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Biochemical Characterization of Phospholipids, Sulfatide and Heparin as Potent Stimulators for Autophosphorylation of GSK-3β and the GSK-3β-Mediated Phosphorylation of Myelin Basic Protein *In vitro*

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The stimulatory effects of SH (sulfatide and heparin) and two phospholipids (PI and PS) on autophosphorylation of GSK-3β and the GSK-3β-mediated phosphorylation of myelin basic protein (MBP) and two synthetic MBP peptides (M86 and M156) were comparatively examined *in vitro*. It was found that (i) both PI and SH highly stimulated the GSK-3β-mediated phosphorylation of MBP, but not glycogen synthase, and two MBP peptides through their direct binding to these substrates and (ii) both PI and heparin, as compared with sulfatide, highly stimulated autophosphorylation of GSK-3β. The K_m value of MBP for GSK-3β was highly reduced and the V_{max} value was significantly increased in the presence of these acidic modulators, which augmented further phosphorylation of MBP by the kinase. Under our experimental condition, similar stimulatory effects of PI and heparin were observed with the GSK-3β-mediated phosphorylation of tau protein (TP) *in vitro*. These results presented here suggest that these two phospholipids and SH may function as effective stimulators for autophosphorylation of GSK-3β and for the GSK-3β-mediated high phosphorylation of SH-binding proteins, including MBP and TP, in the highly accumulated levels of these acidic and sulfated modulators in the brain.

Key words: glycogen synthase, glycogen synthase kinase 3, heparin, myelin basic protein, phospholipid, sulfatide, tau-kinase, tau protein.

Abbreviations: AD, Alzheimer's disease; A-kinase, cAMP-dependent protein kinase; CDK-5, cycline-dependent protein kinase-5; CH-3S, cholesterol-3-sulfate; CK1, casein kinase 1; C-kinase, Ca^{2+}/phospholipid-dependent protein kinase; DTT, dithiothreitol; GS, glycogen synthase; GSK-3β, glycogen synthase kinase-3β; MBP, myelin basic protein; PI, phosphatidylinositol; PS, phosphatidylserine; QCM, quarts crystal microbalance; SH, sulfatide and heparin; TP, tau protein.

Glycogen synthase kinase 3 (GSK-3) is a serine (Ser)/threonine (Thr) protein kinase encoded by two isoforms [GSK-3α (~51 kDa) and GSK-3β (~47 kDa)], which possess similar biochemical characteristics and substrate specificities (*1*, *2*). GSK-3 was originally identified as a protein kinase that phosphorylates the rate-limiting enzyme, glycogen synthase (GS), in glycogen synthesis (*3*, *4*). Initially, GSK-3 was implicated in muscle energy storage and metabolism. However, the recent findings that it has a wide array of substrates, including cytoplasmic enzymes and nuclear transcriptional factors and signalling molecules (*5*, *6*) led to the conclusion that GSK might play a more generalized role in cellular regulation.

Recently, we have characterized two sulfated lipids [sulfatide and cholesterol-3-sulfate (CH-3S)] as potent stimulators of casein kinase 1 (CK1)-mediated *in vitro* phosphorylation of sulfatide- and CH-3S-binding proteins, such as high mobility group protein 1 [HMG1 (*7*)], C-kinaseη (*8*), hC3a (*9*) and bovine fibroblast growth factor-binding protein [FGF-BP (*10*, *11*)]. We have also demonstrated that direct binding of sulfatide and CH-3S to these basic functional proteins results in their conformational changes, which presumably facilitate their high phosphorylation by CK1 *in vitro* (*7–12*). Furthermore, our studies also revealed that (i) C-kinaseη is activated through its phosphorylation by CK1 in the presence of CH-3S (*8*) and (ii) two basic brain proteins [myelin basic protein (MBP) and tau protein (TP)] in the presence of sulfatide or CH-3S are highly phosphorylated by CK1 *in vitro* (*12*). However, there are no reports regarding the stimulatory effects of SH (sulfatide and heparin) and phospholipids, such as PA, PI and PS, on the GSK-3β-mediated phosphorylation of the sulfatide- and CH-3S-binding proteins *in vitro* and *in vivo*.

Since sulfatide, a major lipid component of myelin sheath, is exclusively synthesized in oligodendrocytes in the central nervous system and is present predominantly in the myelin sheath surrounding axons (*13*), it may play an important biological role in the diverse functional and regulatory processes involved in cell growth,

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cell adhesion, neuronal plasticity and cell morphogenesis (*14*). The altered level of sulfatide in human brain tissues may be involved in the pathogenesis of various neuronal diseases, since accumulation of sulfatide in arylsulfatase A-deficient brain causes metachromatic leukodystrophy (*15*). Although sulfatide exists not only in oligodendrocytes and Schwann cells but is also present in the cytoplasm of neurons (*16*, *17*), the physiological significance of sulfatide in neuronal signal transduction in mammalian brain is presently unclear. On the other hand, it has been reported that CH-3S (i) is widely distributed in various body fluids (e.g. the circulating concentration of CH-3S in human plasma: ~2.0 μM (*18*) and in tissues and cells, including erythrocytes, platelets, skin, hair, adrenals, lung and brain (*19*, *20*); (ii) is a potent inhibitor of several enzymes, such as 3-hydroxyl-3-methylglutaryl CoA reductase (*21*), phosphatidyl inositol-3-kinase (*22*), proteases [trypsin, chemotrypsin and pronase (*23*) and plasma thrombin and plasmin (*24*)] and (iii) acts as an endogenous regulator of mammalian cholesterol biosynthesis (*25*). Although CH-3S may function as an effective activator for the CK1-mediated phosphorylation of two CH-3S-binding proteins (HMG1 and C-kinaseη) *in vitro* (*7*, *8*), the physiological effect of CH-3S on the GSK-3β-mediated phosphorylation of cellular CH-3S-binding proteins involved in the regulation of neuron cells remain to be elucidated.

