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Phosphorylation of Ser^{246} Residue in Integrin-linked Kinase 1 by Serum- and Glucocorticoid-induced Kinase 1 is Required to Form a Protein-protein Complex with 14-3-3

Jaesun Chun and Sang Sun Kang[1]*

Department of Biology Education, Korea National University of Education, Chungbuk 363-791, Korea; [1]School of Science Education Chungbuk National University, Chungbuk 361-763, Korea

Abstract: Integrin-linked kinase 1 (ILK1) regulates several protein kinases, including PKB/Akt kinase and glycogen synthase kinase β. ILK1 is also involved distinctively in the cell morphological and structural functions by interacting with the components of the extracellular matrix or integrin. According to the information of serum- and glucocorticoid-induced kinase 1 (SGK1) substrate specificity (R-X-R-X-X-(S/T)-ϕ; ϕ indicates a hydrophobic amino acid), two putative phosphorylation sites, Thr^{181} and Ser^{246}, were found in ILK1. We showed that ILK1 fusion protein and two fluorescein-labeled ILK1 peptides, FITC-^{174}RTRPRNGTLN^{183} and FITC-^{239}CPRLRIFSHP^{248}, were phosphorylated by SGK1 *in vitro*. We also identified that 14-3-3 θ, ε and ζ among several 14-3-3 isotypes (β, γ, ε, η, σ, θ, τ and ζ) formed protein complex with ILK1 in COS-1 cells. Furthermore, the phosphorylation of Ser^{246} by SGK1 induced the binding with 14-3-3. It was also demonstrated that 14-3-3-bound ILK1 has reduced kinase activity. Thus, these data suggest that SGK1 phosphorylates Thr^{181} and Ser^{246} of ILK1 and the phosphorylation of its Ser^{246} makes ILK1 bind to 14-3-3, resulting in the inhibition of ILK1 kinase activity.

Key words: ILK1, SGK1, 14-3-3 isotypes, protein phosphorylation, protein-protein interaction

Integrin-linked protein kinase 1 (ILK1) was first identified with the β1 integrin subunit by using yeast two-hybrid screening (Dedhar et al., 1999; Hannigan et al., 1996). Structurally, ILK1 contains an N-terminal ankyrin repeat domain, a C-terminal kinase domain, and a pleckstrin homolgy (PH) motif. The kinase activity of ILK1 can be modulated by growth factors and cell components in the extracellular matrix (Dedhar et al., 1999; Hannigan et al., 1996). Novak and his colleagues reported that ILK1 overexpression results in translocation of β-catenin to the nucleus, formation of a complex with the LEF-1 transcription factor and activation of promoters containing the LEF-1-binding site (Novak et al., 1998; Novak and Dedhar, 1999). It was also demonstrated that ILK1 directly phosphorylates GSK-3 and PKB/Akt on Ser^{473}, whereas a kinase-deficient form of ILK1 severely inhibits PKB Ser^{473} phosphorylation (Delcommenne et al., 1998; Persad et al., 2001). These data suggest that ILK1 is a receptor-proximal effector of PI3K signaling, which regulates PKB through phosphorylation on Ser^{473} of PKB. Furthermore, ILK1 is capable of interacting with several other focal adhesion proteins including β1 intergrin, LIM-domain containing protein PINCH, calponin homology-containing CH-ILKBP (also known as actopaxin, or α-parvin), affixin (also known as β-parvin) and paxillin (Dedhar et al., 1999; Liu et al., 2000; Nikolopoulos et al., 2001; Tu et al., 1999; Tu et al., 2001; Wu et al., 2001; Yamaji et al., 2001).

14-3-3 proteins are intracellular, dimeric, phosphoserine-binding molecules that have been identified in eukaryotic organisms and they are found primarily in the cytoplasmic compartment of cells (Fu et al, 2000; Tzivion et al, 2001; van Hemert et al., 2001; Yaffe et al., 2001). There are eight mammalian members of the 14-3-3 family encoded by separate genes (β, γ, ε, η, σ, θ, τ, and ζ). Mammalian 14-3-3 proteins regulate tyrosine and tryptophan hydroxylases in neurotransmitter synthetic pathways. 14-3-3 proteins bind to PKC, PDK1, and Ask1 to inhibit their activities (Hashiguchi et al., 2000; Tzivion et al., 2002; van Hemert et al., 2001). 14-3-3 interacts with Raf-1 for Ras-dependent activation of Raf-1 and cell cycle protein phosphatase cdc25c for promoting cytoplasmic translocation of cdc25c

* To whom correspondence should be addressed.
Tel: 82-43-271-0526, Fax: 82-43-261-3278
E-mail: jin95324@chungbuk.ac.kr

(Sanchez et al., 1997). By binding to apoptosis-promoting protein BAD, 14-3-3 prevents BAD from binding to Bcl-X_L (Zha et al., 1996). Binding of 14-3-3 to its partners depends on phosphorylation of the Ser or Thr residue in the specific binding motif ($RX_{1-2}S/T*X_{2-3}S/T$ or $RX_{2-3}S/T*XP$; * indicates the residue to be phosphorylated) (Tzivion et al., 2002; Yaffe et al., 2001). Thus, it is important to characterize protein kinases that phosphorylate the Ser or Thr residue in the motifs and thereby create 14-3-3-binding sites.

Serum- and glucocorticoid-induced kinase 1 (SGK1) is a Ser/Thr protein kinase, which is transcriptionally regulated by serum and/or glucocorticoids in mammary epithelial cells and Rat-2 fibroblasts (Waldegger et al., 1997; Wester et al., 1993). SGK1 is also transcriptionally regulated by corticosteroids in several cell types (Bhargava et al., 2001; Loffing et al., 2001; Maiyar et al., 2003). Recent findings suggest that SGK1 is an important gene in the early action of corticosteroids on epithelial sodium re-absorption. SGK1 encodes a 49-kD protein kinase that shares 45-55% homology with the catalytic domain of protein kinase C, the cAMP-dependent protein kinase A, Akt1, and the ribosomal protein S6 kinase (Maiyar et al., 2003; Waldegger et al., 1997). It has been well documented that SGK1 transcripts are expressed in a variety of adult rat tissues, with the high expression in the thymus, lung, and ovary and with the detectable levels in the mammary gland and several other tissues. The cellular and tissue context strongly influence the expression of SGK1 depending on cell types and extra cellular stimuli (Leong et al., 2003; Sheppard et al., 2002).

