Journal of Cellular Biochemistry 104:1724–1739 (2008)

Importance of Autophosphorylation at Ser186 in the A-Loop of Salt Inducible Kinase 1 for Its Sustained Kinase Activity

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Abstract Autophosphorylation is an important mechanism by which protein kinases regulate their own biological activities. Salt inducible kinase 1 (SIK1) is a regulator in the feedback cascades of cAMP-mediated gene expression, while its kinase domain also features autophosphorylation activity. We provide evidence that Ser186 in the activation loop is the site of autophosphorylation and essential for the kinase activity. Ser186 is located at the +4 position of the critical Thr residue Thr182, which is phosphorylated by upstream kinases such as LKB1. The relationship between phosphorylation at Ser186 and at Thr182 in COS-7 cells indicates that the former is a prerequisite for the latter. Glycogen synthase kinase-3β (GSK-3β) phosphorylates Ser/Thr residues located at the fourth position ahead of the pre-phosphorylated Ser/Thr residues, and inhibitors of GSK-3β reduce the phosphorylation at Thr182. The results of an in vitro reconstitution assay also indicate that GSK-3β could be the SIK1 kinase. However, overexpression and knockdown of GSK-3β in LKB1-defective HeLa cells suggests that GSK-3β alone may not be able to phosphorylate or activate SIK1, indicating that LKB1 may play a crucial role by phosphorylating SIK1 at Thr182, possibly as an initiator of the autophosphorylation cascade, and GSK-3β may phosphorylate SIK1 at Thr182 by recognizing the priming-autophosphorylation at Ser186 in cultured cells. This may also be the case for the other isoform SIK2, but not for SIK3. J. Cell. Biochem. 104: 1724–1739, 2008.

Key words: SIK; LKB1; GSK-3β; signal transduction; autophosphorylation; activation loop

Kinase cascades are regulated by a variety of mechanisms, including phosphorylation relays, not only from upstream to downstream but also through feedback actions or autophosphorylation. Phosphorylation of the activation loop (A-loop), an important region in the catalytic domain, induces a structural change of the catalytic site and switches on the enzymatic activity [Toker, 1998].

Salt-inducible kinase-1 (SIK1) is a serine/threonine protein kinase isolated from adrenals of rats fed with a high Na^+- or K^+-diet [Wang et al., 1999]. The induction of SIK1 mRNA was also detected in PC12 pheochromocytoma when membrane depolarization was induced by K^+ treatment [Feldman et al., 2000]. SIK1 belongs to a family of sucrose nonfermenting-1 protein kinase/AMP-activated protein kinases (AMPK) that is activated by metabolic/energy stresses [Hardie and Carling, 1997]. Although SIK1 mRNA is not induced by salt stresses in cultured adrenocortical cells [Lin et al., 2001], it is rapidly induced when the cells are treated with adrenocorticotropin. The induced SIK1 then represses the transcription(s) of steroidogenic enzymes by inhibiting cAMP-responsive elements (CREs) on the promoters [Doi et al., 2002]. A gene database search found two more isoforms of SIK1 and SIK2, with a high

Grant sponsor: The Charitable Trust Osaka Cancer Researcher Fund; Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: The Ministry of Health, Labor and Welfare Japan; Grant sponsor: The New Energy and Industrial Technology Development Organization (NEDO) of Japan; Grant sponsor: The Japan Human Science Foundation.

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Received 18 November 2007; Accepted 25 January 2008
DOI 10.1002/jcb.21737

expression level in adipose tissue [Horike et al., 2003], and SIK3, which shows ubiquitous expression [Takemori and Okamoto, 2008].

The mechanism by which SIKs repress cAMP-induced gene expression is phosphorylation of a CRE-binding protein (CREB) specific co-activator TORC (transducer of regulated CREB activity) [Katoh et al., 2004; Screaton et al., 2004]. When the cAMP signal is initiated, protein kinase A (PKA) phosphorylates a Ser residue, Ser577, in the C-terminal region of SIK1, which then attenuates the TORC phosphorylation activity of SIK1 [Doi et al., 2002; Katoh et al., 2004]. Ser577 is also an autophosphorylation site [Katoh et al., 2006], indicating that SIKs auto-regulate their TORC-inactivation activity by means of autophosphorylation.

Recently, the class II histone deacetylase HDAC5 (histone deacetylase 5) was also identified as a new SIK substrate in neurons of *C. elegans* [van der Linden et al., 2007] and in muscles of mice [Berdeaux et al., 2007]. The phosphorylation of SIK1 at Ser577 is also important for SIK1's HDAC5-phosphorylation activity, suggesting that the kinase activity of SIK1 and its auto-regulation may play important roles in a variety of signaling cascades.

The tumor suppressor kinase LKB1 was identified as a major upstream activator of AMPK family kinases [Hong et al., 2003; Spicer et al., 2003; Woods et al., 2003] and phosphorylates the Thr residue, Thr182, in the A-loop of SIK1 [Lizcano et al., 2004]. On the other hand, inactivation of SIKs by the kinase inhibitor staurosporine (STS) has been found to enhance the phosphorylation of the LKB1-phosphorylatable Thr residues of SIK1 and SIK2, but not of SIK3 or AMPKα1 [Katoh et al., 2006], which suggests that an as yet unidentified mechanism other than LKB1 may contribute to the differential activation of SIK family kinases. In addition to these Thr-phosphorylation(s), the kinase domain of recombinant SIK1 shows autophosphorylation [Lin et al., 2001]. These data indicate that multiple phosphorylation(s) may regulate the catalytic activity of SIK1.

In the study reported here, we identified a new autophosphorylation site in the A-loop of SIK1, Ser186, which is located at the +4 position of the LKB1-phosphorylatable Thr182. Glycogen synthase kinase-3β (GSK-3β) was found to phosphorylate Thr182 in a phospho-Ser186 dependent manner, which in turn may depend on the LKB1 cascade.

MATERIALS AND METHODS

Cell Culture, Chemicals, Antibodies and Plasmids

COS-7 cells and HeLa cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) and antibiotics at 37°C in an atmosphere of 5% CO_2–95% air.

A library composed of 80 kinase inhibitors was purchased from BioMol International (Plymouth Meeting, PA). STS, H89, KN93, bisindolylmaleimide, PD98059, Wortmannin, SB203580, KT5823 and Hypericin were purchased from Calbiochem (San Diego, CA), Indirubin-3'-monoxime (Indi), Ro31-8220 from Sigma–Aldrich, and Lactacystin from MBL Co., Ltd. (Nagano, Japan).

An anti-hemagglutinin (HA)-tagged IgG, GSK-3β antibodies and 14-3-3 antibody were obtained from Roche Diagnostics (Indianapolis, IN), Cell Signaling Technology (Danvers, MA) and Santa Cruz (Santa Cruz, CA), respectively. The phospho-Thr182 SIK1 IgG is described elsewhere [Katoh et al., 2006]. Phospho-Ser186 SIK1 IgG was prepared using the peptide PLSTWCGpSPPYAAPE (SIK1 A-loop, pS is phospho-Ser).

cDNAs for rat SIK1 [Lin et al., 2001], mouse SIK2 [Horike et al., 2003] and rat AMPKa1 [Crute et al., 1998] have been previously described. cDNAs for Human SIK3 (KIAA0999) and microtubule-associated protein/microtubule affinity regulating kinase 4 (MARK4: KIAA1860) were gifts from the Kazusa DNA Institute (Chiba, Japan) [Katoh et al., 2006]. Mouse LKB1 cDNA [Katoh et al., 2006] was cloned into a pEBG vector, and pEBG-LKB1 was co-transformed into COS-7 cells with pCMVsport6-mouse MO25α (IMAGE: 3994267) and pCMVsport6-human STRADα (IMAGE: 5769892) [Boudeau et al., 2003]. A pCMVsport6-mouse GSK-3β cDNA (IMAGE: 2647729) was purchased from Invitrogen (Carlsbad, CA). Site-directed mutagenesis was used to introduce a *Bgl*II site at 5′ of the GSK-3β cDNA. The primers used were: for GSK-3β *Bgl*II-F, 5-GGTGATTCAAGAAGAA-GATCTATGTCGGGGCGACCG; for GSK-3β *Bgl*II-R, 5-CGGTCGCCCCGACATAGATCTT-CTTCTTGAATCACC. The resultant full-length

mouse GSK-3β cDNA was digested with *Bgl*II and *Not* I (in the multi-cloning site) and ligated into the *Bam*HI–*Not*I site of the pEBG vector.

