investigations of the incorporation of phosphate into myelin basic protein (Miyamoto & Kakiuchi, 1974; Carnegie et al., 1974; Steck & Appel, 1974; Chou et al., 1976; Petrali et al., 1980b). However, this incorporation has been studied only in the context of phosphorylation derived from various protein kinases. Thus far, with one exception (Smith et al., 1976), only acid hydrolysis of ^{32}P-phosphorylated meylin basic protein has been performed to liberate and examine the phosphorylated species. This of course precludes isolation of any acid-labile linkages such as certain phosphodiesters, phosphoramidates, and acyl phosphates and leaves only phosphohydroxy amino acids to be isolated. Also, the diverse set of enzymatic conditions used by investigators may indicate either a multiple range of protein kinases or, more interestingly, the existence of enzymes that incorporate phosphorylated molecules (such as phospholipids) into myelin basic protein. This study was undertaken to explain the lipophilic character of peptides obtained from myelin basic protein.

Materials and Methods

Cyclic AMP dependent protein kinase catalytic subunit, trypsin type IX from hog pancreas, protease type VI from *Streptomyces griseus*, carboxypeptidase Y, phospholipase D type I from cabbage, and phospholipase C type IX from *Clostridium perfringens* (*Clostridium welchii*) were from Sigma. [γ-^{32}P]ATP was from ICN. Purified bovine myelin basic protein was a kind gift from Dr. Fred Westall of the Salk Institute. Myelin basic protein was phosphorylated by the catalytic subunit of cyclic AMP dependent protein kinase as described by Yang et al. (1982). Myelin was prepared from bovine brain white matter as described by Norton and Poduslo (1973).

Phosphorylation of Myelin. Freshly prepared or thawed myelin (80–100 mg, dry weight) was phosphorylated in a 2-mL reaction volume containing 50 mM Tris-HCl,[1] pH 7.4, 10 mM $MgCl_2$, 0.2% Triton X-100, and 50 M [γ-^{32}P]ATP for 15 min at 37 °C.

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[1] Abbreviations: HPLC, high-performance liquid chromatography; PPI, polyphosphoinositide; PhIP, phosphatidylinositol 4-phosphate; $PhIP_2$, phosphatidylinositol 4,5-bisphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PEI, poly(ethylenimine); TLC, thin-layer chromatography; MBP, myelin basic protein.

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Extraction of ^{32}P-Phosphorylated Lipid. Lipid was extracted from ^{32}P-phosphorylated myelin twice with 40 mL of ether/ethanol (3:2 v/v), once with 40 mL of ether, and finally with 40 mL of acetone. The combined lipid extract was dried with rotary evaporation and resuspended in 20 mL of chloroform/methanol (2:1 v/v). The lipid was washed successively with two-thirds of its volume of chloroform/methanol/1 M HCl/85% H_3PO_4 (93:48:46.7:0.3 v/v/v/v) and chloroform/methanol/0.1 M HCl (3:48:47 v/v/v) and then neutralized with NH_4OH (Gonzalez-Sastre et al., 1971).

Purification of ^{32}P-Phosphorylated Myelin Basic Protein. The delipidated protein precipitate was homogenized in a 2-mL solution containing 2% SDS, 2% 2-mercaptoethanol, 0.125 M Tris-HCl, pH 6.8, and 10% glycerol and then applied onto a preparative SDS–polyacrylamide gel (16.5 cm × 14.0 cm × 0.18 cm). Electrophoresis was perfomed at 60 V through the stacking gel and 150 V through the resolving gel. The position of the ^{32}P-phosphorylated myelin basic protein was determined either by KCl staining (Hager & Burgess, 1980) or by autoradiography of the wet gel. The gel containing ^{32}P-phosphorylated myelin basic protein was cut out and pulverized in a glass–Teflon tissue homogenizer. After centrifugation, the supernatant was lyophilized. The dried product was resuspended in water and precipitated with 4 volumes of ice-cold acetone in order to remove SDS (Hager & Burgess, 1980). The acetone wash was repeated.