A recent review (*26*) concerning the biological and physiological properties of MBP notes that: (i) MBP is the second most abundant basic protein [four human isoforms (~21.5, 20.2, 18.5 and 17.2 kDa)] in central nervous system myelin, contributing to the formation and completion of the myelin sheath (*26*); (ii) a major MBP isoform (~18.5 kDa, *pI* = approx. 10 in humans) is an excellent substrate for C-kinase (*27*), A-kinase (*28*), mitogen-activated protein kinase [MAP-kinase (*29*, *30*)] and GSK-3β (*31*) *in vitro*; (iii) GSK-3β is one of protein kinases responsible for the regulation of MBP *in vivo* (*31*) and (iv) the direct binding of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-biphosphate to MBP results in the induction of its conformational changes (*32*). Both MBP and TP are known to be heparin-binding basic proteins (*33*), which may function as effective phosphate acceptors for GSK-3β in the presence of two sulfated lipids (sulfatide and CH-3S) or glycosaminoglycans, such as heparin *in vitro* (*33*).

Therefore, the present *in vitro* study has been carried out to (i) compare the relative potency of SH (sulfatide and heparin) and two phospholipids (PI and PS) as potent stimulators for the GSK-3β-mediated phosphorylation of MBP and two synthetic MBP peptides (M86 and M156) corresponding to the sequence of MBP; (ii) identify the consensus phosphorylation sites in MBP and two MBP peptides and (iii) determine the binding affinities of the GSK-3β substrates with SH and phospholipids.

MATERIALS AND METHODS

Chemicals—[γ-^{32}P]ATP (3,000 Ci/mmol) was obtained from GE Healthcare Biosciences UK Limited (Buckinghamshire, HP7 9NA, England); dithiothreitol (DTT) from Wako Pure Chemical Ind. (Osaka, Japan); purified bovine myelin basic protein (MBP), bovine serum albumin (BSA), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), heparin (porcine intestinal mucosa), sulfatide (galactosylceramide-3-*O*-sulfate, bovine brain), cholesterol-3-sulfate (CH-3S), Commassie Brilliant Blue R-250 (CBB R-250) and GSK-3β (bovine muscle, specific activity: 14,629 units/mg) from Sigma Chemical (St Louis, USA); and 5-iodoindirubin-3′-monoxime (a specific GSK-3β inhibitor) from Calbiochem (San Diego, USA). Two synthetic MBP peptides [M86 (positions 86–98: VHFFKNIVTPRTP) containing a potent phosphorylation site (Thr-Pro-Arg-Thr^{97}-Pro) and M156 (positions 156–169: GGRDSRSGSPMARR) containing a consensus phosphorylation site (Ser^{160}-Arg-X-X-Ser) for GSK-3β] and another MBP peptide M107 (positions 107–117: GLSLSRFSWAG) lacking a phosphorylation site for GSK-3β were obtained from Thermo Fisher Scientific (Ulm, Germany).

Assay for GSK-3β Activity—The *in vitro* assay for GSK-3β activity was performed, as described in previous reports (*1*, *4*). Briefly, purified MBP or MBP peptide (2 μg each) was incubated at 30°C in a total volume of 50 μl containing 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 3 mM Mn^{2+}, GSK-3β (~50 ng) in the presence or absence of phospholipids (PI or PS), SH or CH-3S. The reaction was initiated by the addition of 5 μM [γ-^{32}P]ATP (500 cpm/pmol) and continued for 30 min. After phosphorylation of MBP or synthetic MBP peptides, the reaction was terminated by the addition of 0.5 ml of 20% trichloroacetic acid (TCA). The precipitate was subjected to SDS–PAGE and the ^{32}P-labelled MBP was visualized by autoradiography. After phosphorylation of MBP peptides, the reaction was terminated by the addition of 0.5 ml 20% TCA and 0.25 ml 0.1 M sodium pyrophosphate containing BSA (~1 mg/ml) and 10 mM EDTA. The TCA-insoluble precipitates were trapped on a glass membrane filter (Advantec GF/75, Tokyo, Japan), and then washed successively with 2% TCA and 100% ethanol. After drying, the ^{32}P-radioactivity on the membrane filter was measured with a liquid scintillation spectrophotometer, as described in our previous reports (*7*–*12*).

Determination of the Binding Affinities of MBP and Synthetic MBP Peptides with PI and SH by a QCM—The binding affinities were determined by using a quarts crystal microbalance (QCM, Initium, Tokyo, Japan), as described in our previous reports (*10*, *11*). Briefly, MBP or synthetic MBP peptides (2 μg each) were indirectly immobilized on a QCM plate by aminocoupling techniques. The plate was soaked in 8 ml of 40 mM Tris–HCl (pH 7.6) containing 0.15 M NaCl and 200 μl blocking reagent (Initium, Tokyo, Japan) at 30°C until equilibrium was attained. Three compounds (sulfatide, CH-3S and PI) were prepared as 10 mg/ml or 10 mM stock solutions in dimethyl sulfoxide. These three stock solutions were used for GSK-3β assay and the binding assay with MBP as well as MBP peptides after dilution with distilled water. Eight microlitres of 10 mg/ml heparin, 10 mM sulfatide, 10 mM CH-3S or 10 mg/ml PI solutions were added to the buffer in the cuvette. The resonance frequency of the QCM was defined as the 0 position after equilibrium. The frequency change in the QCM responding to SH or PI was recorded for specific time points.