To obtain knockdown GSK-3β HeLa cells, we constructed an mi-RNA cassette on an expression plasmid. A *Bsa*I linker (5′-TGCTGGAGACCTTATGGTCTCA/5′-CCTGTGAGACCATAAGGTCTCC; underlined sections show recognition sites) was ligated into the cloning site of pcDNA6.2-GW/miR vector (Invitrogen). The region containing a GFP-tag and the *Bsa*I-cloning site was transferred into the pDONR221 vector by means of BP-clonase, and the resultant vector was named pDONR-GW/miR. Oligonucleotides for mi-RNA against human GSK-3β mRNA (5′-TGCTGAGTTGGTGTATACTCCAGCAGGTTTTGGCCACTGACTGACCTGCTGGAATACACCAACT/5′-CCTGAGTTGGTGTATTCCAGCAGGTCAGTCAGTGGCCAAAACCTGCTGGAGTATACACCAACTC) were ligated into the *Bsa*I site of pDONR-GW/miR. The region containing the GFP-tag and miRNA was then transferred again into the plasmid vector pcDNA3.1/DEST by means of LR-reaction.

Immunoprecipitation and In Vitro Kinase Assay

Immunoprecipitation was performed as described previously [Lin et al., 2001]. Briefly, cells (5×10^5) plated on a 10-cm dish were transformed with 3–6 μg of expression plasmids (pSVL-HA or pEBG) for HA- or GST-tagged wild type and mutant SIKs by using 10 μl of LipofectAMINE 2000 (Invitrogen). After 36–48 h incubation, cells were lysed in 0.7 ml of lysis buffer [Lin et al., 2001]. The HA-tagged SIK protein was immunoprecipitated with an anti-HA-tag IgG (2 μg) and protein G-Sepharose (30 μl). Aliquots of purified HA-SIK1 were then subjected to western blot analyses with the anti-SIK1 IgG and to in vitro kinase assays. A glutathione column was used to purify GST fusion proteins. Purified SIKs were incubated with 5 μg of GST-Syntide2, -p300 peptide or -TORC2 peptide in the presence 0.5 μCi (18.5 kBq) of [γ-^{32}P]-ATP at 30°C for 30 min. The kinase reaction was stopped by adding 3× SDS sample buffer (150 mM Tris–HCl (pH 6.8), 6% SDS, 30% glycerol, and 0.1% bromophenol blue) and heating at 100 C for 5 min. The aliquots were subjected to 15% SDS–PAGE, and the phosphorylated peptides were visualized on an autoradiogram.

Phosphorylation activities of LKB1 and GSK-3β were measured in an Mg-buffer [50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 100 μM ATP]. When synthetic peptides [WT, DFGFGNFYKPGEPLSTWCGSPPYAAPEKKKK; pS186, DFGFGNFYKPGEPLSTWCGpSPPYAAPEKKKK (pS is phospho-Ser186)] were used as substrates of GSK-3β [γ-^{32}P]-ATP was diluted with cold ATP at the final concentration of 1 μM. The phosphorylation reactions were stopped by the addition of 0.5 M EDTA, and aliquots of the reaction mixtures were spotted onto P-81 cellulose papers. After washing with 75 mM phosphoric acid, radio counts were measured as Cherenkov radiation.

Phospho-Amino Acid Analysis

The method used for the phospho-amino acid analysis is described elsewhere [van der Geer and Hunter, 1994]. Briefly, radio-labeled GST-SIK1 (1-354) and GST-SIK2 (1-348) peptides were separated from free ATP by filtration with a Microcon 50 spin column (Millipore, Billerica, MA). After washing with 6 M HCl, the peptides were resuspended in 100 μl of 6 M HCl and then incubated at 100 C for 6 h. The hydrolysates were dried with a SpeedVac concentrator (Thermo Scientific, Milford, MA), and pellets were dissolved in 20 μl of sterilized water supplied with 0.5 μg each of cold phosphoserine, phosphothreonine, and phosphotyrosine as standards (Sigma). Phosphoamino acids were separated on cellulose-thin layer chromatography plates (Merck, Darmstadt, Germany) with the running buffer (0.5% (v/v) pyridine and 5% (v/v) acetic acid, pH 3.5) at 1,500 V for 1 h. During the electrophoresis, the plates were kept at 16°C with an isoelectric focusing plate. The positions of the phospho-amino acids were visualized by ninhydrin staining and the signals of ^{32}P-labeled amino acids were visualized autoradiographically.

Modeling of a Three-Dimensional Structure of SIK1

The three-dimensional structure of the SIK1 kinase domain was modeled from the primary sequence of the rat SIK1 kinase domain by means of the Swiss Model (http://swissmodel.expasy.org/swiss-model.html) [Schwede et al., 2003] which predicts and constructs three-dimensional structures based on sequence homologies. The illustration was prepared with