Proteolysis of ^{32}P-phosphorylated myelin basic protein was usually carried out with carboxypeptidase Y or trypsin, followed by carboxypeptidase Y. While carboxypeptidase Y is considered an exopeptidase, commercial preparations usually also contain small amounts of an endopeptidase (yeast proteinase A) (Hayashi et al., 1973), which may account for detection of the lipoprotein with the yeast enzyme alone. Hydrolysis by trypsin (1 mg) was performed in 2 mL of 20 mM Tris-HCl, pH 7.4, while hydrolysis by carboxypeptidase Y (1 mg) was performed in 2 mL of 20 mM 1,4-piperazinebis(2-ethanesulfonic acid) (PIPES), pH 6.8. Samples were incubated at 37 °C. At the end of proteolysis, 40 mL of ether/ethanol (3:2 v/v) was added; after centrifugation the supernatant was dried with rotary evaporation and resuspended in 100 μL of chloroform/methanol (2:1 v/v).

Purification of the Lipophilic Fragment by Reverse-Phase HPLC. The lipophilic fragment was purified by reverse-phase HPLC using a μBondapak C_{18} column (Waters Associates) eluted first with 50% methanol (v/v) and then with 100% methanol. The column was held at room temperature, and the flow rate was 0.5 mL/min. Fractions of 100 drops were collected. ^{32}P radioactivity eluted off with 100% methanol was subjected to phospholipase C, phospholipase D, or mild base (0.1 N NaOH, 27 °C, 10 min, then neutralized with Dowex 50W H^+ form) treatment.

Extraction of ^{32}P-Phosphorylated Lipid. Myelin was phosphorylated as described above. Lipid was extracted from the ^{32}P-phosphorylated myelin twice with an equal volume of chloroform/methanol/concentrated HCl (38:19:3 v/v/v). The organic layers were pooled and washed successively with two-thirds volume of chloroform/methanol/methanol/1 N HCl/85% H_3PO_4 (3:48:46.7:0.3 v/v/v/v), chloroform/methanol/0.1 N HCl (3:48:47 v/v/v) and chloroform/methano./0.1 N HCl (3:48:47 v/v/v) and then neutralized with NH_4OH (Gonzalez-Sastre et al., 1971).

Anion-Exchange HPLC System. The ^{32}P-labeled compound was analyzed by using a SAX anion-exchange column under two systems. System I was described by Yang et al. (1982). The sample was injected by a Model 100 solvent delivery system (Altex). Separation was performed on a column (4.6 mm i.d. × 250 mm length) packed with Partisil-10 SAX anion-exchange resin (Whatman). The elution buffer was 10 mM KH_2PO_4, pH 3.0, in 15% (v/v) methanol. Fractions of 1 min were collected, and radioactivity was counted on Beckman LS-100 scintillation counter. System II uses a different elution buffer as follows: 50 mM KH_2PO_4, pH 3.0, in 10% methanol, which was changed to 200 mM KH_2PO_4 in 10% methanol at fraction 25 and finally to 400 mM KH_2PO_4, pH 3.0, at fraction 61.

Phospholipase C and Phospholipase D Treatment. PPI was suspended in 2 mL of buffer containing 15 mM Tris-HCl, pH 7.4 (for phospholipase C), and 15 mM MES, pH 5.6 (for phospholipase D). Then, 1.5 units of enzyme was added, and the digest was carried out for 1 h at 37 °C. The solution was extracted twice with an equal volume of ether. The resulting solution was dried by rotary evaporation and analyzed either by anion-exchange HPLC or by PEI–cellulose TLC.

Mild Acid and Mild Base Treatment of Lipopeptides. Mild acid treatment was performed in a 1.5-mL plastic vial that contained 0.1 N HCl. The vial was heated in a boiling water bath for 10 min and then neutralized with 0.1 N NaOH. The mild base treatment was carried out in 0.1 N NaOH at 37 °C for 10 min and then neutralized with 0.1 N HCl.

Thin-Layer Chromatography System. Thin-layer chromatography was performed on system I, cellulose MN-300 plates (Macherey-Nagel) developed in 1-butanol/2-propanol/formic acid/water (3:1:2:2 v/v/v/v) (Ushiro & Cohen, 1980), or system II, silica gel 60 (Merck) eluted with chloroform/methanol/15 N NH_4OH/water (90:90:7:22 v/v/v/v) (Schact, 1978).

Results

After incubation of myelin with [γ-^{32}P]ATP, an aliquot was removed and analyzed by SDS gel electrophoresis and autoradiography. As shown in Figure 1, myelin basic protein was the major ^{32}P-phosphorylated protein. Other minor phosphorylated proteins include the W_1 protein of the Wolfgram doublets, which corresponds to 2′,3′-cyclic nucleotide 3′-phosphohydrolase, and a minor 21 000-dalton component of basic protein that migrates on SDS gel slightly faster than proteolipid (PLP). In the purification of ^{32}P-phosphorylated myelin basic protein by preparative SDS gel electrophoresis, the Bromophenol Blue dye was allowed to run out of the gel for 10 min in order to remove radioactive substances such as ^{32}P-labeled ATP, phospholipid, and inorganic phosphate near the dye front.