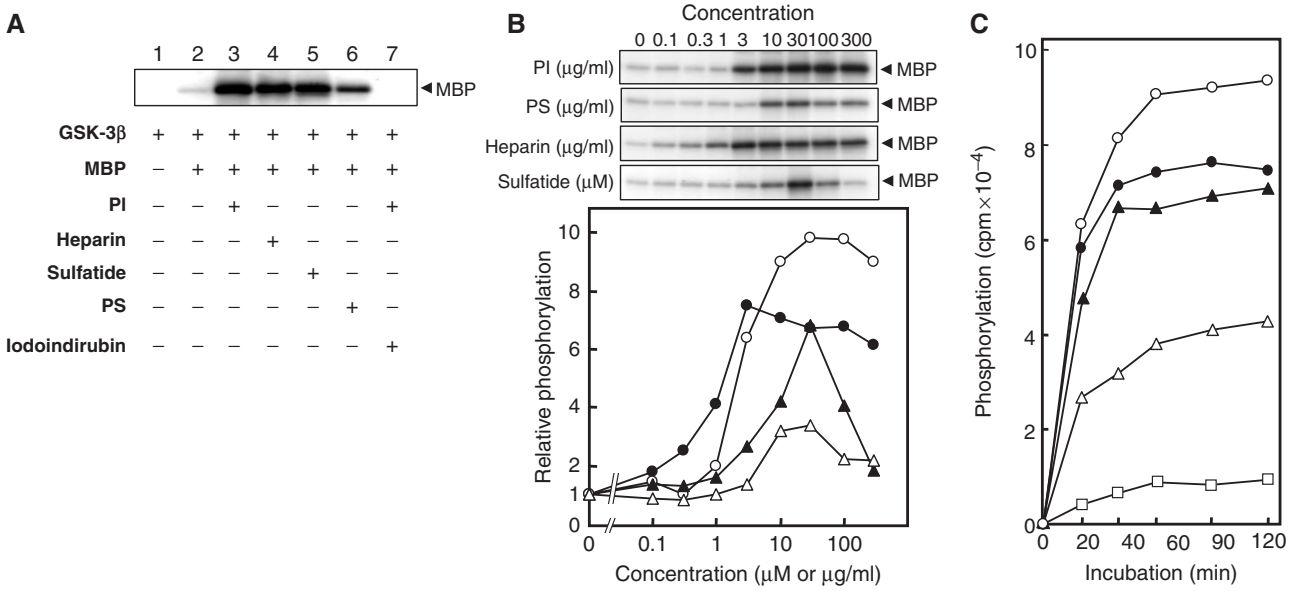

Fig. 1. **Characterization of two phospholipids (PI and PS) and SH as effective stimulators for the GSK-3β-mediated phosphorylation of MBP *in vitro*.** (A) MBP (~2 μg) was incubated for 30 min at 30°C with GSK-3β (~50 ng) and 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated compounds [PI, PS, heparin, sulfatide and iodoindirubin (a specific GSK-3β inhibitor)]. ^{32}P-Labelled MBP in the reaction mixtures was detected by autoradiography after SDS–PAGE. Lane 1, GSK-3β alone; lane 2, MBP incubated with GSK-3β; lane 3, MBP incubated with GSK-3β in the presence of 30 μg/ml PI, 3 μg/ml heparin (lane 4), 30 μM sulfatide (lane 5) or 30 μg/ml PS (lane 6); and lane 7, MBP (~2 μg) incubated with GSK-3β in the presence of 30 μg/ml PI and 1 μM iodoindirubin. (B) Both MBP and GSK-3β were incubated for 30 min at 30°C with 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of either PI (open circle), heparin (filled circle), sulfatide (filled triangle) or PS (open triangle). Each autoradiogram was scanned with a spectrophotometer and the degree of MBP phosphorylated by GSK-3β was determined by densitometer. The phosphorylation rate 1 represents the phosphorylation of MBP by GSK-3β in the absence of these four compounds (PI, PS, heparin and sulfatide). (C) The phosphorylation kinetics of MBP by GSK-3β in the absence (open square) or presence of one of these four compounds [30 μg/ml PI (open circle), 3 μg/ml heparin (filled circle), 30 μM sulfatide (filled triangle) or 30 μg/ml PS (open triangle)] were determined after incubation for the indicated periods at 30°C. ^{32}P-Labelled MBP in the reaction mixtures was detected by SDS–PAGE. MBP band visualized by CBB staining on the gel and the band corresponding to MBP was excised. The [^{32}P]radioactivity of ^{32}P-labelled MBP in gel pieces was determined with a liquid scintillation counter, as described in 'Materials and Methods' section.

Two-Dimensional Phosphopeptide Mapping of ^{32}P-Labelled MBP in vitro—After full phosphorylation of MBP by GSK-3β with 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the absence or presence of a modulator, the ^{32}P-labelled MBP band was visualized by CBB R-250 staining on the gel and the band corresponding to MBP was excised, as described originally by Kurzer *et al*. (*34*). Briefly, the gel slices containing ^{32}P-labelled MBP were incubated overnight at room temperature in 1 ml of 50% acetonitrile containing 50 mM ammonium bicarbonate. The destained gel slices were incubated with 100% acetonitrile for 30 min at room temperature, and then dried by Speed-Vac. 30 μl trypsin (~0.1 μg/ml) in 50 mM ammonium bicarbonate was added to each gel slice and incubated for 45 min at 0°C. The gel slices were rehydrated in 60 μl of 50 mM ammonium bicarbonate for 18 h at 37°C. The dried sample was resuspended in pure water and spotted onto a thin layer cellulose plate. First-dimensional thin layer high-voltage electrophoresis was performed at 1,000 V for 60 min in electrophoresis buffer (pH 1.9) containing 7.8% acetic acid and 2.2% formic acid. The second dimension was chromatographically separated using phosphochromatography buffer consisted of *n*-butanol (37.5%, v/v), pyridine (25%, v/v) and glacial acetic acid (7.5%, v/v) in deionized water.