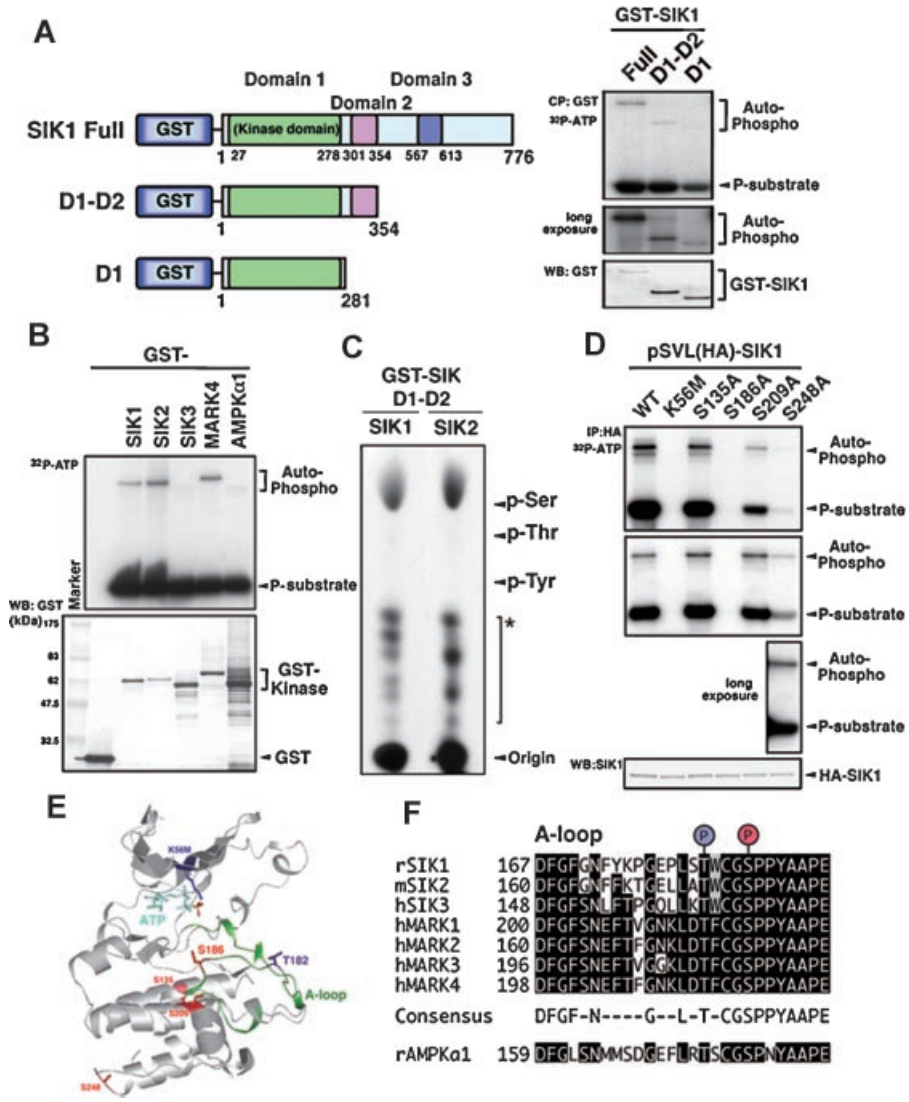

Fig. 1. Kinase domains of SIK1 and SIK2 possess autophosphorylation sites. **A**(left): The primary structure of SIK1 enzymes used in this study. D1: kinase domain 1, D2: domain 2, Full: full-length SIK1. A(right): Expression plasmids (3 μg) for GST-tagged SIK1 and their truncated enzymes were transformed into COS-7 cells. After 36 h incubation cells were harvested for purification of GST-SIK1 with a glutathione column (CP) as described in Materials and Methods Section. Aliquots of GST-SIK1 solution were subjected to western blotting (WB) using an anti-GST antibody (**lower panel**) and to in vitro kinase assays using [γ-^{32}P]-ATP with GST-Syntide2 as a peptide substrate (**upper panel**). To intensify the autophosphorylation signals, a prolonged exposure was employed (**middle panel**). **B**: COS-7 cells were transformed with 3 μg each of pEBG-SIK1 (1-354), -SIK2 (1-348), -SIK3 (1-340), -MARK4 (1-380) and AMPKα1 (2-312) plasmids. The levels of the enzymes expressed are shown in a western blotting panel (**lower panel**). The in vitro kinase reaction used GST-p300 (84-94aa) peptide as substrate (**upper panel**), because AMPKα1 could not phosphorylate Syntide2 sufficiently. When the radio-labeled mixtures were subjected to SDS–PAGE separation, the volume of the mixture was normalized with the radio levels of p300-peptide. **C**: Auto-labeled GST-SIK1 (1-354) and -SIK2 (1-348) enzymes were hydrolyzed with 6 N HCl at 100 C for 6 h. The resultant products were separated on a thin layer chromatography plate with non-labeled phospho-serine (p-Ser), phospho-threonine (p-Thr) and phospho-tyrosine (p-Tyr) as standards, and the position of individual phospho-amino acids were visualized by using a ninhydrin reaction (arrow heads). * indicates partially hydrolyzed products. **D**: COS-7 cells were transformed with expression plasmids for HA-tagged wild-type (full-length) and mutant SIK1(s) (K56M, S135A, S186A, S209A, and S248A). The HA-tagged SIK1 proteins were immunoprecipitated (IP) with an anti-HA-tag IgG. For adjustment of the amount of SIK1 protein used for the in vitro kinase assay (**upper panel**), Western blot analyses were performed using an anti-SIK1 IgG (**lower panel**). To determine the ratio between peptide phosphorylation and autophosphorylation activity, the volume of the radio-labeled mixture was normalized with the peptide phosphorylation activity (**second panel**), and the mixture was then given a long exposure to S248A mutant (**third panel**). The images in *A, B* and *D* show representative data from two independent experiments, and that in *C* from experiments in triplicate. **E**: A three-dimensional model of the SIK1 kinase domain. The A-loop is shown in green and ATP is indicated by sky-blue marks (the phospho-group at the γ-position is indicated by red and yellow marks). Ser residues that were disrupted in (D) are shown in red (Ser186 is shown in a larger font). Thr182 is shown in purple. Lys56, a Lys residue essential for the kinase activity, is blue. **F**: Alignment of amino acid sequences of the A-loops of SIK- and MARK-family kinases. Conserved residues are marked with black areas, and the critical Thr residues phosphorylated by LKB1 and the Ser residue suspected of being autophosphorylation sites are indicated by phospho-symbols.

the MolFeat v2.2 program (FiatLux Co., Ltd., Tokyo, Japan).

RESULTS

The Kinase Domain of SIK1 Contains an Autophosphorylation Site

Autophosphorylation is the enzymatic activity that was first discovered by us in SIK1 [Lin et al., 2001]. To explore the importance of autophosphorylation of SIK1, we decided to identify where the autophosphorylation sites were located.

SIK1 polypeptide was truncated from the C-terminal and expressed in COS-7 cells. After purification, the autophosphorylation and peptide phosphorylation activities were measured by using radio-labeled ATP. (In this study, the term "kinase activity" refers to both autophosphorylation and peptide phosphorylation.) As shown in Figure 1A, the autophosphorylation signal of full-length SIK1 was stronger than that of the truncated enzymes, suggesting that some of autophosphorylation sites may be located in the C-terminal region. When domain 2 (D2) was removed, a significant decrease in the kinase activity was observed. However, a faint signal of autophosphorylation was seen on an X-ray film after a long exposure, indicating that at least one autophosphorylation site may exist in the kinase domain of SIK1.

To examine whether autophosphorylation in the kinase domain is a common feature of SIK-family kinases, peptide fragments for D1–D2 of SIK2 and SIK3 were also prepared as GST-fusion proteins and subjected to an in vitro kinase assay. In addition to these SIK-family kinases, MARK4 and AMPKα1 were used as, respectively, a representative kinase of the MARK family and one of two AMPKs. As shown in Figure 1B, SIK1, SIK2 and MARK4 showed autophosphorylation activity, but little if any was displayed by SIK3 and AMPKα1.

Next, we tried to determine which amino acid was autophosphorylated. The kinase domains (D1–D2) of SIK1 and SIK2 were incubated with ^{32}P-ATP in the absence of substrate, hydrolyzed with hydrochloric acid and separated on thin-layer chromatography (Fig. 1C). The radio-labeled amino acids of both SIK1 and SIK2 migrated to the position of phospho-Ser, suggesting that Ser residues were autophosphorylated and that the sites may be conserved between SIK1 and SIK2.

Fortunately, only four Ser residues in the D1–D2 region were conserved in SIK1 and SIK2 and were therefore substituted with Ala. To foreclose the possibility that the GST-tag acted as an acceptor of phosphate groups, we planned to change the tag to the HA-tag. However, the kinase defective mutant K56M was hardly recovered by immunoprecipitation in the form of D1–D2 or D1 alone, probably due to a relatively long incubation for immuno-reactions. Because full-length SIK1 is more stable than the D1–D2 protein [Lin et al., 2001], we generated the mutants as full-length proteins.

Although the mutants of S135A, S209A and S248A showed weaker kinase activities than wild type SIK1, their ratios of autophosphorylation to peptide phosphorylation signals were almost equal. In contrast, the S186A mutant and a negative control mutant, K56M, did not show any activity (Fig. 1D).

A model of the three-dimensional structure for the SIK1 kinase domain (Fig. 1E) suggests that the hydroxyl group of Ser186 is located near the phospho-group at the γ-position of ATP. In addition, Ser186 is positioned at +4 of the critical Thr residue, Thr182, which is phosphorylated by LKB1 (Fig. 1F) [Lizcano et al., 2004]. These Ser and Thr residues are commonly conserved in the A-loops of AMPK-related kinases. Hence, we speculated that Ser186 was a critical residue for the kinase activity of SIK1 and a candidate for the autophosphorylation site.

Importance of Ser Residues in the A-Loop of SIKs for Kinase Activities

To examine the importance of Ser186 for the kinase activity of SIK1, we generated SIK1 mutants with various substitutions at Ser186. As shown in Figure 2A, substitutions with a negatively charged residue (Asp) or residues possessing small side chains (Cys or Gly) also disrupted the kinase activities. In contrast, substitution with the other phosphorylatable residue (Thr) did not impair kinase activities (Fig. 2B). Thin-layer chromatography of radio-labeled amino acids for SIK1 peptides, the wild type and S186T mutant, indicated that the 186 position is the sole autophosphorylation site in the kinase domain of SIK1 (Fig. 2C), thus also eliminating the worrying problem of the GST-tag.

The importance of Ser residues in the A-loops of SIK2 and SIK3 was also examined. Neither

SIK2 mutants S179A and S179E nor the negative control mutant K49M showed any kinase activity (Fig. 2D). However, the substitution of Ser167Ala for SIK3 did not eliminate the peptide phosphorylation activity (Fig. 2E). Because the kinase domain of SIK3 did not show autophosphorylation activity (Fig. 1B), the importance of Ser167 in SIK3 for its kinase activity might be different from its importance for SIK1 and SIK2. When Glu was substituted for Ser167 of SIK3, the resultant mutant, S167E, did not show any peptide phosphorylation activity, suggesting that the position at 167 of SIK3 may require a small side chain, but not a phospho- or carboxyl-group.

Thr and Ser Phosphorylations in the A-Loop Require SIK1's Kinase Activity

Ser186 is located near the critical Thr residue, Thr182, in the A-loop (Fig. 1E,F). Western blot analyses with an anti-phospho-Thr182 antibody showed that the antibody hardly reacted with the peptides of SIK1 mutants possessing substitutions at Ser186. This seems to suggest that the S186X mutants were less phosphorylated at Thr182. However, the close distance between Thr182 and Ser186 is another possible reason for this diminished reactivity, since the structural disarray at Ser186 resulting from the substitution may interfere with the antibody's reaction with phospho-Thr182.