However, SGK1 has been also identified to promote cell survival, the same function as Akt1 (Brunet et al., 2001; Lang et al., 2001; Park et al., 1999). A few substrate proteins of SGK1 have been characterized. They indicated that SGK1 plays a role in concert with Akt1 to propagate the effects of PI3K, including cell survival and cell cycle progression (Brunet et al., 2001; Lang et al., 2001). We also characterized that SGK1 phosphorylated MEKK3 and inhibited its kinase activity (Chun et al., 2002; Chun et al., 2003). However, many physiological substrates of SGK1 and their functional modulation by SGK1 remain yet to be identified. The consensus SGK1/Akt1 substrate site has been known as R-X-R-X-X-(S/T)-ϕ, where ϕ is any hydrophobic amino acid. The arginine residues were conserved at positions -5 and -3 relative to those of Ser/Thr residues to be phosphorylated (Chun et al., 2003; Kobayashi et al., 1999; Park et al., 1999). Thus, proteins containing such amino acid sequences seem to be potent substrates for SGK1 and Akt1 though it is not clear that every substrate protein of Akt1 is also a substrate of SGK1.

Based on the SGK1 substrate consensus sequence information, the putative SGK1 phosporylation sites, Thr^{181} and Ser^{246}, of ILK1 (Hannigan et al., 1996; Kobayashi and Cohen, 1999; Waldegger et al., 1997) were noted. Thus, whether the phosphorylation of Thr^{181} and Ser^{246} of ILK1 would affect the intrinsic kinase activity of ILK1 was investigated *in vivo* and *in vitro*. It was observed that two synthetic ILK1 peptides (FITC-^{174}RTRPRNGTLN^{183} and FITC-^{239}CPRLRIFSHP^{248}) and recombinant ILK1 were phosphorylated by SGK1, and that the kinase activity of phospho-ILK1 on the ILK1 substrate peptide was inhibited. Phosphorylation of Ser^{246} of ILK1 facilitated binding with 14-3-3. Using co-immunoprecipitation analyses, 14-3-3 θ, ε and ζ isotypes among several 14-3-3 isoforms (β, γ, ε, η, σ, θ, τ and ζ) were identified to be responsible for the protein complex formation with ILK1 in COS-1 cell. Thus, our observations suggest that SGK1 phosphorylates both Thr^{181} and Ser^{246} of ILK1 and the phosphorylation of its Ser^{246} by SGK1 inhibits its kinase activity through facilitating the association of 14-3-3 to ILK1, especially.

MATERIALS AND METHODS

Cell culture

COS-1 was purchased from ATCC. Media and supplements were obtained from GIBCO-BRL. The cell line was maintained in Dulbecco's Modified Essential Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL potassium penicillin, 100 mg/mL streptomycin, 2 mM glutamine and 20 mM sodium bicarbonate. The cells were incubated at 5% CO_2 with 95% humidity and in a 37°C chamber. The growth medium was changed every 3 days.

Antibodies

The monoclonal antibody against the hexahistidine epitope was purchased from InVitrogen. Antibodies against GFP, SGK1, 14-3-3, and ILK1 were purchased from Santa Cruz Biotechnology, Upstate Biotechnology, and Cell Signaling Technology.

DNA constructs and site-directed mutagenesis

ILK1 was cloned in pcDNA3.1/NT-GFP-TOPO (InVitrogen) from the human kidney cDNA library by PCR using two primers (forward primer; 5'-ATGGACGACATTTTCACT-3' and reverse primer; 5'-CTACTTGTCCTGCATCTT-3'). ILK1 was subcloned into prokaryotic expression vector pTrcHis-TOPO (InVitrogen) and eukaryotic expression vector pEGFP-C2 (BD Bioscience). 14-3-3 θ was cloned into a prokaryotic expression vector pET100/D-TOPO (InVitrogen, Carlsbad, CA) by PCR using two primers (forward primer; 5'-CACCATGGAGAAGACTGAGCTGATC-3' and reverse primer; 5'-TTAGTTTTCAGCCCCTTCTGC-3'), and it was subcloned into the eukaryotic expression vector pcDNA3 flag (C-term; InVitrogen).