RESULTS

Stimulatory Effects of SH, PI and PS on the GSK-3β-Mediated Phosphorylation of MBP in vitro—We reported recently that both sulfatide and CH-3S highly stimulate the CK1-mediated phosphorylation of sulfatide- and CH-3S-binding proteins, including MBP and TP, *in vitro* (*12*). Similar stimulatory effects of these four agents on the GSK-3β-mediated phosphorylation of MBP were examined *in vitro*. It was found that (i) MBP was slightly phosphorylated by GSK-3β in the absence of PI and SH (lane 2, Fig. 1A); (ii) the phosphorylation of MBP was highly stimulated when incubated with these four agents (lanes 4–6) and (iii) this phosphorylation was completely inhibited by 1 μM 5-iodoindirubin-3′-monoxime (a specific GSK-3β inhibitor) (lane 7, Fig. 1A). However, CH-3S functioned as a poor stimulator for the GSKβ-mediated phosphorylation of MBP, but PA had no effect on phosphorylation (data not shown). As shown in Fig. 1B, maximum phosphorylation was observed with 30 μg/ml PI, 3 μg/ml heparin, 30 μM sulfatide and 30 μg/ml PS, respectively. The phosphorylation of MBP by GSK-3β increased linearly up to 20 min, and reached a plateau in 90 min (Fig. 1C). Among these four agents, PI was the most effective modulator for the GSK-3β-mediated phosphorylation of MBP *in vitro* (Fig. 1C). The phosphoamino acid

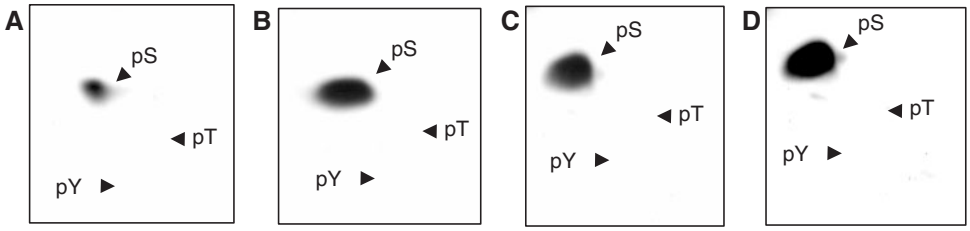

Fig. 2. **Detection of phosphoamino acids of MBP fully phosphorylated by GSK-3β in the presence of PI or SH.** The ^{32}P-labelled phosphoamino acids of MBP fully phosphorylated by GSK-3β in the absence (A) or presence of 30 μg/ml PI (B), 3 μg/ml heparin (C) or 30 μM sulfatide (D) were detected by two-dimensional TLC electrophoresis and followed by autoradiography, as described in 'Materials and Methods' section.

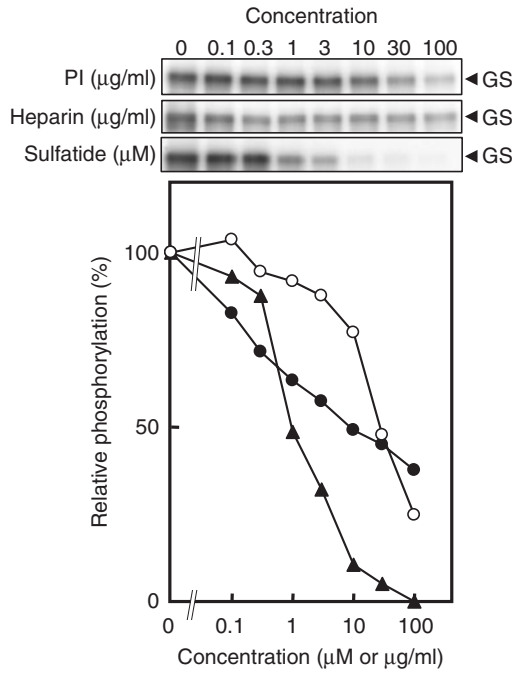

Fig. 3. **The inhibitory effects of PI and SH on the GSK-3β-mediated phosphorylation of GS.** GS (~2 μg) was incubated for 30 min at 30°C with GSK-3β (~50 ng) and 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of either PI (open circle), heparin (filled circle) or sulfatide (filled triangle). ^{32}P-Labelled GS in the reaction mixtures was detected by autoradiography after SDS–PAGE. Hundred percent represents the phosphorylation of GS by GSK-3β in the absence of these three compounds (PI, heparin and sulfatide).

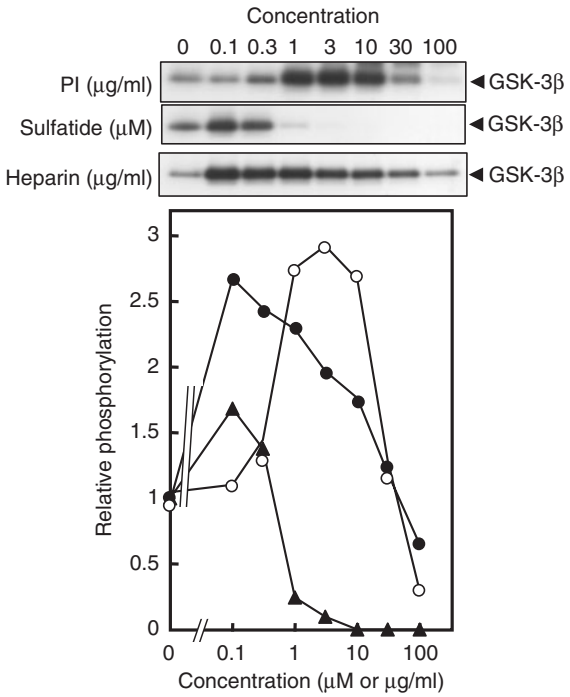

Fig. 4. **The stimulatory effects of PI and SH on autophosphorylation of GSK-3β.** GSK-3β (~50 ng) was directly incubated for 30 min at 30°C with 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of either PI (open circle), heparin (filled circle) or sulfatide (open triangle). ^{32}P-Phosphorylated GSK-3β in the reaction mixtures was detected by autoradiography after SDS–PAGE.

analysis under optimum assay conditions revealed that GSK-3β preferentially phosphorylated Ser-residues on MBP (Fig. 2). However, the phosphorylation of GS by GSK-3β was inhibited by SH as compared to PI (Fig. 3). These results suggest that (i) SH and two phospholipids (PI and PS) act as effective stimulators for the GSK-3β-mediated phosphorylation of MBP, but inhibitors for GS and (ii) the stimulatory effect of PI is higher than those observed with other agents *in vitro*.