To ascertain the above possibilities, we compared the phosphorylation level at Thr182 of several kinase defective SIK1 mutants. As shown in Figure 3A, the anti-phospho-Thr182 antibody did not react with any kinase-detective mutants (upper panel), as was also the case for the anti-phospho-Ser186 antibody (middle panel). Because the K56M mutant possesses an intact sequence of the A-loop, we concluded that the reduced reactivity of antibodies was due to a

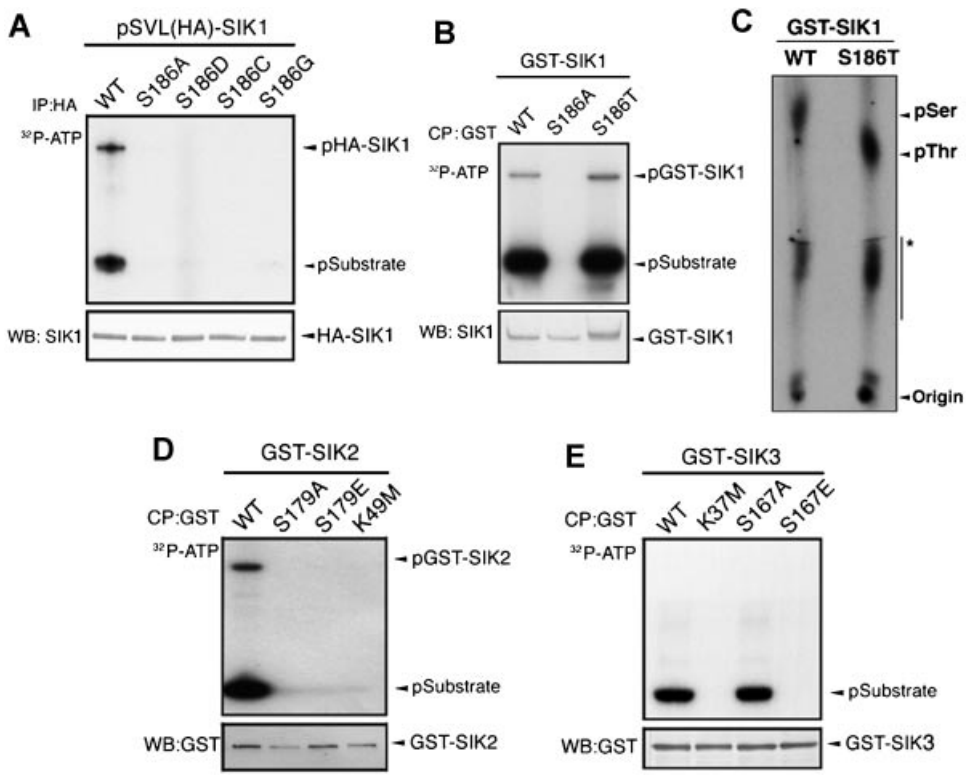

Fig. 2. Importance for SIK kinase activities of Thr and Ser residues in the A-loop(s) of SIK1-3. **A**: An in vitro kinase assay of HA-tagged SIK1 (full-length) mutants with substitutions at Ser186 was performed as described in the legend for Fig. 1D. IP and WB: immunoprecipitation and Western blotting, respectively. **B**: An in vitro kinase assay of the GST-tagged SIK1 kinase domain (1-354) containing Ser with Thr substitution at the autophosphorylation site. CP: column purification. **C**: Thin-layer chromatography of radio-labeled amino acids prepared from SIK1 kinase domains (1-354) of wild type and S186T was performed as described in the legend for Fig. 1C. **D**: Ala or Glu was substituted for Ser179 residue of GST-SIK2. K49M is a kinase defective SIK2. **E**: Ala or Glu was substituted for Ser167 of the SIK3 kinase domain (1-340). K37M is a kinase defective SIK3. These panels constitute representative sets from triplicate experiments.

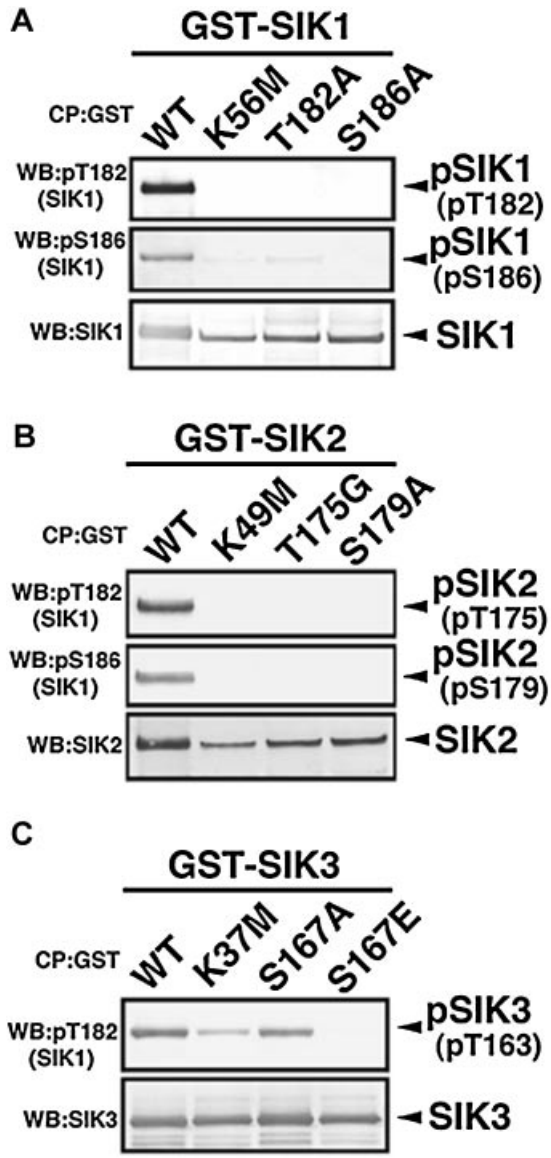

Fig. 3. Phosphorylation in the A-loops of SIK-family kinases depends on their kinase activity. **A**: GST-SIK1s (full-length) were overexpressed in COS-7 cells and purified by glutathione columns. SIK1 protein was detected by means of western blot using the anti-SIK1 IgG (**bottom**), and the same amount of protein was subjected to western blots with the phospho-specific antibodies. The levels of Thr-phosphorylation of SIK2 (full-length) (**B**) and SIK3 (1-340) (**C**) were also examined.

decrease in the level of phosphorylation, and that the phosphorylation at Thr182 may depend on the kinase activity of SIK1.

A similar phenomenon was observed for SIK2 (Fig. 3B), but it was slightly different from that for SIK3 (Fig. 3C) because Thr163 of a kinase defective K37M mutant was somewhat phosphorylated and the active S167A mutant was phosphorylated at Thr163. Because there appeared to be less accumulation of kinase defective mutants of SIK1 and SIK2 in cells, we decided not to normalize protein levels for subsequent analyses.

Ro-318220 and Indirubin-3′-Oxime Inhibit Phosphorylation at Thr182 of SIK1 in COS-7 Cells

In addition to these results, we previously demonstrated that treatment of COS-7 cells with STS increased the level of phosphorylation of SIK1at Thr182 and of SIK2 at Thr175, but not of SIK3 at Thr163 or of AMPKα1 at Thr172 [Katoh et al., 2006]. Putting these findings together suggests that, in addition to LKB1, multiple upstream cascades differentially phosphorylate the Thr-residues of the SIK isoforms in COS-7 cells.

To re-characterize the upstream cascade of SIK1, GST-fusion SIK1 was overexpressed and the cells were treated with various kinase inhibitors in a small library. As shown in a representative result (Fig. 4A), two inhibitors, Ro-318220 and indirubin-3′-oxime (Indi), inhibited the phosphorylation at Thr182. These inhibitions were accompanied by a decrease in the levels of phospho-Ser186 and kinase activity. In addition, the protein levels of GST-SIK1 in Ro-318220- and Indi-treated cells were also reduced. The level of phospho-Thr182 declined quickly, within 15 min, whereas the protein levels decreased slowly (Fig. 4B), indicating that the SIK1 protein may be degraded after dephosphorylation.

Results of preliminary experiments indicated that the Indi-dependent degradation of SIK1 protein was sensitive to the proteasome inhibitor lactacystin (not shown). Although the mechanism by which lactacystin blocked the Indi-dependent degradation of SIK1 was not clear, we decided to use lactacystin to examine the specific levels of phospho-SIK isoforms. As shown in Figure 4C, phosphorylation at Thr182 of SIK1 and at Thr175 of SIK2 was highly sensitive to Indi, but that at Thr163 of SIK3 and at Thr172 of AMPKα1 was only slightly sensitive.