After extraction from the gel, treatment of the ^{32}P-labeled myelin basic protein with carboxypeptidase Y or trypsin yields a fast-migrating spot of radioactivity (Figure 2) as shown by thin-layer chromatography (system I). This spot appears only after approximately 12 h or more of proteolysis. Myelin basic protein (from Dr. Fred Westall, Salk Institute) phosphorylated by the catalytic subunit of cyclic AMP dependent protein kinase and subsequently subjected to carboxypeptidase Y treatment fails to produce the fast-migrating fragment (Figure 2). The fragment obtained after proteolysis is considered to be lipophilic since it is easily extractable into organic solvent and since 100% methanol is required to elute it from a reverse-phase high-performance liquid chromatographic column. The purified lipophilic fragment does not comigrate on system II thin-layer chromatography with any ^{32}P-phosphorylated lipid isolated from myelin previously incubated with [γ-^{32}P]ATP (Figure 3).

It has been established that there is more than one myelin basic protein in both rat (Agrawal et al., 1981, 1982) and cattle

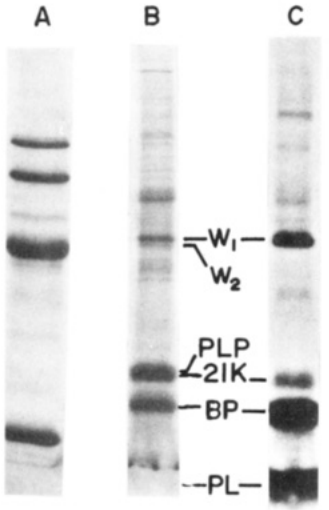

FIGURE 1: SDS gel electrophoresis and autoradiography of ^{32}P-phosphorylated myelin proteins. A twenty-microliter aliquot of the phosphorylation reaction mixture was analyzed by SDS gel electrophoresis. After the gel was stained with 25% methanol, 10% acetic acid, and 0.05% Coomassie brilliant blue and then destained with 10% methanol and 7.5% acetic acid, the gel was dried onto Whatman 3MM paper, and autoradiography was performed on Du Pont Chronex 4 X-ray film with a screen intensifier at −70 °C for 3 h: (A) Coomassie brilliant blue staining of the molecular weight standards phosphorylase *a*, bovine serum albumin, ovalbumin, and myoglobin; (B) Coomassie brilliant blue staining of myelin proteins; (C) autoradiography of ^{32}P-phosphorylated myelin proteins. Abbreviations: W_1 and W_2, Wolfgram doublets; PLP, proteolipid; 21K, minor component of basic protein; BP, myelin basic protein; PL, phospholipid.

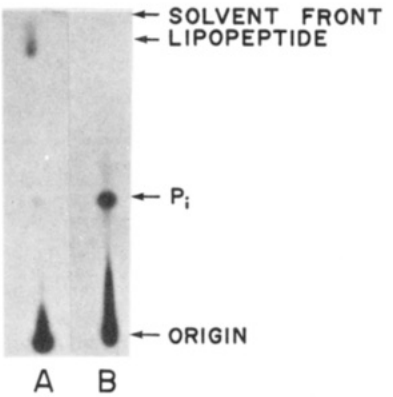

FIGURE 2: Carboxypeptidase Y digestion of ^{32}P-phosphorylated myelin basic protein. System I thin-layer chromatography of 24-h carboxypeptidase Y digestion of (A) myelin basic protein phosphorylated in myelin by endogenous kinase and (B) myelin basic protein (from Dr. Fred Westall) phosphorylated with catalytic subunit of protein kinase. Autoradiography was performed on Du Pont Chronex 4 X-ray film with a screen intensifier at −70 °C for 6 h.

