A Novel Consensus Phosphorylation Motif in Sulfatide- and Cholesterol-3-sulfate-Binding Protein Substrates for CK1 in Vitro

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A novel phosphorylation motif for casein kinase 1 (CK1) in response to two sulfated lipids [sulfatide and cholesterol-3-sulfate (CH-3S)] was determined, using three functional proteins [myelin basic protein (MBP), tau protein (TP), and RhoA (a small GTPase)] and five synthetic MBP peptides as phosphate acceptors for the kinase in vitro. It was found that (i) MBP, p8 (positions 38–118) cleaved from MBP, and a synthetic peptide M103 were effectively phosphorylated by CK1 in the presence of SCS; (ii) sulfatide in comparison with CH-3S highly enhanced autophosphorylation of CK1; (iii) SCS had a high binding affinity with MBP and peptide M103, but not other MBP peptides lacking K-G-R; and (iv) a novel consensus phosphorylation motif (K/R-X-K/R-X-X-S/T) for CK1 was identified among several SCS-binding proteins (SCS-BPs) and three CK1 isoforms (δ, ε, and γ). The binding of SCS to two basic brain proteins (MBP and TP) resulted in the high stimulation of their phosphorylation by three CK1 isoforms (α, δ, and ε), but not CK1γ. In contrast, an acidic protein (RhoA) was effectively phosphorylated by CK1δ in the presence of SCS, and also highly phosphorylated by CK1γ in the presence of sulfatide. Our results presented here suggest that (i) sulfatide may function as an effective phosphate acceptor for autophosphorylation of CK1; and (ii) cellular SCS-binding proteins, containing novel phosphorylation motifs for CK1, may be preferentially phosphorylated by CK1 with isoform specificity at the highly accumulated level of SCS in the brain.

Key words casein kinase 1; myelin basic protein; tau protein; RhoA; sulfatide; cholesterol-3-sulfate

Casein kinase 1 (CK1: EC 2.7.11.1) is a ubiquitous and highly conserved 2nd messenger-independent multi-functional serine (Ser)/threonine (Thr) protein kinase (approx. 37–51 kDa), which is widely distributed in living organisms from yeast to mammals.1–4) Two consensus phosphorylation motifs [D/E-X-X-S/T and S(p)/T(p)-X-X-S/T] for CK1 on various functional acidic proteins have been characterized.3) A recent review4) noted the following: (i) at least seven CK1 isoforms (α, β, γ1–3, δ, and ε) and their various splice variants have been identified in a variety of mammalian cell sources; (ii) the CK1 family members phosphorylate various functional proteins, such as cellular enzymes, transcriptional proteins, cytoskeletal and non-cytoskeletal proteins, viral proteins and oncogene products, in vitro and in vivo; and (iii) CK1 may play an important role in the regulatory mechanisms involved in cell proliferation, Wnt signaling and circadian rhythms. However, the precise physiological significance of the CK1-mediated phosphorylation of various functional and regulatory factors involved in these biological processes remains to be elucidated.

Recently, we reported that (i) two sulfated lipids [sulfatide and cholesterol-3-sulfate (CH-3S), SCS] highly stimulate the CK1-mediated phosphorylation of several basic proteins, such as high mobility group protein 1 (HMG1),5) C-kinase-CK1-mediated phosphorylation of several basic proteins, and cholesterol-3-sulfate (CH-3S), SCS] highly stimulate the CK1δ-mediated phosphorylation of several basic proteins, including these two basic brain proteins [MBP and tau protein (TP)], and the novel phosphorylation motifs for CK1 among SCS-BPs remain to be elucidated.

Therefore, the present in vitro study has been carried out to (i) characterize SCS as a potent stimulator for autophosphorylation of CK1δ and the CK1δ-mediated phosphorylation of three functional proteins [MBP, TP and RhoA (a small GTPase)] and five synthetic MBP peptides; (ii) identify a novel phosphorylation site for CK1δ in several SCS-BPs; and (iii) determine the substrate specificities of four CK1 isoforms (α, δ, ε and γ) using TP (a basic protein, pI=approx. 8.2) and RhoA (an acidic protein, pI=approx. 5.8) as their phosphate acceptors in the presence of SCS.