Stimulatory Effects of PI and SH on Autophosphorylation of GSK-3β—To investigate the stimulatory effects of SH and PI on autophosphorylation of GSK-3β *in vitro*, the kinase was directly incubated with 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the absence or presence of an agent under autophosphorylation conditions. Maximum autophosphorylation of GSK-3β was observed when incubated with 3 μg/ml PI, 0.1 μg/ml heparin and 0.1 μM sulfatide, respectively (Fig. 4). Both PI and heparin stimulated 2.5- to 3.0-fold phosphorylation of GSK-3β, whereas sulfatide stimulated 1.7-fold, as compared with the control (absence of the stimulators, Fig. 4). However, no significant stimulation was observed with CH-3S (data not shown). These results put together suggest that both PI and SH, but not CH-3S, are effective stimulators for autophosphorylation of GSK-3β *in vitro*.

Kinetics of SH- and PI-Induced Autophosphorylation of GSK-3β and MBP Phosphorylation by GSK-3β—To understand the SH- and PI-induced high stimulation of

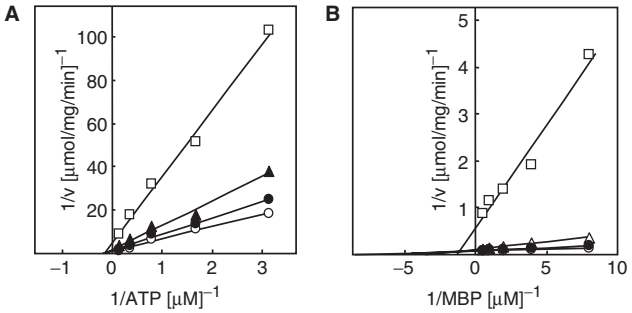

Fig. 5. **The kinetics for autophosphorylation of GSK-3β and for the GSK-3β-mediated phosphorylation of MBP in the presence of PI or sulfatide.** (A) The autophosphorylation kinetics for GSK-3β were determined by incubation for 30 min at 30°C with the indicated concentrations of [γ-^{32}P]ATP in the presence or absence of the optimum concentrations of these three stimulatory compounds *in vitro*. ^{32}P-Labelled GSK-3β in the reaction mixtures was determined by the membrane filter method, as described in the 'Materials and Methods' section. (B) The kinetics for the GSK-3β-mediated phosphorylation of MBP were determined after the incubation (for 30 min at 30°C) of various concentrations of MBP and GSK-3β (~50 ng) in the presence or absence of the optimum concentrations of these three compounds *in vitro*. The radioactivity of ^{32}P-phosphorylated MBP in the reaction mixtures was determined by a liquid scintillation counter, as described in the 'Materials and Methods' section. Lineweaver–Burk plots of ATP or MBP for GSK-3β in the absence (open square) or presence of 30 μg/ml PI (open circle), 3 μg/ml heparin (filled circle) or 30 μM sulfatide (filled triangle).

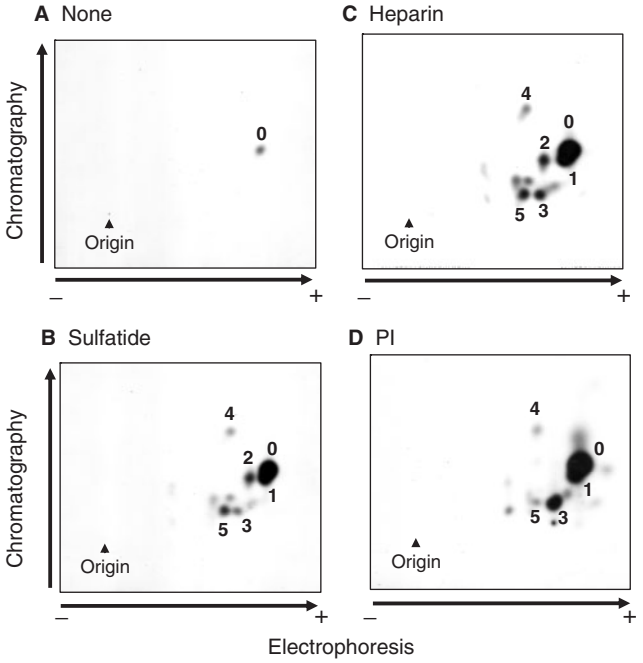

Fig. 6. **Peptide mapping of MBP phosphorylated by GSK-3β the presence of either SH or PI.** Both MBP (~2 μg) and GSK-3β (~50 ng) were incubated with 5 μM [γ-^{32}P]ATP (500 cpm/pmol) for 120 min at 30°C in the presence or absence of 3 μg/ml heparin, 30 μM sulfatide or 30 μg/ml PI. ^{32}P-Labelled MBP was resolved on an SDS–12.5% PAGE minigel. After digestion of ^{32}P-labelled MBP with trypsin in the gel, the resulting polypeptides were separated by TLC electrophoresis in the buffer (pH 1.9; first dimension) and chromatography buffer (second dimension). The origin indicated is the position where the phosphopolypeptides are loaded. (A) without stimulators; (B) in the presence of 3 μg/ml heparin; (C) 30 μM sulfatide or (D) 30 μg/ml PI.

autophosphorylation of GSK-3β and the GSK-3β-mediated of MBP, the phosphorylation kinetics of such reactions were determined. No significant change in the K_m value for ATP in autophosphorylation of GSK-3β was observed, whereas the V_{max} value for ATP shifted considerably (Fig. 5A). In the presence of PI, an increased autophosphorylation appears to be due to decrease the K_m value for GSK-3β, which shifted from 6.32 to 5.51 μM and the V_{max} value for ATP shifted from 0.21 to 0.55 μmol/mg/min in the presence of 0.1 μM sulfatide, 0.79 μmol/mg/min in the presence of 0.1 μg/ml heparin and 0.97 μmol/mg/min in the presence of 2.0 μg/ml PI, respectively (Fig. 5A). In the presence of 30 μg/ml PI, the K_m value for MBP of GSK-3β shifted from 0.82 to 0.13 μM and the V_{max} value shifted from ~1.85 to 17.48 μmol/mg/min (Fig. 5B). Similar shifts in the K_m and V_{max} values were also seen in the presence of heparin or sulfatide (Fig. 5B).