To generate mutant ILK1 T181A/S246A construct,

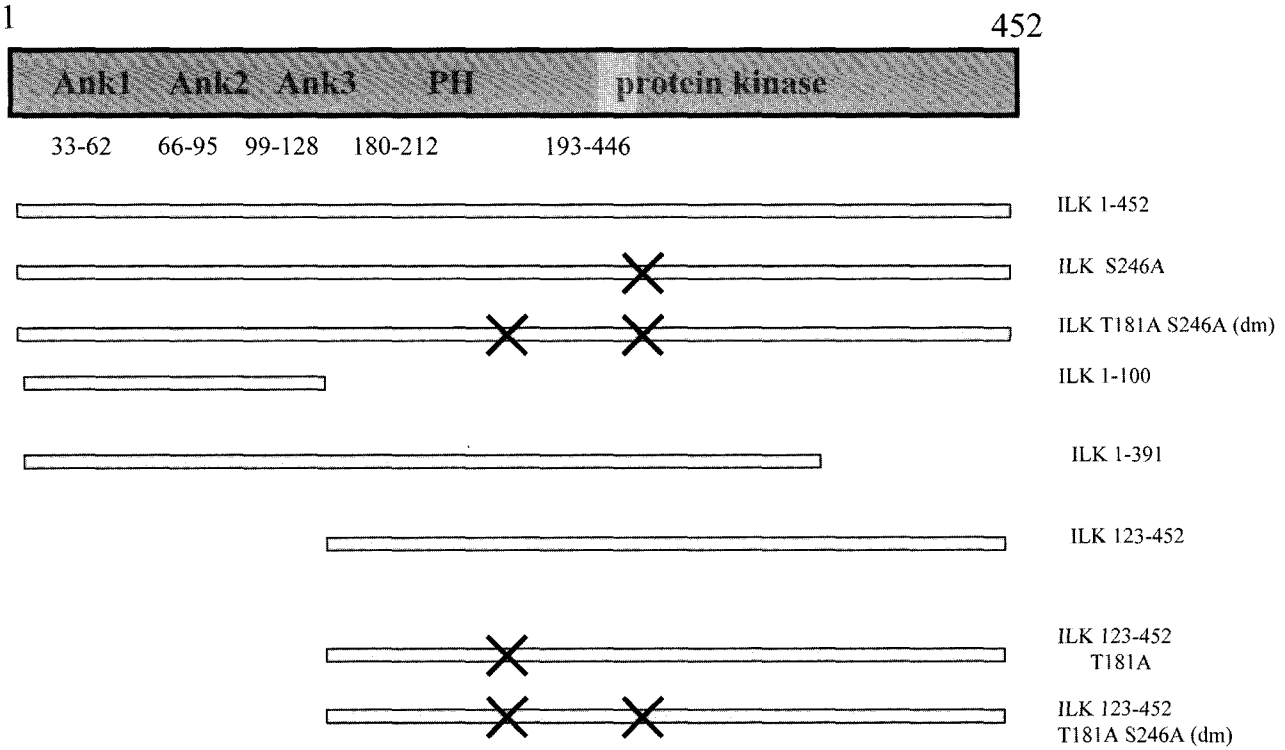

Fig. 1. Integrin linked kinase 1 domains and mutants. Functional domains and mutants of ILK1. There are three ankyrin-binding domains (Ank1, Ank2, and Ank3), a pleckstrin homolgy (PH) domain, and a protein kinase domain. Thr^{181}, Ser^{246} or (dm, both Thr^{181} and Ser^{246}) was replaced with alanine by site-directed mutagenesis (X indicates Thr^{181} or Ser^{246} residue positions). Two site-directed mutants and four deletion mutants with wild-type ILK1 were prepared.

two mutagenic primers (5'-ACCCTCCTCGGCCCCGA AATGGAGCTCTGAACAAA-3' for T181A and 5'-CCC CGGCTCAGGATATCCGCGCATCCAAATGTGCTCC-3 for S246A) and QuikChange Multi Mutagenesis Kit (Stratagene, West Cedar, Tx) was used, following the manufacturer's instructions. The mutation was confirmed by DNA sequencing. Deletion mutants and point mutants of ILK1 (ILK1 1-100, ILK1 1-139, ILK1 123-452, ILK1 123-452, ILK1 S246A, and ILK1 T181AS246A) were cloned into pTrcHis-TOPO as illustrated in Fig. 1.

Transfection and immunoprecipitation

Wild-type and mutant EGFP-ILK1 (S246A) constructs and GST-SGK1 (a gift from Dr. Alessi) were transfected or co-transfected into COS-1 cells using FuGENE6 (Roche Molecular Biochemicals) according to manufacturer's instructions. Cells (2×10^7) were lysed in 1 mL RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) for 10 min at 4°C. Cell lysates were then centrifuged at 12,000 g for 15 min at 4°C and the supernatant was pre-cleaned using a protein A agarose bead (Upstate Biochem). Anti-GFP and anti-14-3-3 antibodies were used to precipitate ILK1 and 14-3-3, respectively. The antibodies were incubated with the pre-cleaned supernatant for 16 h at 4°C and then precipitated using protein A agarose beads. To purify GST-SGK1, glutathione-Sepharose beads were used. The beads were washed three times with 1 mL of RIPA lysis buffer and then washed twice with PBS again. The final pellet was used for SGK1 or ILK1 assay or Western blot as described.

Hexahistidine-tagged wild-type and mutant ILK1 were also expressed in *Escherichia coli* XL1, and purified using Ni^{2+}-NTA agarose and glutathione-Sepharose beads following the manufacturer's instructions. Purified proteins were used for ILK1 kinase assay and pull down experiments. For pull down experiments, the hexahistidine-tagged wild-type or mutant ILK1 fusion proteins was pretreated with 0.5 μg of active SGK1 in 50 μL protein kinase assay buffer (20 mM HEPES pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 0.2 mM EGTA, and 20 mM ATP) for 1 h at 30°C, and the bead was washed three times with protein kinase assay buffer at 4°C. Then, the final washed bead was incubated with COS1 cell lysate for 1 h at 4°C to bind with 14-3-3. After washing three times with RIPA buffer, the bead was western blotted with 14-3-3 antibody.

Phosphorylation of recombinant ILK1

The purified and preheated ILK1 123-452 and ILK1 123-452 S246A proteins (30 pmol) were incubated with 0.5 μg of active SGK1 in 50 μL protein kinase assay buffer (20

mM HEPES pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 0.2 mM EGTA, 20 mM ATP, and 1 μCi [γ-^{32}P]-ATP) for 1 h at 30°C. To confirm the protein phosphorylation, the kinase reaction mixture was treated with 10 ng of alkaline phosphatase for 1 h at 30°C. The reaction mixture was loaded on 10% SDS-PAGE gel, and the dried gel was exposed on X-ray film.