MATERIALS AND METHODS

Chemicals [γ-32P]ATP (3000 Ci/mmol) was obtained from GE Healthcare Biosciences UK Limited (Buckinghamshire, HP7 9NA, England); dithiothreitol (DTT) from Wako Pure Chemical Ind. (Osaka, Japan); bovine serum albumin (BSA), CK1-7 (a CK1 specific inhibitor), sulfatide (galactosyl-ceramide-3-O-sulfate, bovine brain), cholesterol-3-sulfate (CH-3S) and phosphatidylinositol (PI) from Sigma Chemical (St. Louis, U.S.A.).

CK1 Substrate Proteins and Synthetic MBP Peptides

Purified bovine MBP, recombinant human TP (TP-441) and α-casein were obtained from Sigma Chemical (St. Louis, U.S.A.); and human recombinant RhoA from Cytoskeleton Inc. (Denver, U.S.A.). Five synthetic MBP peptides [M86 (positions 86–98), M88 (positions 88–104), M103 (positions 103–117), M103/S109A (replacement of Ser-109 with...
Ala) and M107 (positions 107—117) were obtained from Thermo Fisher Scientific (Ulm, Germany).

**CK1 Isoforms** Human recombinant CK1α (pI= approx. 9.6, specific activity: 16670 units/mg) and CK1ε (pI=approx. 9.7, specific activity: 33330 units/mg) were obtained from Oxford Biomedical Research (Oxford, MI, U.S.A.); human recombinant CK1γ2 (pI= approx. 9.1) specific activity: 21438 units/mg) from Upstate, Inc. (Lake Placid, NY, U.S.A.); and rat recombinant CK1δ (pI= approx. 9.8, specific activity: 17500 units/mg) and purified rat liver CK1δ (pI= approx. 9.8, specific activity: 17500 units/mg) from Promega Co. (Madison, U.S.A.).

**Assay for the Phosphorylation of SCS-BPs and Synthetic MBP Peptides by CK1 in Vitro** The in vitro assay for CK1 was performed, as originally described in our previous reports. Briefly, three SCS-BPs (MBP, TP and RhoA) or five synthetic MBP peptides (approx. 2 μg each) were separately incubated for 30 min at 30°C in standard reaction mixtures (50 μl), comprising 40 mM Tris-HCl (pH 7.6), 2 mM DTT, 3 mM MnCl₂⁺, CK1δ or other CK1 isoforms (approx. 5 units each) and 5 μM [γ-32P]ATP (500 cpm/pmol) in the presence or absence of either sulfatide or CH-3S.

**2D-P-Labeled SCS-BPs or MBP peptides in the reaction mixtures were detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography, as described in our previous reports.** After incubation (for 30 min at 30°C), the phosphorylation of these protein substrates by CK1 was arrested by the addition of 0.5 ml 20% trichloroacetic acid (TCA) and 0.25 ml 0.1M sodium pyrophosphate containing BSA (approx. 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 32P-radioactivity on the membrane filter was measured with a liquid scintillation spectrometer, as described in our previous reports.7—9,

**Two-Dimensional Phosphopeptide Mapping of 32P-Labeled TP in Vitro** After full phosphorylation of TP by CK1δ with 5 μM [γ-32P]ATP (500 cpm/pmol) in the absence or presence of 3 μM sulfatide, the 32P-labeled TP band was excised, as visualized by Coomassie brilliant blue (CBB) R-250 staining and then dried by Speed-Vac. 30 μl of 100% acetonitrile was added to each gel slice 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 1000 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.

**Determination of the Binding Affinities of MBP and MBP Peptides with SCS by a QCM** The binding affinities of MBP or synthetic MBP peptides with sulfatide or CH-3S were determined by using a quartz crystal microbalance (QCM; Iinitium, Tokyo, Japan), as described in our previous reports. Briefly, MBP or MBP peptides (approx. 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 at 30°C until equilibrium was attained. Then, 8 μl of 10 mM sulfatide or 10 mM CH-3S (final concentration: 10 μM) 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 SCS was recorded for specific time points.