Involvement of GSK-3β in the Phosphorylation at Thr182 of SIK1

Bisindolylmaleimide I (Bis) is structurally similar to Ro-318220, and both show a similar inhibitory spectrum except for GSK-3β and S6K

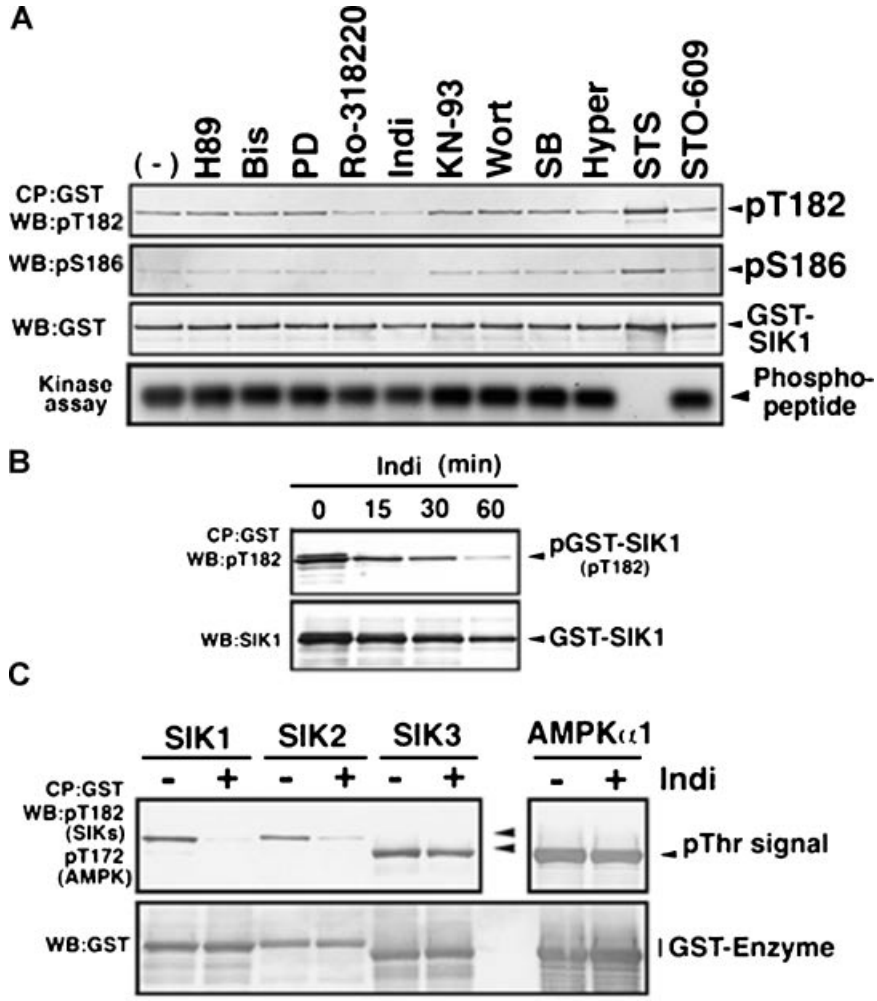

Fig. 4. Ro-318220 and Indi inhibit phosphorylation of SIK1 at Thr182 in COS-7 cells. **A**: COS-7 cells expressing GST-SIK1 (1-354) were treated with representative kinase inhibitors for 1 h. The following inhibitors were used: H89 (1 μM), Bisindolylmaleimide I (Bis: 20 μM), PD98059 (PD: 20 μM), Ro 318220 (20 μM), indirubin-3′-oxime (Indi: 20 μM), KN93 (10 μM), Wortmannin (Wort: 1 μM), SB203580 (SB: 20 μM), Hypericin (Hyper: 2 μM), KT5823 (20 μM), Staurosporine (STS: 10 nM), and STO-609 (25 μM). GST-SIK1 was purified and subjected to western blotting using anti-phospho-Thr182 (**top panel**), anti-phospho-Ser186 (**second panel**) or anti-GST (**third panel**) antibodies. The purified GST-SIK1 enzyme was also subjected to an in vitro kinase assay using a TORC2 peptide (SALNRTSSDSALHDD) (**bottom panel**). **B**: Time-dependent reduction by Indi of the level of phospho-Thr182 SIK1. **C**: COS-7 cells that had been expressing GST-SIK1 (1-354), -SIK2 (1-348), -SIK3 (1-340) or -AMPKα1 (2-312) were treated with Indi (20 μM) for 30 min in the presence of lactacystin (10 μM). The phosphorylation at Thr residues was detected with an anti-phospho-Thr182 SIK1 or an anti-phospho-Thr172 AMPKα1 antibody (**upper panels**). An anti-GST antibody was used for the detection of GST-fusion enzymes (**lower panel**).

[Hers et al., 1999]. However, Bis did not inhibit phosphorylation of SIK1 at Thr182 (Fig. 4A). In contrast, Ro-318220 and Indi show no structural similarity, but both are known to inhibit cyclin-dependent kinase (CDK) family kinases and GSK-3β [Leclerc et al., 2001; Polychronopoulos et al., 2004]. On the other hand, STS strongly inhibits CDKs but upregulates GSK-3β [Koivisto et al., 2003; Shankar et al., 2004], suggesting that GSK-3β may be a candidate for the upstream kinase of SIK1 in COS-7 cells.

Another finding, namely that autophosphorylation at Ser186 is the prerequisite for phosphorylation at Thr182 (Fig. 3A), also supports the notion of involvement of GSK-3β in the phosphorylation of Thr182, because GSK-3β recognizes phospho-primed peptides and phosphorylates Ser and Thr residues located at four amino acids ahead of the phospho-primed residues (**S**/**T**-X-X-X-pS/pT: letters in bold indicate the phosphorylation site of GSK-3β, pS/pT is the phospho-primed residue) [Fiol et al.,