Non-radioactive protein kinase assay

SGK1 or ILK1 assay was performed using fluorescein-conjugated SGK1 substrate peptides (FITC-^{174}RTRPRNGTLN^{183} and FITC-^{239}CPRLRIFSHP^{248}), the control peptides (FITC-^{174}RTRPRNGALN^{183} and FITC-^{239}CPRLRIFAHP^{248}), and ILK1 substrate peptide (FITC-^{336}SMADVKFSFQ^{345}), which were purchased from Peptron Co. The fluorescein-labeled oligopeptide (0.8 μg) was incubated with 10 μL of cell lysates, active ILK1 or SGK1 in 20 μL of protein kinase reaction buffer (20 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, 1 mM ATP) at 30°C for 30 min. The reactions were stopped by heating at 95°C for 10 min. The phosphorylated peptide was separated on 0.8% agarose gel at 100 V for 15 min. The phosphorylated products gained two more negative charge, and they were migrated to the anode. After electrophoresis, the gel was photographed on a transilluminator. The optical density of phosphorylated product was measured by densitometry (Kang et al., 1999; Kwon et al., 2000).

Immunofluorescence microscopy

COS-1 cells were plated about 30% of confluency on the Microscope cover glass (Fisher) in 6-well plates (Corning). Wild-type EGFP-ILK1 and mutant EGFP-ILK1 (S246A) constructs were transfected into COS-1 cell using FuGENE6 (Roche Molecular Biochemicals) as manufacturer's instructions. Cells were serum starved for 36 h and subsequently treated with 10% calf serum for 15 h. Cell confluency was kept not to exceed 60%. Cells were fixed, permeabilized, and processed for direct (ILK1) or indirect (14-3-3) immunofluorescence microscopy as described previously with minor modifications (Han et al., 2000; Shin et al., 2005). Cells were blocked in 5% BSA in PBS for 1 h and incubated with 1 : 1000 diluted rabbit anti-14-3-3 θ antibodies (Santa Cruz Biotech.) for 2 h at room temperature. For the indirect (14-3-3) immunofluorescence microscopy, the washed slides were incubated for 1 h at room temperature with 1 : 200 diluted goat anti-rabbit Alexa Flour 568 (Molecular Probes Inc.). Slides were washed and then mounted with Dako fluorescent mounting medium (Dako Co.) and examined using the optics at Center for Research Facilities of Chungbuk National University.

RESULTS

Phosphorylation of the recombinant ILK1 proteins and the ILK1 peptides by SGK1

SGK1 recognizes the amino acid sequence, R-X-R-X-X-(S/T)-ϕ, where ϕ is any hydrophobic amino acid (Chun et al., 2003; Kobayashi et al., 1999; Park et al., 1999). Using this information, we noticed two putative SGK1 phosphorylation sites in ILK1 (Dedhar et al., 1999; Hannigan et al., 1996). They are Thr^{181} in the stretch of ^{174}RTRPRNGTLN^{183} and Ser^{246} in the stretch of ^{239}CPRLRIFSHP^{248}. To determine whether SGK1 phosphorylates ILK1, SGK1 was incubated in the presence of [γ-^{32}P] ATP with ILK1 123-452 and ILK1 123-452 T181A S246A purified from recombinant *E. coli*. As shown in Fig. 2A, the recombinant wild type ILK1 123-452 protein was phosphorylated by SGK1, whereas the mutant ILK1 123-452 T181AS246A, with both Thr^{181} and Ser^{246} replaced with alanine, was not phosphorylated by SGK1. To ascertain the phosphorylation, the reaction mixture was treated with alkaline phosphatase, after which the phosphorylated protein bands disappeared (Fig. 2A, middle lane). To rule out the possibility that an unknown kinase associated with the reaction preparation may have phosphorylated ILK1, we performed the kinase assay with the kinase dead SGK1 (inactivated with heat), and observed that the kinase dead SGK1 did not phosphorylate ILK1 fusion protein (Fig. 2A). To further determine that Thr^{181} and Ser^{246} residues of ILK1 were phosphorylated by SGK1, the fluorescein-labeled ILK1 peptides, FITC-^{174}RTRPRNGTLN^{183} and FITC-^{239}CPRLRIFSHP^{248}, were incubated with SGK1. As shown in Fig. 2B and C, the amount of phosphorylated ILK1 peptide increased with increased SGK1. Without SGK1, there was no detectable amount of phosphorylated product (Fig. 2B and C, right lane). To verify the phosphorylation of FITC-^{174}RTRPRNGTLN^{183} and FITC-^{239}CPRLRIFSHP^{248} in Fig. 2B and C, we used FITC-^{174}RTRPRNGALN^{183} and FITC-^{239}CPRLRIFAHP^{248} peptide for their controls. We did not observe any phosphorylation product of FITC-^{174}RTRPRNGALN^{183} and FITC-^{239}CPRLRIFAHP^{248} peptides with/without SGK1 (Fig. 2D). To further confirm the phosphorylation of ILK1 Thr^{181} and Ser^{246} *in vivo*, EGFP-ILK1 (wt) and EGFP-ILK1 T181A S246A (dm) were transfected in COS-1 cell and ILK1 protein was purified immuno with GFP antibody. Western blot was performed with ILK1 antibody or Anti-phospho Thr/Ser antibody. As shown in Fig. 2E, ILK1 T181A S246A was 10 times less phosphorylated than ILK1 (wt). Therefore, these observations demonstrated that SGK1 phosphorylates both Thr^{181} and Ser^{246} of ILK1.