**RESULTS**

**Characterization of SCS as a Potent Stimulator for the CK1δ-Mediated Phosphorylation of MBP in Vitro** In our initial experiments for the biochemical characterization of the CK1δ-mediated phosphorylation of MBP in vitro, it was confirmed that (i) no phosphorylation of MBP by CK1δ was observed in the absence of sulfatide (lane 2, Fig. 1A); (ii) CK1δ highly phosphorylated MBP in the presence of 10 μM sulfatide (lane 3); and (iii) this phosphorylation was completely inhibited by 1 μM CK1-7 (lane 4). The optimum concentration of sulfatide and CH-3S for the CK1δ-mediated phosphorylation of MBP was found to be approx. 10 μM (Fig. 1B). However, sulfatide inhibited the CK1δ-mediated phosphorylation of α-casein in a dose-dependent manner (Fig. 1B). The maximum phosphorylation of MBP by CK1δ in the presence of 10 μM sulfatide was about 1.7-fold higher than that observed with 10 μM CH-3S (Fig. 1B). In addition, higher binding affinity of sulfatide compared with CH-3S was observed with MBP, but not with BSA (Fig. 1C). These results suggest that SCS may function as an effective stimulator for the CK1δ-mediated phosphorylation of MBP through its direct binding to the basic protein in vitro.

**Stimulatory Effect of SCS on Autophosphorylation of CK1δ in Vitro** To examine the stimulatory effect of SCS on autophosphorylation of CK1 in vitro, rat liver CK1δ (a basic protein, pI= approx. 9.8) was directly incubated with 5 μM [γ-32P]ATP (500 cpm/pmol) in the absence or presence of an agent under autophosphorylation conditions. Maximum autophosphorylation of CK1δ was observed when incubated with 0.3 μM sulfatide (Fig. 2A). Sulfatide stimulated approx. 6.0-fold autophosphorylation of CK1δ, as compared with the control (absence of SCS), whereas no significant stimulation was observed with CH-3S (Fig. 2A). To understand the sulfatide-induced high stimulation of autophosphorylation of CK1δ, the phosphorylation kinetics was determined. In the presence of 0.3 μM sulfatide, the Kₘ value for ATP of CK1δ shifted from 0.48 to 0.34 μM and the Vₘₐₓ value for ATP shifted from 1.3 μmol/mg/min to 11.0 μmol/mg/min (Fig. 2B). These results suggest that sulfatide, but not CH-3S, directly interacts with CK1δ and also stimulates autophosphorylation of CK1δ in vitro.

**Identification of a Novel Phosphorylation Site for CK1 on MBP Peptides** To identify the consensus phosphorylation sites in MBP, the basic protein was digested with V8 protease and the cleaved peptides were separated by C18 re-
verse-phase column chromatography (Fig. 3A). A number of peptides were generated by protease treatment, as determined by trisine-SDS-PAGE analysis of individual fractions (Fig. 3B). The effective substrate activities for CK1\(\delta\) in the individual fractions were detected when incubated at 30 °C for 30 min with CK1\(\delta\) and 5 \(\mu\)M \(\gamma\)-\(^{32}\)P\(\text{ATP}\) in the presence of 3 \(\mu\)M sulfatide. As shown in Fig. 3C, two MBP fragments (p5 in fractions 39—52 and p8 in fractions 63—68) were highly phosphorylated. Our studies also revealed that Ser-residues in p8 and Ser-residues in p5 were the preferred phosphate acceptor sites for CK1\(\delta\), respectively, and the phosphorylation of these two MBP fragments by CK1\(\delta\) was highly stimulated in the presence of sulfatide (Fig. 3C). Further biochemical characterization revealed that the CK1\(\delta\)-mediated phosphorylation of p5, containing a potent phosphorylation motif (D/E-X-X-S/T) for CK1, was slightly stimulated by sulfatide in vitro (data not shown). Determination of the partial N-ter-

Fig. 1. Characterization of SCS as a Potent Stimulator for the CK1\(\delta\)-Mediated Phosphorylation of MBP \emph{in Vitro}

[A] After incubation (for 30 min at 30 °C) of MBP (approx. 2 \(\mu\)g) with CK1\(\delta\) (approx. 5 units) and 5 \(\mu\)M \(\gamma\)-\(^{32}\)P\(\text{ATP}\) (500 cpm/pmol), \(^{32}\)P-labeled MBP in the reaction mixtures was detected by SDS-PAGE followed by autoradiography. Lane 1, CK1\(\delta\) alone; lane 2, MBP incubated with CK1\(\delta\); lane 3, lane 2 + 10 \(\mu\)M sulfatide; and lane 4, lane 2 + 1 \(\mu\)M CK1-7 and 10 \(\mu\)M sulfatide. [B] Both MBP and CK1\(\delta\) were incubated for 30 min at 30 °C with 5 \(\mu\)M \(\gamma\)-\(^{32}\)P\(\text{ATP}\) (500 cpm/pmol) in the presence of the indicated concentrations of sulfatide (●) or CH-3S (○). The phosphorylation of \(\alpha\)-casein by CK1\(\delta\) was determined in the indicated concentrations of sulfatide (△). The rate 1 represents the maximum phosphorylation of MBP by CK1\(\delta\) in the presence of 10 \(\mu\)M sulfatide and the phosphorylation of \(\alpha\)-casein by CK1\(\delta\) in the absence of SCS. [C] The binding affinities of MBP (a) and BSA (b) with 10 \(\mu\)M sulfatide (—) or 10 \(\mu\)M CH-3S (····) were determined at room temperature, using a QCM.