(Yang, unpublished results). When isolated and subjected to similar proteolysis, a lipophilic fragment could be derived from all the myelin basic proteins examined (data not shown). Purification of this lipophilic fragment from either rat or bovine myelin basic protein with reverse-phase HPLC gives two peaks of radioactivity (lipopeptide I and II) after elution with 100% methanol (Figure 4). The lipophilic material released by trypsin digestion was indistinguishable from that released by other treatments although the yield was somewhat lower (data not shown). Different proteolysis conditions were tested, and it appeared that the two-protease system in which ^{32}P-phosphorylated myelin basic protein was digested with trypsin first, then extracted with $CHCl_3$/methanol (2:1 v/v), and subsequently digested with carboxypeptidase Y gave the best yield.

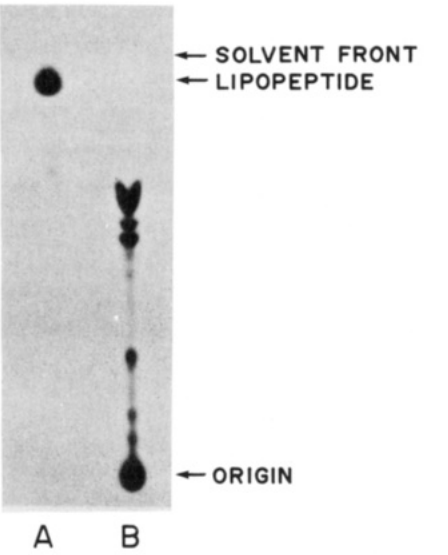

FIGURE 3: Comparison of lipophilic fragment and myelin lipid. System II thin-layer chromatography of (A) reverse-phase HPLC purified lipophilic fragment, from myelin basic protein, and (B) ^{32}P-phosphorylated myelin lipid.

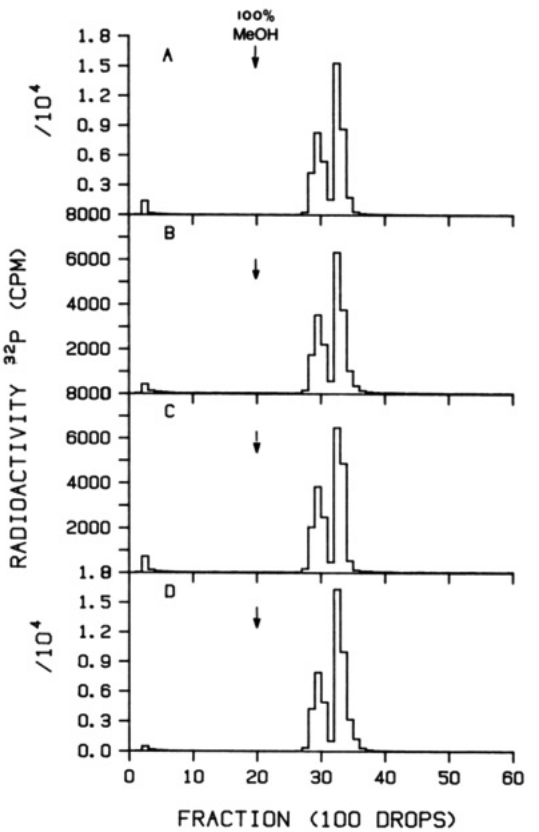

FIGURE 4: Purification of lipopeptides from myelin basic protein in bovine brain white matter and rat brain. Myelin basic protein was purified and digested by a two-protease system as described. The lipopeptides released from (A) small MBP from rat, (B) large MBP from rat, (C) large MBP from bovine brain, and (D) small MBP from bovine brain were extracted with 40 mL of ether/ethanol (3:2 v/v) and purified by reverse-phase HPLC using a μBondapak C_{18} column as outlined. Fractions of 100 drops were collected, and the radioactivity was measured by a liquid scintillation counter with open channel without scintillation cocktail added.

Treatment of the lipopeptides with mild base (Figure 5) or with phospholipase C (Figure 6) markedly reduces the hydrophobic nature of the purified fragment, while treatment with phospholipase D (Figure 6) produces an as yet unidentified phosphorylated species. All the lipopeptides lost hy-