SH- and PI-Induced Unique Phosphorylation Sites on MBP for GSK-3β—To characterize the SH- and PI-induced high phosphorylation of MBP by GSK-3β *in vitro*, phosphopeptide mapping was done after incubation for 120 min at 30°C of MBP with GSK-3β and 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence or absence of one of these three stimulators. ^{32}P-Labelled MBP was subjected to SDS–PAGE, after the MBP band was exhaustively in-gel digested with trypsin. Autoradiography detected only one phosphorylated fragment (spot 0) when phosphorylation was carried out in the absence of any stimulators (Fig. 6A). Two major spots (spots 0 and 1) and three minor spots (spots 2–5) were detected in ^{32}P-labelled MBP extensively phosphorylated by GSK-3β in the presence of 30 μM sulfatide *in vitro* (Fig. 6B). Comparison of the phosphopeptide patterns derived from sulfatide-, heparin- and PI-treated samples revealed some interesting differences. Three common major spots (0, 1 and 3) were detected in MBP fully phosphorylated by GSK-3β in the presence of 30 μM PI (Fig. 6D). Under the same experimental conditions, these three spots were also detectable in the presence of sulfatide or heparin (Fig. 6B and C). The intensity of spot 5 in sulfatide- and heparin-treated samples was much higher than that observed with PI-treated sample, whereas spot 4 was commonly inducible by these three stimulators. All these results put together suggest that the direct binding of these acidic stimulators to MBP may expose unique potent phosphorylation sites for GSK-3β *in vitro*.

MBP-Derived Peptides for the Effective Substrates of GSK-3β—To identify the consensus phosphorylation sites on MBP, the basic protein was digested with V8 protease and the cleaved peptides were separated by C18 reverse-phase column chromatography (Fig. 7A). A number of peptides were generated by protease treatment, as determined by tricine–SDS–PAGE analysis of individual fractions (Fig. 7B). The effective substrate activities for GSK-3β in the individual fractions were detected when incubated for 30 min at 30°C with the kinase and

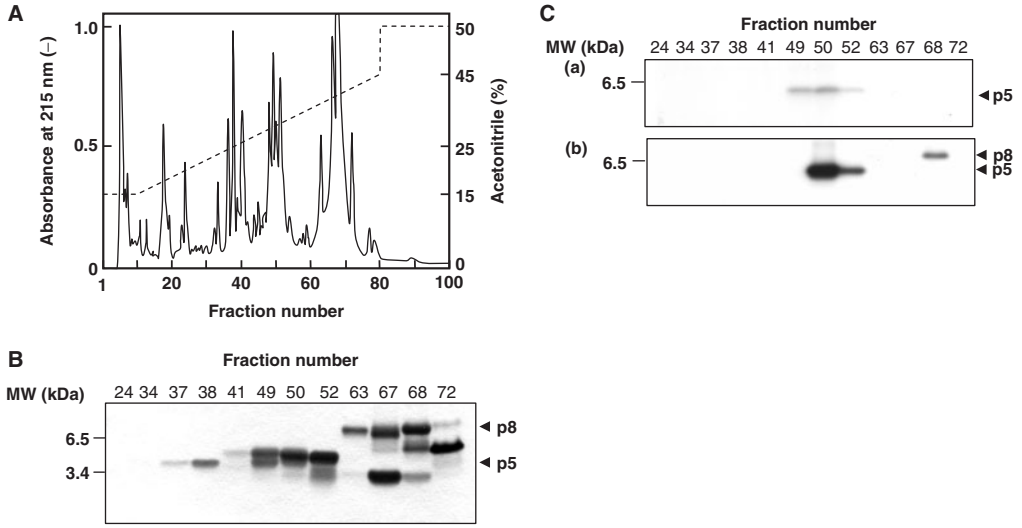

Fig. 7. **Separation of the fragments cleaved from MBP digested with V8 protease by TSK-gel column chromatography and detection of MBP fragments phosphorylated by GSK-3β in the presence of PI.** (A) MBP (~0.1 mg) was digested with V8 protease (~2 μg) overnight at 37°C. The fragments formed were applied onto TSK-gel ODS 120T HPLC column, which was previously equilibrated with 0.1% trifluoro-acetic acid. Elution was carried out with a linear gradient from 0 to 50% acetonitrile in 0.1% trifluoro-acetic acid. (B) After drying by Speed–Vac concentrator, the MBP fragments were detected by CBB R-250 staining after tricine–SDS–PAGE. (C) The MBP fragments in the indicated fractions were incubated separately with GSK-3β (~50 ng) and 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the absence (a) or presence (b) of 10 μg/ml PI. After incubation 30 min at 30°C, ^{32}P-labelled polypeptides in the reaction mixtures were detected by autoradiography following tricine–SDS–PAGE.

5 μM [γ-^{32}P]ATP in the presence of 10 μg/ml PI. As shown in Fig. 7C, two MBP fragments (p5 in fractions 39–52 and p8 in fractions 63–68) were highly phosphorylated. Only Thr-residues on p8 and Ser-residues on p5 were the preferred phosphate acceptor sites for GSK-3β, respectively, and the phosphorylation of these two MBP fragments by GSK-3β was highly stimulated by PI (Fig. 8).

The partial N-terminal amino acid sequences and Peptide Mass Finger Print Analysis revealed the residues (positions 38–118 of MBP) of p8 and the residues (positions 119–169) of p5, respectively. Therefore, we prepared two synthetic peptides [M86 (positions 86–98 of p8) containing a potent GSK-3β phosphorylation site (Thr-Pro-Arg-Thr^{97}-Pro) and peptide M156 (positions 156–169 of p5) containing a potent GSK-3β phosphorylation site (Ser^{160}-Arg-X-X-Ser)] as phosphate acceptors for the kinase. As expected, GSK-3β-mediated phosphorylation of these two MBP peptides were highly dependent on the presence of SH or PI (Fig. 9). Under the given experimental conditions, PI had a high stimulatory effect, as compared with SH *in vitro*.