Table 1. A Novel Consensus Phosphorylation Motif (R/K-X-R/K-X-X-S/T) for CK1 in Seven Distinct SCS-BPs and Three CK1 Isoforms (\(\delta\), \(\varepsilon\) and \(\gamma\))

<table>
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<tr>
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<td>GRNRRGSKASAD</td>
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<td>CK1(\gamma)</td>
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Fig. 2. The Stimulatory Effects of SCS on Autophosphorylation of Rat Liver CK1\(\delta\) \emph{in Vitro}

[A] Rat liver CK1\(\delta\) (approx. 50 ng) was directly incubated for 30 min at 30 °C with 5 \(\mu\)M \(\gamma\)-\(^{32}\)P\(\text{ATP}\) (500 cpm/pmol) in the presence of the indicated concentrations of either sulfatide (●) or CH-3S (○). \(^{32}\)P-Phosphorylated CK1\(\delta\) in the reaction mixtures was detected by autoradiography after SDS-PAGE. The rate 1 represents autophosphorylation of CK1\(\delta\) in the absence of SCS. [B] The autophosphorylation kinetics for rat liver CK1\(\delta\) were determined by incubation for 30 min at 30 °C with the indicated concentrations of \(\gamma\)-\(^{32}\)P\(\text{ATP}\) in the presence or absence of the optimum concentration (0.3 \(\mu\)M) of sulfatide \emph{in vitro}. \(^{32}\)P-Labeled CK1\(\delta\) in the reaction mixtures was determined by the membrane filter method, as described in the Materials and Methods. Lineweaver–Burk plots of ATP for CK1\(\delta\) in the absence (○) or presence (●) of sulfatide.
minal amino acid sequences and Peptide Mass Fingerprinting (PMF) identified the amino acid residues (positions 38—117 of p8). On the basis of characterization for the novel phosphorylation sites for CK1 on p8, three synthetic MBP peptides were prepared: M86 (positions 86—98 of p8), M88 (positions 88—104) and M103 (positions 103—117). Two other MBP peptides were also prepared: M103/S109A (replacement of Ser-109 with Alanine) and fragment M107 (positions 107—117 of p8) lacking Lys (K)-Gly (G)-Arg (R) (Fig. 4A). High phosphorylation of peptide M103 by CK1 was observed when incubated with 5 μM [γ-^32P]ATP (500 cpm/pmol) in the presence of 3 μM sulfatide (Fig. 4B).

In contrast, CH-3S induced high phosphorylation of peptide M103 by CK1, but did not significantly affect the CK1-mediated phosphorylation of the other four peptides (M86, M88, M103/S109A and M107) (Fig. 4B). In addition, higher binding affinities were observed with sulfatide than with for two peptides (M103 and M103/S109A), but not for peptide M107 lacking KGR (Fig. 4C). These results show that (i) the hexa-residues (KGRGLS) on p8 and peptide M103 may be a potent phosphorylation site for CK1 in the presence of SCS; and (ii) the three residues (KGR) in the CK1 phosphorylation site on peptide M103 may be responsible for the binding with SCS.