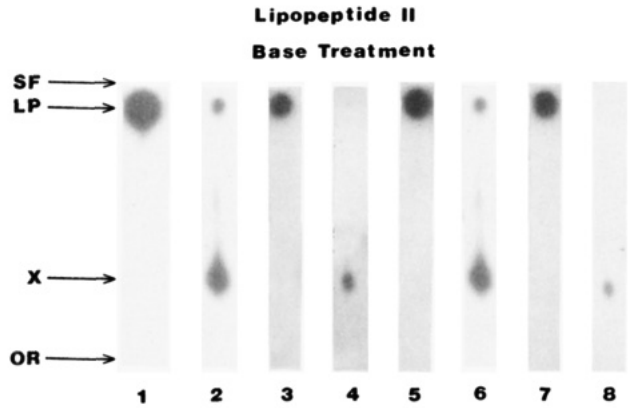

FIGURE 5: Base treatment of lipopeptide II from bovine myelin basic protein and rat myelin basic protein. Lipopeptide II from small bovine MBP (lanes 1 and 2), large bovine MBP (lanes 3 and 4), small rat MBP (lanes 5 and 6), and large rat MBP (lanes 7 and 8) was subjected to base treatment as described under Materials and Methods and analyzed by using the TLC system outlined before. Radioactive spots were detected by autoradiography. Lanes 1, 3, 5, and 7 were untreated controls while lanes 2, 4, 6, and 8 were base-treated as outlined in the text. Abbreviations: SF, solvent front; LP, lipopeptide; OR, origin.

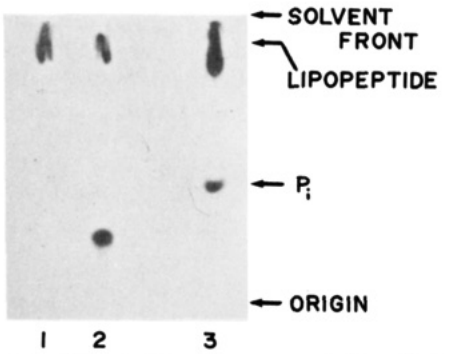

FIGURE 6: Phospholipase C and D treatment of purified lipophilic fragment. Autoradiography of the system I thin-layer chromatography performed on purified lipophilic fragment (1), phospholipase C treated lipophilic fragment (2), and phospholipase D treated lipophilic fragment (3).

drophobic character when treated with mild acid (0.1 N HCl, 10 min, 100 °C).

After limited acid hydrolysis (2 N HCl, 110 °C, 30 min) and then separation on HPLC using an SAX anion-exchange column under system I (Yang et al., 1982), both lipopeptide I and lipopeptide II gave rise to a peak that does not correspond to any phosphoamino acid standard (data not shown). However, using buffer of higher ionic strength (system II; see Materials and Methods), most of the radioactivity would be released from the column (Figure 7), and both inositol monophosphate and inositol bisphosphate could be identified; the peak at fraction 49 (Figure 7) was not identified.

Discussion

Myelin basic protein is a highly basic protein with a p*I* of about 10.6. However, it can be extracted into chloroform/methanol (2:1 v/v), which indicates its lipophilic nature although only 31% of its amino acids are hydrophobic and 40% of its amino acids are hydrophilic. The possibility of an electrostatic interaction between the basic protein and polyphosphoinositides has been suggested (Gonzalez-Sastre, 1970). However, in this study, during which myelin basic protein was treated successively by extraction with organic solvents followed by SDS gel electrophoresis, the likelihood of nonspecific, noncovalent lipid association with the myelin basic protein is very small indeed (Schlesinger, 1981). In any case a small

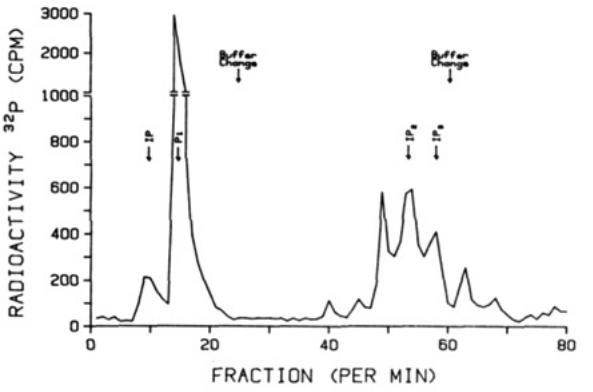

FIGURE 7: HPLC analysis of lipopeptide that had been subjected to partial acid hydrolysis. Lipopeptide II was partially hydrolyzed. The hydrolysate was then analyzed by HPLC using an SAX anion-exchange column as described under Materials and Methods. The radioactivity of each fraction was measured on a liquid scintillation counter with open channel without scintillation cocktail added. Abbreviations: IP, inositol phosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; P_i, inorganic phosphate.