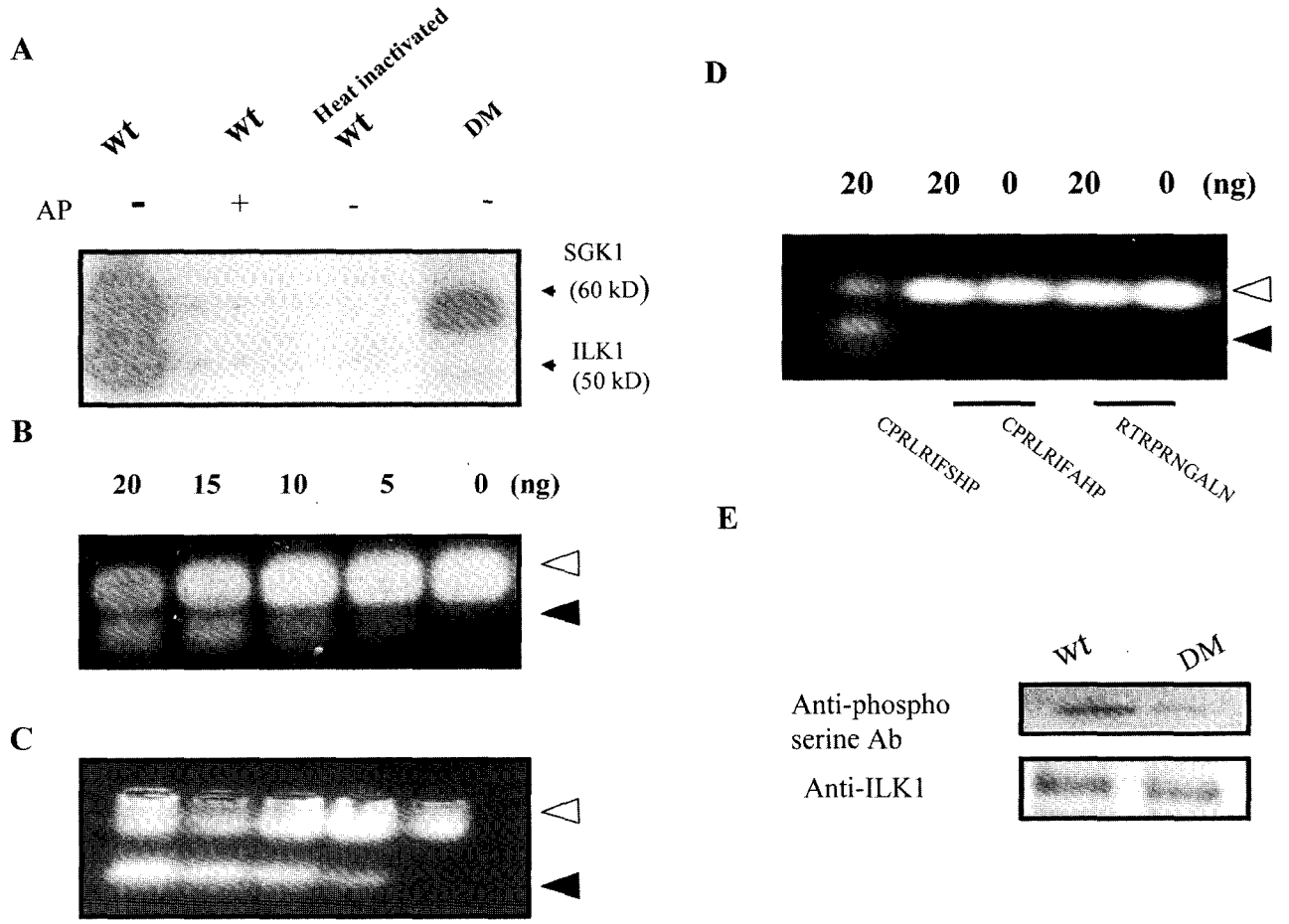

Fig. 2. Phosphorylation of recombinant ILK1 proteins and ILK1 peptides by SGK1. To determine whether SGK1 phosphorylates ILK1, SGK1 was incubated in the presence of [γ -^{32}P] ATP with wild-type ILK1 123-452 (wt) and ILK1 123-452 (T181A S246A, dm) purified from recombinant *E. coli*. Alkaline phosphatase (AP) was treated to confirm the phosphorylation of the proteins. The kinase dead SGK1 (heat inactivated) fails to phosphorylate ILK1 substrates. The phosphorylated ILK1 and SGK1 are indicated (A). To specify phosphorylation sites in ILK1, two synthetic ILK1 peptides, FITC-^{174}RTRPRNGTLN^{183} (B) and FITC-^{239}CPRLRIFSHP^{248} (C) were incubated with the indicated amounts of SGK1. To control the phosphorylation of FITC-^{174}RTRPRNGTLN^{183} (B) and FITC-^{239}CPRLRIFSHP^{248} (C), we used FITC-^{174}RTRPRNGALN^{183} and FITC-^{239}CPRLRIFAHP^{248} as the negative controls (D). To define the phosphorylation product, we used FITC-^{239}CPRLRIFSHP^{248} peptide (the right lane of Fig. 1D). The upper lane indicates the amount of SGK1 and the bottom lane indicates the FITC conjugated peptide sequences. The phosphorylated product (◀) and unphosphorylated substrate (◁) are marked. To verify the phosphorylation of ILK1, EGFP-ILK1 (wt) and EGFP-ILK1 T181A S246A were transfected and immunopurified ILK1 protein with GFP antibody. The western blot was performed with ILK1 antibody or an anti-phospho Thr/Ser residue antibody (E). Figures represent three independent experiments.

Formation of protein-protein complex between ILK1 and 14-3-3 in COS-1 cell

It has been reported that 14-3-3 binding partners contains the specific binding motif like R-X$_{(2-3)}$-pS-X-P and R-X$_{(1-2)}$-pS-X$_{(2-3)}$-S, where pS indicates a phospho-serine (Yaffe et al., 2001; Tzivion et al., 2002). A 14-3-3 binding motif was predicted around Ser^{246} of ILK1, that is, R-I-F-pS-H-P (Hannigan et al., 1996; van Hemert et al., 2001). Therefore, immunoprecipitation experiments were performed to determine whether ILK1 forms a protein complex with 14-3-3 in COS-1 cell, and it was observed that ILK1 and 14-3-3 precipitate together (Fig. 3A). It has been reported that there are eight known 14-3-3 isotypes (β, γ, ε, η, σ, θ, τ, and ζ). Thus, we tested which 14-3-3 isotypes are responsible to bind ILK1 in COS1 cell. As shown in Fig. 3B, we found that ILK1 prefers to form protein complexes with 14-3-3 ε, ζ, or θ. Together, these results suggested that ILK1 forms a protein complex with 14-3-3 in COS1 cell. Next, we determined that the phosphorylation of Ser^{246} (not Thr^{181}) residue in ILK1 is responsible to bind 14-3-3. For this purpose, His six conjugated several deletion proteins (ILK1 1-100, ILK1 1-391, ILK1 123-452, ILK1 123-452 T181A, ILK1 123-452 T181A S246A) were prepared and phosphorylated with SGK1 as described in Material and Method. Using these proteins and COS 1 cell lysates, we performed the pull down assay. As shown in Fig. 3C, ILK1 1-391 and ILK1 123-452 T181A, of which Ser246 was intact, pulled down 14-3-3 from COS1 cell lysate.