**A Novel Phosphorylation Motif for CK1 in Several SCS-BP Substrates** A computer database search and our previous reports revealed that several SCS-BPs (HMG1, PKCη, hC3a, FGF-BP, MBP and peptide M103, Table 1) contain a common consensus CK1 phosphorylation motif (K/R-X-K/R-X-X-S/T), which is different from the two canonical phosphorylation motifs [S(p)/T(p)-X-X-S/T and D/E-X-X-S/T] for CK1; and (ii) two functional proteins [human TP (an isoform, 441 amino acid residues) and human RhoA (196 amino acid residues)] possess multiple novel consensus phosphorylation sites for CK1. As expected, SCS highly stimulated the CK1-mediated phosphorylation of TP (Fig. 5A). The maximum phosphorylation of TP by CK1 was observed when incubated with 3 μM sulfatide or 3 μM CH-3S (Fig. 5A). In the presence of 3 μM sulfatide or 3 μM CH-3S, the phosphorylation of TP by CK1 increased up to 60 min and reached a plateau within 180 min (Fig. 5B). The sulfatide-induced stimulation of the CK1-mediated phos-
...of CK1 was determined after incubation for the indicated periods at 30 °C. The [32P]radioactivity of 32P-labeled RhoA in gel pieces was determined with a liquid scintillation counter, as described in Materials and Methods. [C] The kinetics for the CK1-mediated phosphorylation of RhoA were determined (approx. 5 units) in the presence or absence of 3 μM sulfatide in vitro. The radioactivity of 32P-phosphorylated RhoA in the reaction mixtures was determined by autoradiography after SDS-PAGE. [D] Both RhoA (approx. 2 μg) and CK1δ (approx. 5 units) were incubated with 5 μM [γ-32P]ATP (500 cpm/pmol) for 30 min at 30 °C in the presence of the indicated concentrations.

Fig. 6. Characterization of the CK1δ-Mediated Phosphorylation of RhoA in the Presence of SCS in Vitro

[A] Both RhoA (approx. 2 μg) and CK1δ (approx. 5 units) were incubated with 5 μM [γ-32P]ATP (500 cpm/pmol) for 30 min at 30 °C in the presence of the indicated concentrations of sulfatide (●) or CH-3S (○). 32P-Labeled RhoA in the reaction mixtures was detected by autoradiography after SDS-PAGE. [B] The phosphorylation kinetics of RhoA by CK1δ in the absence (Δ) or presence (●) of 3 μM sulfatide or 3 μM CH-3S (○) were determined after incubation for the indicated periods at 30 °C. The [32P]radioactivity of 32P-labeled RhoA in gel pieces was determined with a liquid scintillation counter, as described in Materials and Methods. [C] The binding affinities of RhoA (a) with 3 μM sulfatide (●) or 3 μM CH-3S (○) were determined at room temperature, using a QCM; and BSA (b) used as a negative control for binding affinity.

The Isoform Specificity of CK1 for the Phosphorylation of TP and RhoA in Vitro

A comparison was made of the isofor specificity of CK1 for the phosphorylation of two functional proteins [a basic protein (TP) and an acidic protein (RhoA)] in vitro. The optimum concentrations of SCS highly stimulated the phosphorylation of TP by three...
CK1 isoforms (α, δ and ε), but not CK1γ, with different phosphorylating abilities (Fig. 7A). As shown in Fig. 7B, high phosphorylation of RhoA was observed with two CK1 isoforms (δ and γ), but not with the others (α and ε). The sulfatide-induced stimulation of the CK1δ-mediated TP phosphorylation was slightly higher than that observed with CH-3S (Fig. 7A). The CK1γ-mediated phosphorylation of RhoA was preferentially stimulated by sulfatide, but not by CH-3S (Fig. 7B). These results suggest that (i) SCS effectively stimulates the phosphorylation of the two basic brain proteins (MBP and TP) by CK1δ; and (ii) sulfatide highly stimulates the phosphorylation of an acidic protein (RhoA) by CK1γ in vitro.

DISCUSSION

The present in vitro study revealed that (i) SCS functioned as an effective stimulator for the CK1δ-mediated phosphorylation of three functional proteins [MBP (Fig. 1B), TP (Fig. 5A) and RhoA (Fig. 6A)] and a synthetic peptide M103 (Fig. 4); (ii) sulfatide, but not CH-3S, highly enhanced autophosphorylation of CK1δ (Fig. 2A); (iii) sulfatide, as compared with CH-3S, had a higher affinity with MBP (Fig. 1C), peptide M103 (Fig. 4C) and RhoA (Fig. 6A); and (iv) sulfatide significantly increased the affinity of TP for CK1δ, and also highly increased the V_{max} value for TP of CK1δ (Fig. 5C). These results show that the direct binding of SCS to CK1δ and two substrates [TP (a basic brain protein) and RhoA (an acidic protein)] may induce their high phosphorylation by CK1δ in vitro.