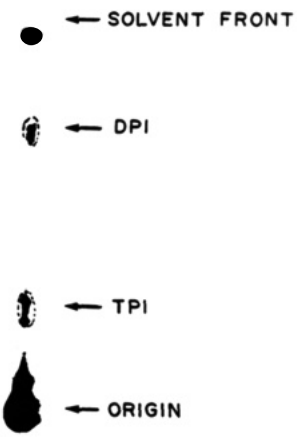

FIGURE 8: Release of polyphosphoinositide after acid treatment of ^{32}P-phosphorylated myelin basic protein. ^{32}P-phosphorylated myelin basic protein was treated with 1 N HCl for 30 min at 37 °C, extracted with ether/ethanol (3:2 v/v), and analyzed by thin-layer chromatography (silica gel) as described under Materials and Methods. Autoradiography of the plate is shown with the positions of standard diphospoinositide (DPI) and triphosphoinositide (TPI) indicated by dotted circles. Standards were detected by iodine vapor. Standards were obtained by a neomycin affinity column according to Schacht (1978).

peptide of highly lipophilic character was released after prolonged proteolysis.

After proteolytic digestion, the radioactivity (^{32}P) released and extracted into organic solvent did not correspond to any ^{32}P-labeled myelin phospholipid on thin-layer chromatography nor to any known phosphoamino acid on either high-performance liquid chromatography or thin-layer chromatography. This "lipophilic" fragment is therefore believed to contain both the peptide and the lipid moiety and is therefore termed lipopeptide. The lability of the lipopeptides to mild base, or to phospholipase C or phospholipase D treatment, indicates the presence of a phosphatidic acid component in the lipid portion of the fragment. There are at least two different lipopeptides released from the myelin basic protein as judged by reverse-phase HPLC, and they behave very similarly under all experimental conditions so far. The difference between the two may lie with the fatty acids attached to the glycerol moiety of phosphatidic acid.

Brain tissue is known to contain more polyphosphoinositides than other tissues. In myelin, polyphosphoinositides are the major lipid that undergoes phosphorylation, and its turnover

rate is also rather high. The purified ^{32}P-labeled myelin basic protein was shown to release ^{32}P-labeled polyphosphoinositides after mild acid treatment (Figure 8), suggesting that the lipid moiety of the lipopeptide is a polyphosphoinositide. Such a conclusion is further supported by the fact that both inositol monophosphate and inositol bisphosphate are present in the acid hydrolysate of lipopeptides I and II.

Very recently, Futerman et al. (1985) have shown the covalent attachment of inositol to the acetylcholinesterase membrane-anchoring domain and have also suggested that phosphatidylinositol may be involved in anchoring this enzyme to the plasma membrane.

The failure of periodate treatment to affect the lipophilic character of the lipopeptide (data not shown) indicates that the lipid moiety is most likely phosphatidylinositol 4,5-bisphosphate. Only one periodate caused cleavage would be generated in the 4,5-bisphosphate, and therefore the lipophilic moiety would not be displaced from the ^{32}P radioactive label. If phosphatidylinositol 4-phosphate is the lipid moiety, then periodate oxidation would cleave the C–C bonds between C_2–C_3 and between C_5–C_6, resulting in the removal of the lipophilic character from the lipopeptides.

The linkage of the phospholipid to myelin basic protein is probably via a phosphodiester bond [see the following paper in this issue (Chang et al., 1986)]. Such a linkage would be expected to be labile to acid as well as to base, which may explain the finding in this study that the lipopeptides are slightly acid sensitive.

The results here indicate the presence of a covalently attached ^{32}P-labeled phosphatidylinositol to myelin basic protein and suggest that ^{32}P incorporation may not be solely due to phosphomonoesters (Carnegie et al., 1974) and phosphoramidates (Smith et al., 1976) of amino acids. A reinvestigation of the regulation of the endogenous kinases is warranted since conditions affecting the activity of these kinases may also influence enzymatic attachment of phospholipid to protein. The presence of calcium ion, cyclic AMP, or phosphatidylserine, which have been demonstrated to stimulate phosphorylation of myelin basic protein (Miyamoto, 1975; Petrali et al., 1980a,b; Sulakhe et al., 1980a,b; Turner et al., 1982a,b), may also affect the enzyme system that links myelin basic protein with phospholipid.

The discovery of the covalent lipid attachment to myelin basic protein may be highly significant since the secondary structure and function of this membrane protein has yet to be elucidated (Martenson, 1981). The possible relationship of this protein modification with disease and disorders of myelin (Norton, 1977b) is intriguing.

Acknowledgments

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