Binding Affinities of SH and PI with MBP and Two Synthetic MBP Peptides—The earlier results (Figs 1, 5 and 7) and an earlier report (*32*) regarding the *in vitro* binding of phospholipids to MBP suggest that the direct binding of PI and SH to MBP and two synthetic MBP peptides (M86 and M156) may induce their high phosphorylation by GSK-3β *in vitro*. To investigate a possibility, the binding affinities of PI and SH with MBP and these two peptides were measured using a QCM. Higher binding affinity of SH, in comparison with PI, was observed with MBP, but not with GS (Fig. 10A). Furthermore, the higher binding affinities of M86 and

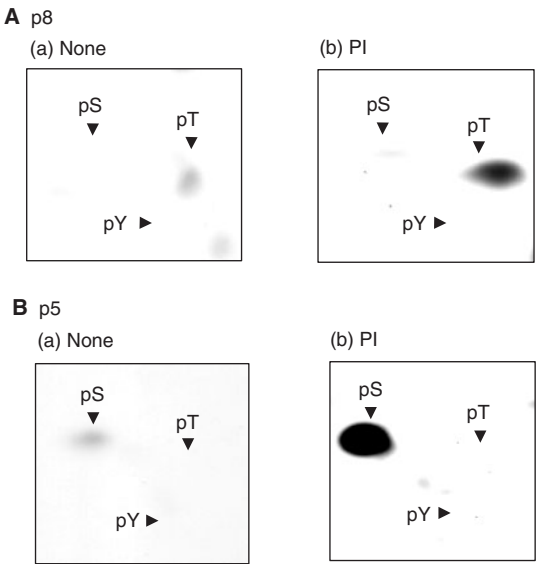

Fig. 8. **Detection of phosphoamino acids of two MBP fragments (p8 and p5) fully phosphorylated by GSK-3β in the presence or absence of PI.** The ^{32}P-labelled phosphoamino acids of two MBP fragments (p8 and p5) fully phosphorylated by GSK-3β in the absence (A) or presence of 10 μg/ml PI (B) were analysed by two-dimensional TLC electrophoresis, as described in 'Materials and Methods' section.

M156 with PI and SH were observed, as compared with another MBP peptide (M107, positions 107–117 of MBP), which used as a negative control (Fig. 10B and C). These results suggest that the direct binding of SH and PI to the

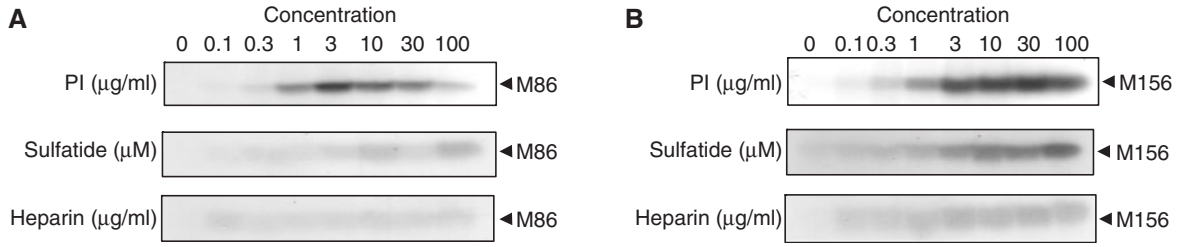

Fig. 9. **The stimulatory effects of PI and SH on the GSK-3β-mediated phosphorylation of M87 and M156.** Two synthetic MBP peptides [(A) M86 (positions 86–98) and (B) M156 (positions 156–169), ~2 μg each) were separately incubated for 30 min at 30°C with GSK-3β (~50 ng) and 5 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of either PI, heparin or sulfatide. Phosphorylation of these two synthetic MBP peptides by GSK-3β was detected by autoradiography after tricine–SDS–PAGE, as described in 'Materials and Methods' section.

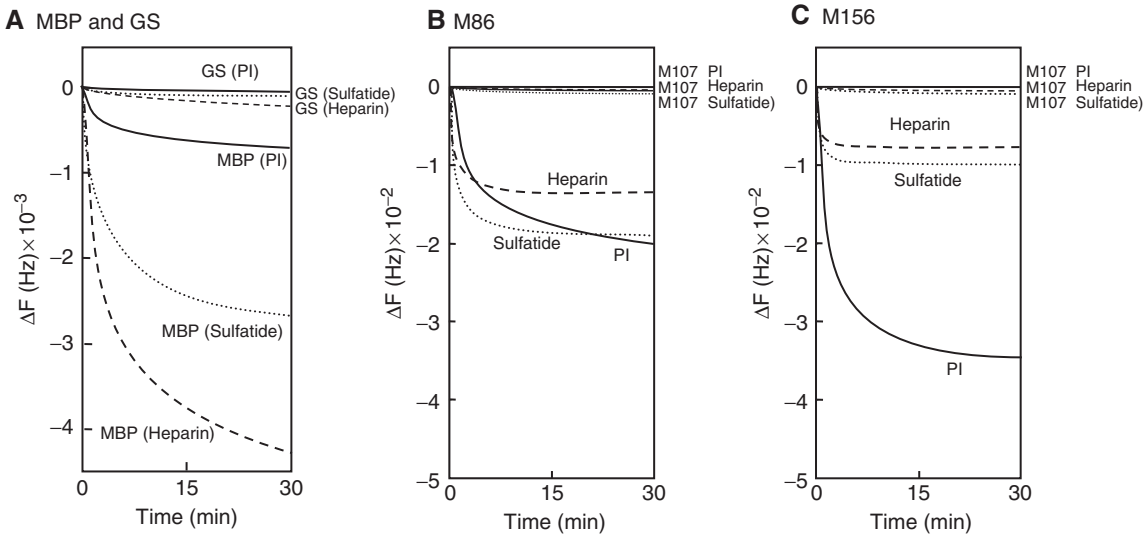

Fig. 10. **The binding affinities of PI and SH with MBP and two synthetic MBP peptides (M86 and M156).** (A) The binding affinities of MBP with either 10 μg/ml PI, 10 μg/ml heparin or 10 μM sulfatide were determined, using a QCM (Initium, Tokyo), in 8 ml of 40 mM Tris–HCl (pH 7.6) at 30°C. GS was used as a negative control for the binding affinity of PI and SH. (B) Binding affinities of 10 μg/ml PI, 10 μg/ml heparin (dashed line) or 10 μM sulfatide (dotted line) with two synthetic MBP fragments [M86 (B) and M156 (C), ~2 μg each] in comparison with MBP peptide M107 as a negative control.

arginine (R)/lysine (K)-residues in the consensus phosphorylation sites for GSK-3β on these two synthetic MBP peptides may induce their high phosphorylation by GSK-3β *in vitro*.