Determination of a novel phosphorylation site for CK1δ and the binding affinity of SCS with five synthetic MBP peptides (Fig. 4) revealed that (i) a peptide M103 (positions 103—117 of MBP) contained a novel CK1δ phosphorylation site (Ser-109) in response to SCS (Fig. 4A); and (ii) the three amino acid residues (KGR) in the novel phosphorylation site for CK1δ on peptide M103 may be responsible for the binding of SCS, since no binding of SCS was detected with peptide M107, which lacks KGR (Fig. 4C). These results show that the hexa-amino acid residues (KGRGLS) on peptide M103 may be a potent phosphorylation site for CK1δ in response to SCS. As summarized in Table 1, there is a novel consensus phosphorylation motif [K/R-X-K/R-X-X-S/T] for CK1δ in at least seven SCS-BPs and three CK1 isoforms (δ, ε and γ). Therefore, it is possible to speculate that (i) sulfatide in comparison with CH-3S may function as a connecting mediator for the physiological interaction between three CK1 isoforms (δ, ε and γ) and their native substrates, such as TP and RhoA; and (ii) this interaction may lead to effectively phosphorylate these SCS-binding proteins with CK1δ isoform specificity in vitro.

The stimulatory effect of SCS on the isoform-specificity of CK1δ for the phosphorylation of TP and RhoA was determined comparatively in vitro. We found that SCS highly stimulates the phosphorylation of TP by three CK1 isoforms (α, δ and ε), but not by CK1γ, which effectively phosphorylated RhoA in the presence of sulfatide (Fig. 7). The substrate specificities of these four CK1 isoforms may be closely correlated with their amino acid sequences, because these three human CK1 isoforms possess high amino acid homologies (approx. 84% identity between δ and ε; and 76% identity between α and δ), but have a low homology with the amino acid sequence of CK1γ (approx. 54% identity to α; 51% to δ; and 47% to ε). As expected, CK1γ highly phosphorylated an acidic protein (RhoA), but not a basic protein (TP), in the presence of sulfatide (Fig. 7B), because the basic amino acid-rich C-termini of CK1 isoforms may provide their distinct properties, including substrate specificity.

The Ser/Thr-residues of a novel consensus phosphorylation motif (K/R-X-K/R-X-X-S/T) for CK1δ on several SCS-BPs (Table 1) is also recognized by RACα Ser/Thr-protein kinase (Akt, named as protein kinase B). Indeed, both MBP and peptide M103 were phosphorylated by Akt, but this phosphorylation was inhibited by SCS in a dose-dependent manner (data not shown). In addition, we reported that SCS inhibits at least three SCS-BPs (MBP, FGIF-BP and C3a) by A-kinase in a dose-dependent manner through its direct binding to these functional proteins in vitro. Interestingly, a database analysis revealed that there are a number of functional proteins, including cytoskeletal proteins (myosin and troponin) and transcriptional proteins (eukaryotic translation initiation factor 4E and p53) and cellular enzymes.

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**Fig. 7. The Stimulatory Effect of SCS on the Phosphorylation of TP or RhoA by Four Distinct CK1 Isoforms in vitro**

TP or RhoA (approx. 2 μg each) was separately incubated with four distinct CK1 isoforms (α, δ, ε and γ). The reaction mixtures were detected by autoradiography after SDS-PAGE. Lane 1, TP or RhoA was incubated with four CK1 isoforms in the absence of SCS; and incubated in the presence of sulfatide (lane 2, fixed column) or CH-3S (lane 3, gray column). The phosphorylation rate 1 (lane 1, white column) represents the phosphorylation of TP or RhoA in the absence of SCS in vitro.
(Ser/Thr-protein phosphatase 2A and 5'-AMP-activated protein kinase), containing multiple novel phosphorylation sites for CK1. Therefore, it is possible to speculate that accumulated high levels of SCS may induce the preferential phosphorylation of various cellular SCS-BPs, containing novel CK1 phosphorylation sites, by three CK1 isoforms (α, δ and ε), but selectively inhibit their phosphorylation by at least two protein kinases (A-kinase and Akt) at the cellular level.