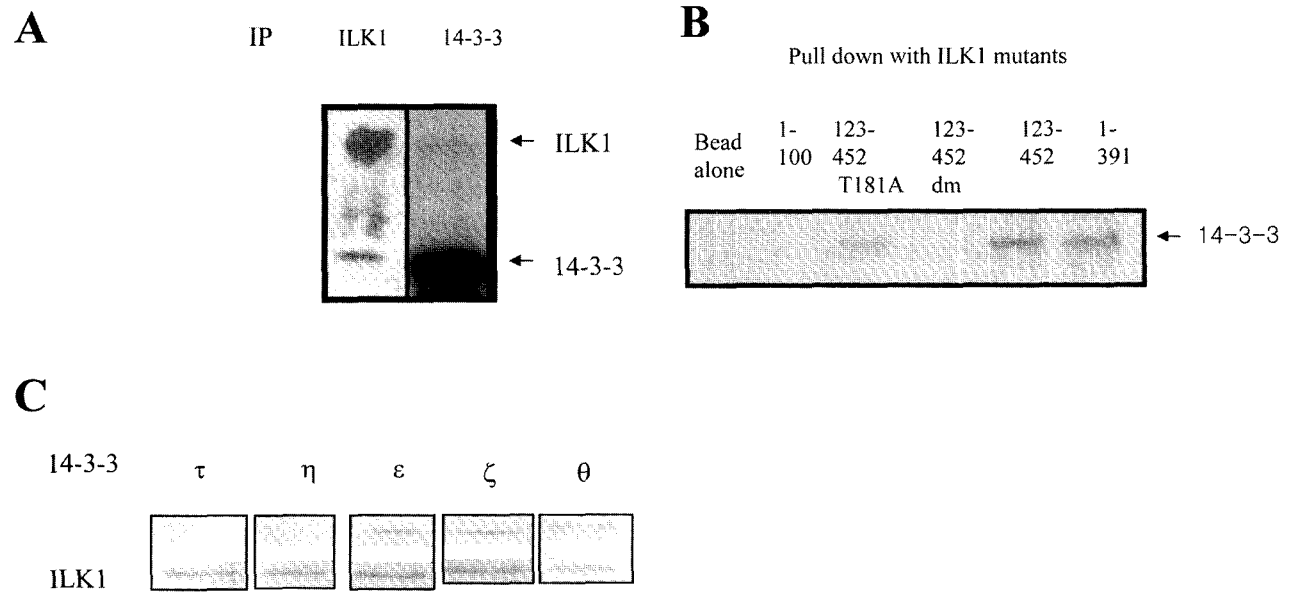

Fig. 3. Formation of a protein-protein complex between ILK1 and 14-3-3. To verify formation of ILK1-14-3-3 complex, ILK1 and 14-3-3 immunoprecipitates were analyzed using both anti-14-3-3 and anti-ILK1 antibodies, respectively (A). From COS-1 cells, ILK1 was precipitated using anti-ILK1 antibody. To define 14-3-3 binding domain in ILK1, hexahistidine-tagged ILK1 mutants were expressed in E. coli and pre-bound to Ni^{2+}-NTA agarose beads. The beads were incubated with COS-1 cell lysates and analyzed using anti-14-3-3 antibody (B). The immunocomplex was analyzed using 14-3-3-isotype specific antibodies as indicated above. In the same samples, ILK1 was quantitated by western blot analysis (C).

However, ILK1 1-100 and ILK1 123-452 T181A S246A (double mutant) failed to recruit 14-3-3. As we expected from the analysis of 14-3-3 binding motif (Fig. 1), these data indicated that ILK1 interacted with 14-3-3, and Ser^{246} residue of ILK1 was critical for this association (Fig. 3C).

Confocal microscopy with EGFP-ILK1 and 14-3-3θ

To test the co-localization ILK1 and 14-3-3θ *in vivo*, we performed confocal microscopy with EGFP-ILK1 and 14-3-3θ. As shown in Fig. 4A, the confocal microscopic images showed that ILK1 and 14-3-3θ localized together in the cytosol. However, we did not observe co-localization of EGFP-ILK1 S246A and 14-3-3θ (Fig. 4B). These results also supported that the phosphorylation of Ser^{246} residue is important for the 14-3-3 binding, consistant with the result of Fig. 3C. Therefore, these data suggested that ILK1 forms a protein complex with 14-3-3 in COS-1 cell and the phosphorylation of ILK1 Ser^{246} residue by SGK1 is required for the protein complex formation with 14-3-3.

14-3-3θ inhibits autophosphorylation activity of ILK1

To understand the biological roles of complex formation between 14-3-3θ and ILK1, we carried out ILK1 protein kinase assay with/without 14-3-3θ *in vitro*. 20 ng of immuno purified ILK1 and ILK1 S246A were pre-incubated with the different amount of 14-3-3θ for 20 min at 30°C, and ILK1 kinase assay was performed using fluorescein-conjugated ILK1 peptide, FITC-^{336}SMADVKFSFQ^{345}, mimicking the ILK1 self-phosphorylation site around Ser^{343} of ILK1 (Delcommenne et al., 1998; Tu et al., 2001). As shown in Fig. 5, the kinase activity of ILK1 was reduced by 14-3-3θ pretreatment. However, ILK1 S246A was constantly active regardless of 14-3-3θ treatment. Therefore, these results demonstrated that 14-3-3θ plays a role to inhibit the protein kinase activity of ILK1 *in vitro*.