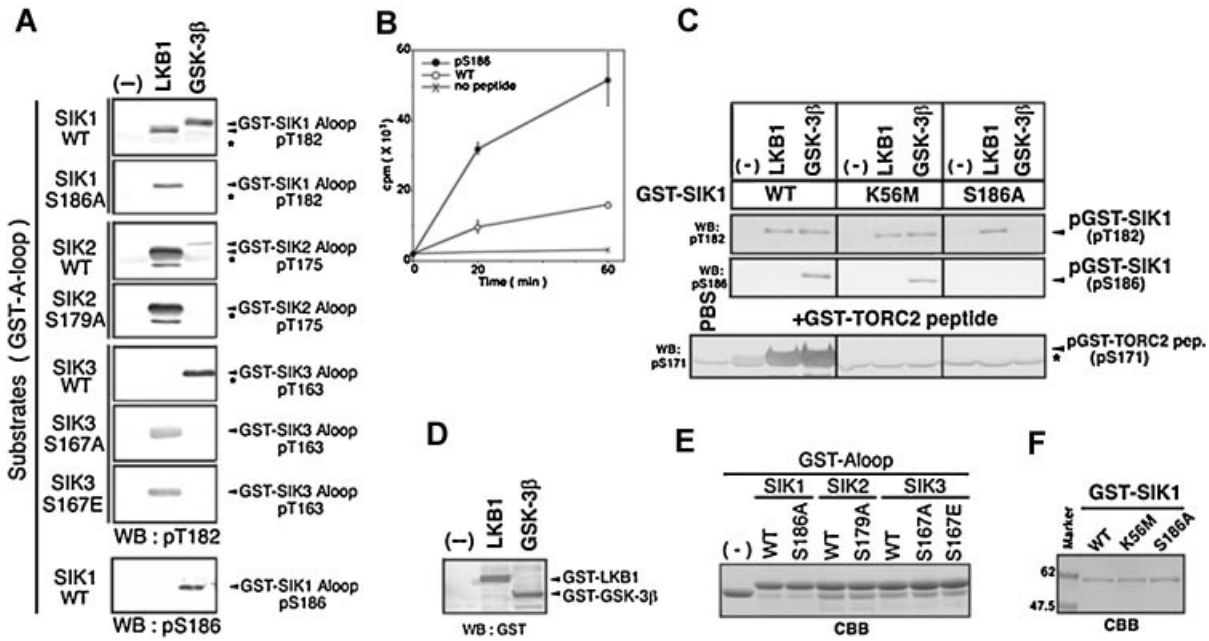

Fig. 5. GSK-3β phosphorylates SIK1 in vitro. **A**: GST-fusion A-loop peptides of SIK1 (163-199), SIK2 (156-192), SIK3 (144-180) and their mutant peptides were prepared in *E. coli*, and GST-LKB1 and GST-GSK-3β enzymes were prepared in COS-7 cells, as shown in (**D**). The levels of phosphorylation at the Thr residues were examined with anti-phospho-Thr182 SIK1 IgG (arrow heads). * indicates the positions of unphosphorylated peptides on the membrane. The level of phospho-Ser186 in the SIK1 A-loop peptide was also examined (**bottom**). **B**: Wild type GSK-3β was incubated with synthetic peptides [WT, DFGFGNFYKP-GEPLSTWCGSPPYAAPEKKKK; pS186, DFGFGNFYKPGEPLST-WCG**pS**PPYAAPEKKKK (**pS**: phospho-Ser186)] in the presence of [γ-^{32}P]-ATP. Radio counts of peptides were measured by using P81-cellulose papers as described in Materials and Methods Section. n = 3. **C**: in vitro reconstitution of the phosphorylation cascade from LKB1 or GSK-3β toward TORC2. Recombinant GST-SIK1 (1-354) and its mutants were prepared in *E. coli*. and activated by LKB1 or GSK-3β. The levels of phospho-Thr182 (**top panel**) and -Ser186 (**middle panel**) were examined by means of western blots using aliquots of the activated-enzyme mixtures. The resultant mixtures were diluted to 1/10 with SIK kinase buffer and further incubated with the GST-TORC2 peptide. The level of the TORC2 phosphorylation was examined by using the anti-phospho-Ser171 TORC2 antibody (**bottom panel**). * indicates a non-specific reaction of the anti-Ser171 IgG with an unknown contaminant in the fraction of the GST-TORC2 peptide. **E**: The quality of A-loop peptides of SIK1-3 was examined by means of SDS–PAGE followed by Coomassie staining. **F**: The levels of recombinant GST-SIK1 (1-354) and its mutants used in (C).

1987]. Considering that Ser186 is located at the +4 position of Thr182, we hypothesized that the autophosphorylation at Ser186 produces the priming site for the phosphorylation by GSK-3β at Thr182.

To examine this possibility, GST-fusion peptides for the A-loop(s) of SIK1-3 and their mutants with substitutions at the Ser(s) were prepared by using *E. coli* as substrate for the GSK-3β enzyme. For positive control reaction, the LKB1 enzyme was also prepared in COS-7 cells. As shown in Figure 5A, all Thr residues in the A-loop peptides, except for the wild type SIK3 peptide, were phosphorylated by LKB1 in vitro. GSK-3β also phosphorylated the Thr residues as long as the +4 Ser residues were not disrupted.

Because the peptides phosphorylated by GSK-3β migrated more slowly in the SDS–PAGE than those phosphorylated by LKB1, we checked for possible co-phosphorylation at Ser186 in the SIK1 peptide. As shown at the bottom of Figure 5A, this peptide when incubated with GSK-3β, but not with LKB1, was phosphorylated at Ser186, suggesting that GSK-3β, in vitro, may first phosphorylate Ser186 and then Thr182 in a +4-priming-dependent manner.

To examine whether the phospho-priming at Ser186 facilitated the phosphorylation at Thr182 by GSK-3β, peptides with phospho-Ser186 were synthesized (Fig. 5B). GSK-3β phosphorylated the phospho-primed peptides more efficiently than the non-primed peptides, indicating that the phosphorylation at Ser186 could accelerate phosphorylation at Thr182 by GSK-3β.

Next, we designed an in vitro system for monitoring the phosphorylation cascade from upstream of SIK1 to TORC. The recombinant

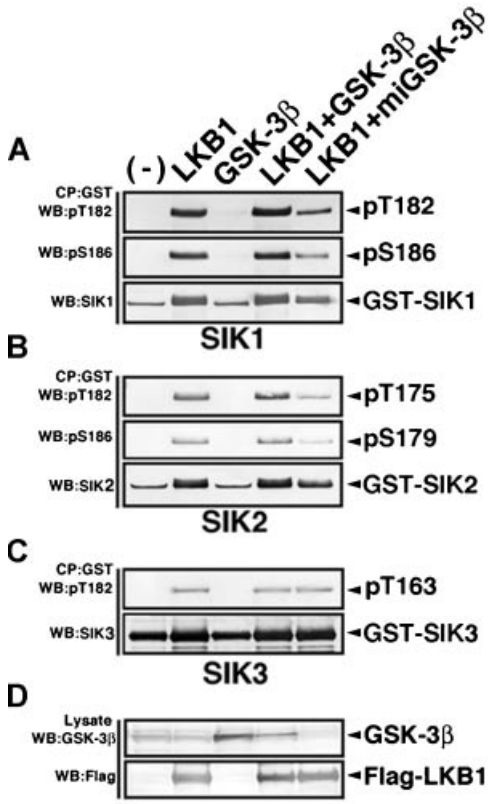

Fig. 6. LKB1 is essential for the regulation of SIK1 by GSK-3β in HeLa cells. HeLa cells were co-transformed with 2 μg of the plasmid for GST-fusion SIK1 (full-length) (**A**), SIK2 (full-length) (**B**) or SIK3 (1-340) (**C**) with or without the expression plasmid for LKB1, pFlag-7-LKB1 (2μg), for GSK-3β, pCMV-Sport6-GSK-3β (2 μg) or for miRNA against human GSK-3β, pcDNA3.1-mihGSK-3β (2 μg). For control transformation, an empty pCMV-Sport6 was used. After 48 h, GST-SIKs were purified by using glutathione columns (CP) and then subjected to western blot analyses (WB) using the indicated antibodies. Levels of GSK-3β and LKB1 in the cell lysates prepared for SIK1 purification were examined (**D**).

SIK1 kinase domain (1-354) prepared in *E. coli* was incubated with LKB1 or GSK-3β in the presence of ATP and then subjected to a phosphorylation assay using a TORC2-peptide. Wild type and kinase defective K56M SIK1s were almost equally phosphorylated by LKB1 and GSK-3β (Fig. 5C), but the S186A SIK1 mutant was phosphorylated only by LKB1, which is in agreement with the results obtained with the A-loop peptides (Fig. 5A). The K56M or the S186A mutant SIK1s could not phosphorylate TORC2 peptide, even when the Thr182 residue had been phosphorylated, indicating that the Ser residue at 186 is indispensable for the kinase activity of recombinant SIK1.

Although autophosphorylation at Ser186 seemed to be essential for the kinase activity of SIK1 in COS-7 cells (Fig. 2), the wild-type recombinant SIK1 activated by LKB1 appeared to exert its kinase activity without autophosphorylation at Ser186. However, we previously detected autophosphorylation in the non-activated recombinant SIK1 kinase domain when radio-labeled ATP was used in the kinase reaction [Lin et al., 2001]. This discrepancy may be a result of a technical problem, in that the active SIK1 protein that is, the SIK1 protein completely folded in *E. coli*, may be composed of a small population of the purified recombinant protein, which could make it difficult to detect phospho-Ser186 in LKB1-activated SIK1. Figure 5D-F show the levels of expressed enzymes or peptides indicated in Figure 5A–C.

LKB1 Is Essential for the GSK-3β-Mediated Regulation of SIK1

Although GSK-3β could phosphorylate Thr182 in vitro irrespective of SIK1 kinase activity (Fig. 5), no phosphorylation at Thr182 was detected in kinase-defective SIK1 in COS-7 cells (Fig. 3). These findings suggested that the GSK-3β-mediated phosphorylation at Thr182 in cultured cells may require initial activation of SIK1. To examine the involvement of LKB1 in the initiation cascade of GSK-3β-mediated activation of SIK1, we utilized LKB1-defective HeLa.

As shown in Figure 6A, LKB1 enhanced the level of phosphorylation of SIK1 at Thr182 and Ser186, which was accompanied by stabilization of the SIK1 protein, while GSK-3β alone had no effect. However, enhanced phosphorylation at Thr182 and Ser186 was observed when LKB1 was co-expressed with GSK-3β. Moreover, knockdown of GSK-3β with the miRNA reduced the LKB1-dependent phosphorylation at Thr182 and Ser186. These results suggest that GSK-3β alone may not be able to activate SIK1 in cultured cells and may exert its function as a modulator only after the activation of SIK1 by LKB1. This was also the case for SIK2 (Fig. 6B), but not for SIK3 (Fig. 6C). Figure 6D shows the levels of GSK-3β and LKB1 in the cell lysates.