Earlier studies concerning the physiological correlation between CK1 and TP have demonstrated that (i) the cellular level of CK1δ is highly elevated in Alzheimer's disease (AD) brain; (ii) two CK1 isoforms (α and δ) are tightly associated with the neurofibrillary lesions of AD brain and other tauropathic neurofibrillary degeneration diseases; (iii) CK1δ directly binds to TP; and (iv) the CK1δ-mediated phosphorylation of TP reduces its binding ability with microtubules and induces their destabilization, which finally leads to apoptosis of neurons. These earlier reports and our previous reports strongly suggest that the preferential phosphorylation of TP by two CK1 isoforms (α and δ) in the high level of SCS may induce the physiological alteration between TP and its associated cytoskeletal proteins (microtubulin, actin, calmodulin and myosin), which are involved in the mechanisms of various neuronal diseases including AD.

Recent reviews have noted that: these small GTPases in the Ras superfamily are molecular switches that control a wide variety of signal transduction pathways relating to the regulation of actin cytoskeleton, cell polarity, microtubule dynamics and membrane transport system; and also play an important role as molecular switches for transmitting the several extracellular signals to the interior of the cell from the cell membrane. In the present study, we found that (i) recombinant human RhoA is a SCS-binding protein (Fig. 6C); (ii) the human RhoA is phosphorylated in vitro by CK1δ (Figs. 6A, B) in a manner similar to the SCS-induced high stimulation of the CK1δ-mediated TP phosphorylation; and (iii) a similar stimulatory effect of SCS on the phosphorylation of RhoA was observed with CK1γ, but not two CK1 isoforms (α and ε) (Fig. 7B). However, no significant phosphorylation of RhoA by CK1γ was detected in the absence of SCS in vitro (Fig. 6B). These results suggest that (i) both CK1γ and CK1δ may be protein kinases responsible for the specific modulation of RhoA in the presence of SCS; and (ii) the preferential phosphorylation of small GTPases by two CK1 isoforms (γ and δ) may be involved in the regulation of the Rho GTPase-mediated signal transduction at the high level of SCS. Although it has been reported that A-kinase phosphorylates Rho GTPase and does not modulate its GTPase activity in vitro, the phosphorylation of RhoA by A-kinase was inhibited by SCS at 3 μm, which highly stimulated the CK1γ-mediated phosphorylation of RhoA (data not shown). Therefore, the SCS-dependent phosphorylation of RhoA by CK1γ may be implicated in the regulation of the actin cytoskeleton, cell cycle progression, cell transformation and membrane trafficking. This possibility is supported by evidence that (i) almost all members of the Rho family contain a common consensus phosphorylation site (K-R-X-K-R-X-X-S/T) for CK1 in response to SCS; (ii) the domains containing the consensus phosphorylation sites for CK1γ on these Rho GTPases are well conserved; and (iii) the Ser-188 on RhoA is the same phosphorylation site for PKA and for CK1γ in the presence of SCS in vitro. Therefore, after further detailed characterization of the CK1γ-mediated phosphorylation of small GTPases in vitro, it should be possible to explain the regulatory mechanism of the novel physiological functions of the Rho family of small GTPases in their physiological correlation with various associated effectors, which are involved in the regulation of the actin cytoskeleton, cell cycle progression, cell transformation and membrane trafficking.

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, it may play an important biological role in the diverse functional and regulatory mechanisms involved in cell growth, cell adhesion, neuronal plasticity and cell morphogenesis. Altered levels of sulfatide in human brain tissues may be involved in the pathogenesis of various neuronal diseases, since a high accumulation of sulfatide in arylsulfatase A deficient brain causes metachromatic leukodystrophy. Although sulfatide exists not only in oligodendrocytes and Schwann cells but is also present in the cytoplasm of neurons, it seems likely that sulfatide may be a key functional mediator for the CK1-mediated signal transduction in neurons in mammalian brain.

To clearly understand the biological significance of the CK1-mediated phosphorylation of various functional SCS-BPs, including TP and RhoA, further analytical studies are required to (i) detect the preferential phosphorylation of these SCS-BPs, containing novel CK1 phosphorylation sites, by CK1 at the accumulated high level of sulfatide in suitable intact cells; (ii) determine the SCS-dependent phosphorylation of TP by CK1δ in its physiological association with cytoskeletal proteins (microtubulin, actin, calmodulin and myosin), which are involved in the mechanisms of various neurofibrillary degeneration diseases; and (iii) also investigate the physiological significance of the SCS-dependent high phosphorylation of small GTPases by CK1γ in the RhoA-mediated signal transduction of cell cycle progression, cell transformation and membrane trafficking.

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