SGK1 inhibits the kinase activity of ILK1 by the induction of 14-3-3 binding

The Ser^{246} residue of ILK1 demonstrated to be a SGK1 target site as well as a 14-3-3 binding site (Fig. 2 and 3). Therefore, the phosphorylation of ILK1 by SGK1 seemed tightly related with protein-protein interaction between ILK1 and 14-3-3θ. To estimate the influence of Ser^{246} phosphorylation by SGK1 on binding between 14-3-3θ and ILK1, COS-1 cells were transfected with EGFP-ILK1, EGFP-ILK1 S246A and GST-SGK1 at different DNA ratio of ILK1 to SGK1 (0:0, 1:1, 1:2, 1:4, and 1:6). To monitor expression level of the transfected SGK1, western blot analyses were performed. Expression of SGK1 increased along with transfected DNA concentration (Fig. 6E and F). In the same cell lysates, SGK1 was partially purified using gluthathione-Sepharose beads and SGK1 activity was measured using fluorescein-conjugated SGK1 substrate peptide (FITC-^{239}CPRLRIFSHP^{248}). The activity of SGK1 also increased in proportion to the transfected DNA (Fig. 6C and D). ILK1 was immuno purified using

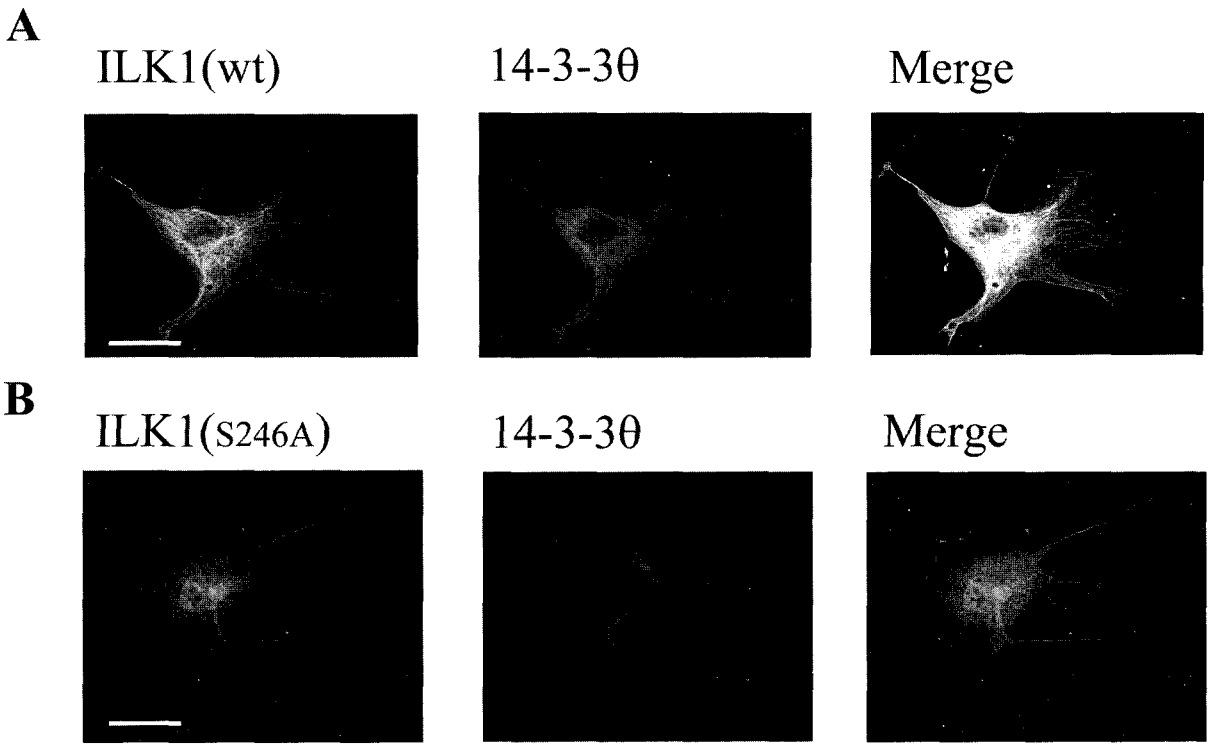

Fig. 4. Confocal microscopy. In COS-1 cell, the transfected EGFP-ILK1 or EGFP-ILK1 S246A (green) was shown directly. 14-3-3 θ (red) was visualized using their appropriate primary antibodies, and Alexa Flour 568-conjugated secondary antibodies. Merged image (yellow) shows coincident distribution of the wild type ILK1 and 14-3-3 θ (A). No merged image (yellow) was showed with the EGFP- ILK1 S246A and 14-3-3 θ (B). Figures represent three or more independent experiments. Scale bars = 10 μm.

anti-GFP antibody and quantitated using anti-ILK1 antibody. The wild type and mutant ILK1 precipitated constantly (Fig. 6G and H). In the ILK1 immuno-complexes, the amount of 14-3-3 proteins was measured