Phosphorylation of the Ser Residue in the A-Loop of SIK1 and SIK2 Is Important for TORC2 Phosphorylation Activity in COS-7 Cells

We previously reported that the loss of LKB1 signaling resulted in the hypo-phosphorylation

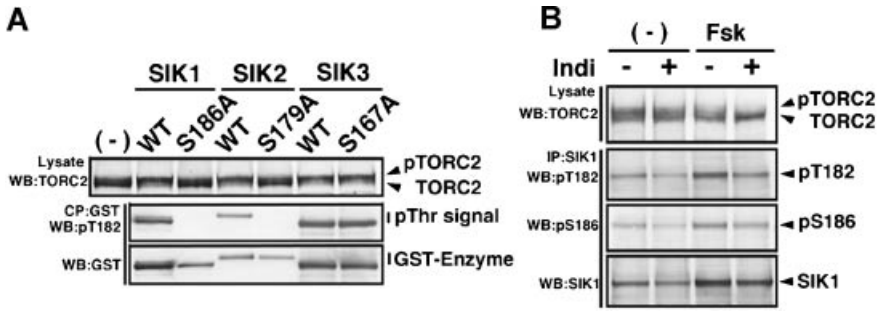

Fig. 7. Ser186-dependent regulation of TORC2. **A**: Cell lysates were prepared from COS-7 cells expressing GST-SIK1 (1-354), -SIK2 (1-348) or -SIK3 (1-340) and used for the detection of phospho-TORC2 (**upper panel**). Phospho-TORC2 (pTORC2) migrated more slowly in the SDS–PAGE than did dephospho-TORC2 (TORC2). The phosphorylation at Thr residues was detected with an anti-phospho-Thr182 SIK1 antibody (**middle panel**) using purified enzymes. An anti-GST antibody was used for the detection of GST-fusion enzymes (**lower panel**). **B**: COS-7 cells were treated with 10 μM of forskolin (Fsk) for 30 min in the presence or absence of Indi (20 μM). Phospho-TORC2 was detected using cell lysates. Phosphorylation levels of endogenous SIK1 were examined after purification by immunoprecipitation with anti-SIK1 antibody.

of TORC2 due to impaired activation of SIK family kinases [Katoh et al., 2006]. To determine the importance of the Ser residue in the A-loop of SIK for the regulation of endogenous substrates, we monitored the levels of TORC2 phosphorylation in COS-7 cells.

First, we tested the ability of wild type and Ser-disrupted SIK1-3 to phosphorylate endogenous TORC2. As expected, overexpression of active SIKs (wild type SIK1-3 and S167A SIK3) increased the ratio of phospho-TORC2/TORC2 (Fig. 7A).

Next, we examined the relation between the phosphorylation levels of endogenous SIK1 and TORC2. Forskolin (Fsk), an agonist of cAMP/PKA signaling, induces TORC2 dephosphorylation and stimulates CREB-mediated transcription. SIK1 is also a CREB target and induced by Fsk, which restores the phosphorylation level of TORC2 as a feedback mechanism in the cAMP/PKA signaling [Koo et al., 2005]. Without Fsk, the GSK-3β inhibitor Indi did not show a significant effect on the phosphorylation level of TORC2 (Fig. 7B), although the phosphorylation levels in the A-loop of SIK1 were reduced. However, a 30-min treatment with Fsk reduced the level of phospho-TORC2, and the dephosphorylation was accelerated by co-treatment with Indi. These results suggested that GSK-3β-mediated phosphorylation of the A-loop of SIK1 might act as an essential step in the feedback regulation of TORC2 in the cAMP signaling. It should also be mentioned here that COS-7 cells showed a slight expression of SIK3, but SIK2 could not be detected.

14-3-3 Binds to the C-Terminal Region of SIK1 in a Ser186-Dependent Manner

Although phosphorylation relay in the A-loop of SIKs could be essential for their kinase activity, we cannot speculate how the phosphorylation switches on the kinase activity, because no information is available about the molecular structures of active (phosphorylated) forms of AMPK-related kinases [Nayak et al., 2006; Panneerselvam et al., 2006]. Especially, to fix the fluctuation of the A-loop in MARK1 and MARK2, the corresponding Thr and Ser have to be substituted with Ala.

One possibility is that the phosphorylation induces structural change in the catalytic site, which may act as the switch for the activity. In addition to this, 14-3-3 proteins were found to bind to the A-loop of SIK1 in a phospho-Thr182 dependent manner, and this binding then enhanced SIK1's kinase activity [Al-Hakim et al., 2005]. 14-3-3 binds to motifs of R/KSXpS/TXP or RXXXpSXP (pS/T represents phospho-Ser/Thr) and modulates a variety of functions of target molecules [Tzivion and Avruch, 2002]. Because Pro188 is located at the +2 position of Ser186 (Fig. 1F), we decided to examine the potential of phospho-Ser186 to act as the acceptor for 14-3-3.

The kinase domain (D1–D2) and full-length SIK1 was overexpressed in COS-7 cells as a GST-fusion protein and purified with glutathione columns, after which 14-3-3 proteins associated with SIK1 were detected by Western blot analyses (Fig. 8). Unexpectedly, 14-3-3 was

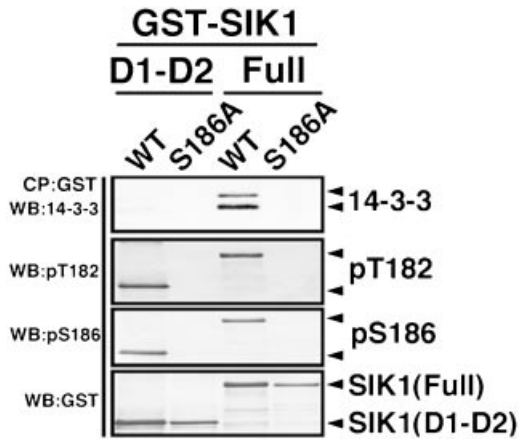

Fig. 8. 14-3-3 binds to the C-terminal region of SIK1 in a Ser186-dependent manner. GST-SIK1 (1-354: D1–D2) and GST-SIK1 (full-length) were expressed in COS-7 cells. After purification of SIK1 by using glutathione columns, the levels of associated 14-3-3 and phosphorylation(s) in the A-loop were examined by means of western blot analyses.

found to bind to only full-length SIK1, and the binding was eliminated as the result of a disruption at Ser186. This finding suggests that 14-3-3 may bind to autophosphorylation sites that are located in the C-terminal region, not in the A-loop, and that the binding of 14-3-3 is insufficient to explain the mechanism by which phosphorylation(s) in the A-loop activates the kinase activities.

DISCUSSION

Autophosphorylation is an important mechanism by which numerous kinases regulate their own biological activities [Hashimoto et al., 1987; Hanson et al., 1989; Rivers et al., 1998; Sato et al., 2002; Eswarakumar et al., 2005]. We also found that a PKA-phosphorylation site, Ser577 in the C-terminal regulatory domain of SIK1, is autophosphorylated and negatively regulates the TORC-phosphorylation activity of SIK1 [Katoh et al., 2006].

Here we report a newly discovered autophosphorylation site, Ser186, at the +4 position of Thr182 in the A-loop of SIK1. Phosphorylation at Thr182 is essential for switching on the SIK1 kinase activity [Lizcano et al., 2004]. The substitution of Ser186 with non-phosphorylatable residues eliminated SIK1 kinase activity both in vivo (Fig. 2) and in vitro (Fig. 5), suggesting that the autophosphorylation at Ser186 may also be essential for kinase activity.

AMPK-related kinases are composed of several families. The primary structure of the kinase domain of MARK/PAR-1 is most similar to that of SIK (Fig. 1F). Like SIK1 and SIK2, two of the MARK family kinases (MARK1 and MARK2) have been found to be phosphorylated at the +4 Ser position of critical Thr residues [Johnson et al., 1996]. Ser phosphorylation is speculated to be inhibitory, because the substitution of Glu for the Ser residue eliminated the kinase activity of MARK2 [Timm et al., 2003]. These findings indicate that the OH-group of Ser seems to be essential for the catalytic activities of MARKs.

The same appears to be true for SIK1 (Fig. 2A) and SIK2 (Fig. 2B). However, the phosphorylation of Ser186 by GSK-3β in vitro did not inhibit the kinase activity of SIK1 (Fig. 5C). Mutation analyses also indicated that the other phosphorylatable residue, Thr (Fig. 2B), but not Ala, Asp, Cys or Gly (Fig. 2A), could substitute for Ser at position 186. These results suggest that the phospho-group at Ser(s) may be required for the kinase activities of SIK1 and SIK2.