DISCUSSION

In the present study, we found that the direct binding of SH and PI to GSK-3β and two SH-binding proteins (MBP and TP) may induce the high stimulation of GSK-3β autophosphorylation and their phosphorylation by GSK-3β *in vitro*. This conclusion is supported by our experimental results that (i) the GSK-3β-mediated phosphorylation of MBP was highly stimulated in the presence of SH or two phospholipids (PI and PS), but not PA (Fig. 1B); (ii) PI as effective as heparin, as compared with sulfatide, highly stimulated autophosphorylation of GSK-3β (Fig. 4); (iii) SH had a binding affinity with MBP, but not GS (Fig. 10A); (iv) polypeptide mapping detected three major spots (0, 1 and 3) of MBP fully phosphorylated by GSK-3β in the presence of SH or PI (Fig. 6B–D) and (v) these four potent acidic stimulators induced a significant increase in the affinity of GSK-3β against MBP (substrate) and also induced a high increase in the V_{max} value for MBP of GSK-3β (Fig. 5B). Under our experimental conditions, heparin extremely stimulated the GSK-3β-mediated phosphorylation of TP (data not shown), as has been demonstrated in earlier reports (*35–37*), whereas these three acidic stimulators inhibited the GSK-3β-mediated phosphorylation of GS in the dose-dependent manner (Fig. 3).

Confirmation here of the GSK-3β-mediated phosphorylation of two MBP fragments (p8 and p5) and two synthetic MBP peptides (M86 and M156, containing a potent phosphorylation site for GSK-3β) and determination of their binding affinities with PI and SH revealed that (i) these four peptides (p8, p5, M86 and M156) functioned as effective phosphate acceptors for GSK-3β in response to PI, as compared with SH (Figs 7C, 8 and 9) and (ii) the R/K-rich residues in the consensus phosphorylation sites for GSK-3β on two MBP peptides (M86 and

M156) may be responsible for the binding of these four acidic stimulators *in vitro*. This explanation is strongly supported by evidence that both phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-biphosphate can directly interact with the R/K-rich residues on MBP (*32*). Therefore, it was concluded that that both PI and SH may function as effective stimulators for the GSK-3β-mediated phosphorylation of MBP through their selective binding to the R/K-rich residues near the consensus phosphorylation sites (Ser-residues) on the basic protein. Indeed, we detected only Ser-residues of MBP molecule fully phosphorylated by GSK-3β in the presence of these acidic stimulators *in vitro* (Fig. 2), although Yu and Yang (*31*) reported that GSK3 phosphorylates Thr-residues in preference to Ser-residues on MBP in the absence of any stimulators *in vitro*. It seems likely that the dissimilarity between their report and our results, using GSK-3β in the presence of PI or HS, may be due to different enzymes and assay conditions with or without suitable stimulators *in vitro*.

A similar sulfatide-induced high stimulation was observed with the CK1-mediated phosphorylation of several sulfatide- and CH-3S-binding proteins (*7–12*) *in vitro*. The stimulatory effects of two phospholipids (PI and PS) and two sulfated lipids (sulfatide and CH-3S) on the phosphorylation of MBP by GSK-3β and CK1 may be resemble, because both protein kinases can recognize phospho-Ser or phospho-Thr nearby phosphorylation sites on their substrate proteins. However, it is presumed that the stimulatory mechanisms of protein phosphorylation by these two protein kinases are different, because no significant effects of PI and sulfatide on autophosphorylation of CK1 are detected *in vitro*.

It has been reported that MBP binds to F-actin as well as G-actin, and that this binding induces G-actin polymerization into actin filaments under non-polymerizing conditions (*30*). MBP also binds to Ca^{2+}-calmodulin Ca^{2+}-dependently (*26*, *38*), and this interaction causes the dissociation of MBP from actin filaments (*38*) and reduction of the affinity of actin to polymerize with MBP *in vitro* (*39*). In addition, MBP-deficient shiver oligodendrocytes, which are lacking the MBP gene, produce membrane sheets with abnormally assembled microtubules and actin-based structures in axon-free cultures (*40*). Thus, MBP is an important factor in the formation of normal cytoskeleton in oligodendrocytes. Therefore, the high phosphorylation of MBP by GSK-3β, as well as by four other protein kinases (A-kinase, C-kinase, MAP-kinase and CK1) may be implicated in the physiological interaction between MBP and cytoskeletal proteins (microtubulin, actin and calmodulin) at the cellular level. Recently, we reported that both sulfatide and CH-3S inhibit the phosphorylation of several sulfatide- and CH-3S-binding proteins, including MBP, FGF-BP and C3a, by A-kinase in a dose-dependent manner through their direct binding to these functional proteins *in vitro* (*9–11*). Similar inhibitory effects of these two agents were also observed with the C-kinase-mediated phosphorylation of C3a and MBP *in vitro* (unpublished observation). Therefore, our experimental results presented here suggest that the phosphorylation of MBP by at least two protein kinases (A-kinase and C-kinase) may be selectively prevented at higher accumulated levels of SH in the brain.

To clearly understand the biological significance of the GSK-3β-mediated phosphorylation of various SH-binding proteins, including MBP and TP, in the high levels of two phospholipids (PI and PS) and SH, further analytical studies are required to (i) detect high phosphorylation of cellular SH-binding proteins by GSK-3β in suitable intact cells and (ii) characterize the regulatory mechanisms of the physiological functions of novel cellular PI- and SH-binding proteins, which may be preferentially phosphorylated by GSK-3β in high-aged rat brains.

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