In contrast, the involvement of the Ser residues in the A-loop of SIK3 may be substantially different. The substitution of Glu for the Ser residue also eliminated the kinase activity of SIK3, but that of Ala showed no effect on the kinase activity. This agrees well with the finding that no autophosphorylation signal was observed in the kinase domain of SIK3 (Fig. 1B). Moreover, we previously reported a unique characteristic of the A-loop of SIK3. The substitution of Glu at Thr163 converted SIK3 into a constitutively active form, although the corresponding substitution of SIK1 and SIK2 (Thr182Glu and Thr175Glu, respectively) killed their kinase activity [Katoh et al., 2006]. These observations suggest that the structural requirements of the A-loop of SIK3 may be quite different from those of SIK1 and SIK2.

During this study we noticed another discrepancy, namely that LKB1 could phosphorylate critical Thr residues of all SIK isoforms in vitro [Lizcano et al., 2004], no matter whether the +4 Ser was intact or not (Fig. 5A). However, LKB1 apparently failed to phosphorylate the Thr residues in SIK1 and SIK2 mutants that had not been phosphorylated at the +4 Ser position in COS-7 cells (Fig. 3). In addition, two small compounds, Ro-318220 and Indi, inhibited the phosphorylation at the Thr residues of SIK1 and SIK2, but not of SIK3 or AMPKα1

(Fig. 4C), suggesting that, in addition to LKB1, as yet unknown kinases may also phosphorylate SIKs in COS-7 cells, and that this phosphorylation may depend on phospho-Ser at the +4 position.

GSK-3β phosphorylates Ser/Thr residues in peptides phospho-primed at the +4 position [Doble and Woodgett, 2003], while GSK-3β is inhibited by Ro-318220 and Indi. We therefore investigated the capability of GSK-3β to phosphorylate Thr182 in vitro and found that, indeed, GSK-3β could phosphorylate Thr182 in a Ser186-dependent manner (Fig. 5B). However, the in vitro phosphorylation of Thr residues in the A-loop peptides for SIK1 and SIK2 did not require phospho-primed Ser residues (Fig. 5A). In addition, the A-loop peptide of SIK3 was efficiently phosphorylated by GSK-3β in vitro, although it might not be the substrate for GSK-3β in COS-7 cells (Fig. 4C). A structural analysis of GSK-3β demonstrated that phospho-priming is not a precondition for the substrate when it is phosphorylated by GSK-3β in vitro [Frame et al., 2001], and this discrepancy may be attributed to the difference in substrate concentration/experimental condition between in vivo and in vitro. This may be one of the reasons for the discrepancy in SIKs' A-loops.

The first effect of the involvement of GSK-3β in SIK cascades was seen in the regulation of the transcription factor CREB [Fiol et al., 1994]. GSK-3β phosphorylates CREB at Ser129 by recognizing phospho-Ser133 as the priming site. Because the substitution of Ala for CREB at Ser129 impairs response to cAMP, GSK-3β has been proposed as an activator of CREB. Recently, this model had to be reconsidered because of findings that overexpression of GSK-3β inhibited CRE-dependent transcription induced by reactive oxygen species in cardiomyocytes [El Jamali et al., 2004] and that it was silenced by serum starvation in glioblastoma cells [Tullai et al., 2007]. Moreover, the inhibitory action of GSK-3β does not depend on the transcriptional activation domain of CREB in neuroblastoma cells [Hansen et al., 2004]. Instead, the co-activator TORC binds to the bZIP domain of CREB and transmits CREB's transcriptional-activity to transcription-machineries. On the other hand, SIKs negatively regulate TORC activities [Screaton et al., 2004], suggesting that, in particular cells, GSK-3β may negatively regulate CREB activity through the activation of SIKs, especially in the cAMP signaling cascade (Fig. 7). Recently, LiCl, other inhibitor of GSK-3β, was also found to be an activator of TORC in cAMP signaling [Boer et al., 2007]. It was, however, concluded that the action of LiCl did not depend on GSK-3β, because neither SB216763 nor SB415286 (other GSK-3 inhibitor) showed significant effect on CRE-dependent reporter activities.

Involvement of LKB1 in the regulation of GSK-3β signaling has been reported [Ossipova et al., 2003; Lin-Marq et al., 2005]. GSK-3β phosphorylates β-catenin in an LKB1-dependent manner, which could be explained by two mechanisms, either indirect activation of the catalytic activity of GSK-3β by dephosphorylation at the inactivating-phosphorylation site Ser9 [Lin-Marq et al., 2005], or activation of priming kinases by LKB1, such as MARK/PAR-1 [Drewes et al., 1997; Spicer et al., 2003]. Combination of these two mechanisms may produce a different regulation of LKB1-dependent signaling cascades including SIKs.

The hypothetical model (Fig. 9) suggested from the present by this phenomenon fails to

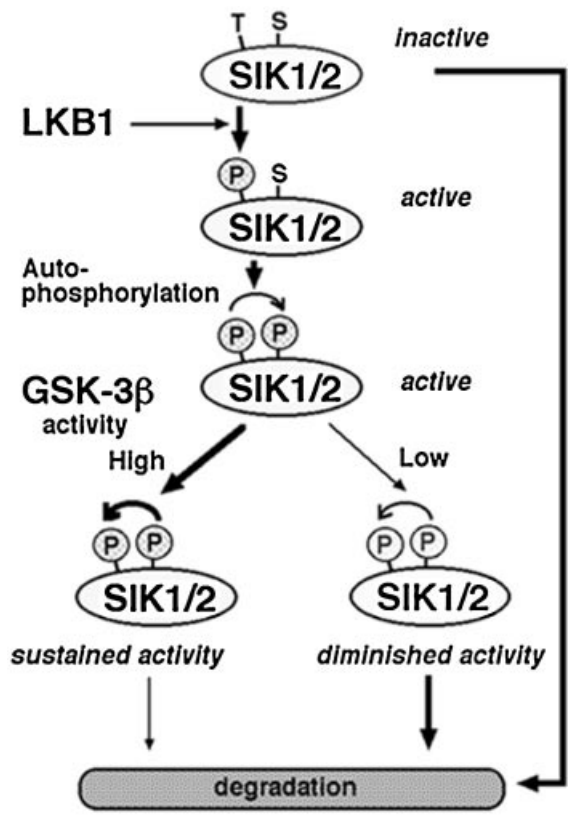

Fig. 9. A hypothetical model of LKB1-mediated regulation of SIK1/2.

clarify several points in this study. One is why the level of phospho-Thr182/Ser186 correlates with the SIK1 protein level. Lactacystin, an inhibitor of the ubiquitin-proteasome system, diminished the degradation of the dephosphoforms of SIK1 and SIK2 (Fig. 4C). Although AMPK-related kinases have a domain similar to the ubiquitin-associated domain, structural analysis of the domain in MARK2 has suggested there is less potential of ubiquitination of the domain [Panneerselvam et al., 2006]. As an alternative possibility, it has been proposed that Snf1lk (SIK1) induces degradation of the TGF-β receptor by phosphorylating Smad7, a transducer of TGF-β signaling, which results in ubiquitination of the TGF-β receptor and recruitment of the TGF-β receptor/SIK1 complex in proteasomes (http://www.diva-portal.org/demo/theses/abstract.xsql?dbid=5891). However, the negative correlation between the kinase activity of SIK1 and the recruitment to proteasomes has not yet been examined.

It was found that once staurosprine was added to the culture medium, it could tightly bind to the ATP pocket of SIKs and inhibit their kinase activity even after the purification of SIK enzymes [Katoh et al., 2006]. Interestingly, staurosporine enhances the phosphorylation of SIK1 at Thr182 and Ser186, although it inhibits autophosphorylation activity. It has also been reported that treatment of HaCaT keratinocytes with low-dose staurosporine upregulates GSK-3β by inhibiting its Ser9-kinases, such as PKA, PKC and AKT [Koivisto et al., 2003; Shankar et al., 2004]. This suggests that, like the in vitro reaction, uncontrolled GSK-3β activity in staurosporine-treated cells would maintain the levels of phosphorylation of SIK1 at Thr182/Ser186 in an autophosphorylation-independent manner. Further clarification of SIK-cascades may contribute to a better understanding of the complex mechanisms involved in physiological regulations.

ACKNOWLEDGMENTS

We would like to thank Dr. Lee A. Witters at Dartmouth College, Hanover, NH, USA, for providing us with the AMPKα1 expression plasmid.

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