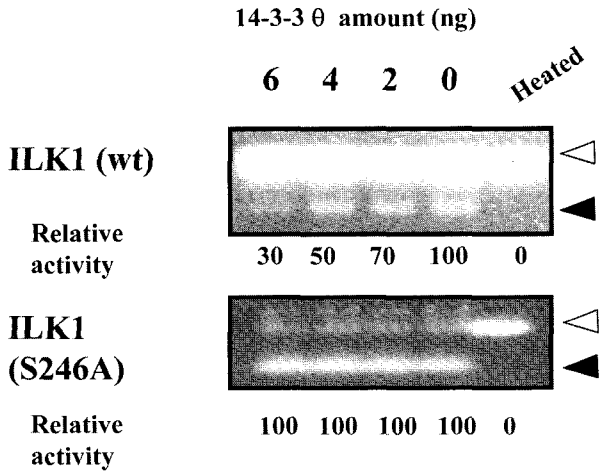

Fig. 5. Binding of 14-3-3 inhibits self-phosphorylation activity of ILK1. 20 ng of ILK1 and ILK1 S246A was pretreated with the indicated amounts of 14-3-3 θ for 20 min at 30°C, and ILK1 kinase assay was performed using ILK1 substrate peptide (FITC-^{336}MADVQFSFQC^{345}). Wild-type ILK1 (A) was inhibited in a 14-3-3 concentration dependent manner but ILK1 S246A (B) was constantly active. Figures represent three independent experiments.

using 14-3-3β monoclonal antibody which recognizes all 14-3-3 subtypes. The wild-type ILK1 recruited 14-3-3 but the mutant failed to precipitate 14-3-3 (Fig. 6I and J). In the same immuno-complexes, ILK1 kinase activity was measured using flourescein-conjugated ILK1 substrate peptide (FITC-^{336}SMADVKFSFQ^{345}). The mutant ILK1 S264A was constantly active (Fig. 6B), regardless of SGK1 activity (Fig. 6D). However, the wild-type ILK1 activity decreased (Fig. 6A), according to SGK1 activity (Fig. 6C). Therefore, in summary, these results suggested that SGK1 phosphorylates both Thr^{181} and Ser^{246} and the phosphorylation by SGK1 at Ser^{246} inhibits ILK1 through recruiting 14-3-3 to ILK1 in vivo (Fig. 7).

DISCUSSION

Identification and characterization of SGK1 substrate proteins may provide clues to understanding how SGK1 contributes to cell survival and death (Brunet et al., 2001; Lang et al., 2001; Park et al., 1999). Cell growth signals activate ILK1, in turns; ILK1 phosphorylates PKB/Akt1 on Ser^{473} (equivalent to Ser^{422} of SGK1) (Dedhar et al., 1999; Delcommenne et al., 1998; Hannigan et al., 1996; Tu et al., 2001). It is interesting that apoptosis signal-regulating kinase 1/2 (ASK1/2) and mitogen activated protein kinase kinase kinase 3 (MEKK3) contain SGK1/Akt1 pho-

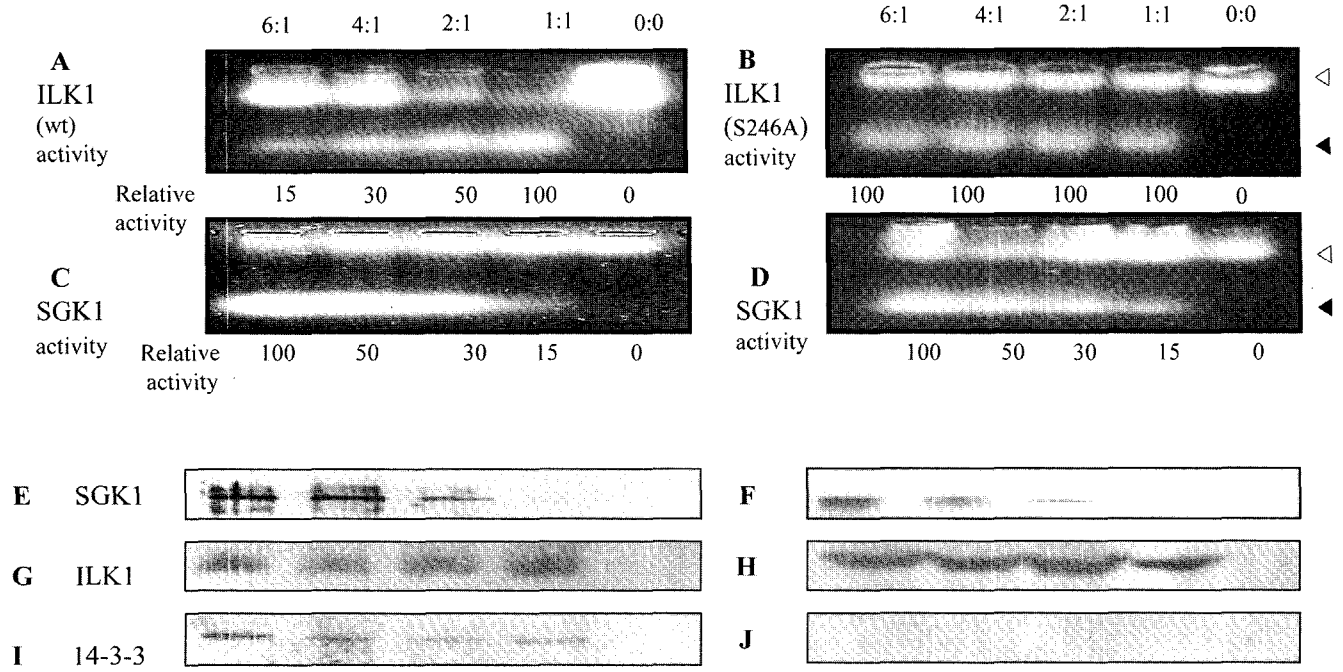

Fig. 6. The association of ILK1 with 14-3-3 in COS cells. COS-1 cells were transfected in different ratio of GST-SGK1 to GFP-ILK1 wt (right panel) or GFP-ILK1 S246A (left panel), as indicated above. In the cell lysates, the expression of SGK1 was monitored using anti-SGK1 antibody (E and F). SGK1 was partially purified using the gluthathione-sepharose beads and the activity was monitored using SGK1 substrate peptide (FITC-^{239}CPRLRIFSHP^{248}) (C and D). ILK1 was precipitated using an anti-GFP antibody and blotted using an anti-ILK1 antibody (G and H). In the ILK1 immunoprecipitates, the amount of 14-3-3 was quantitated using an anti-14-3-3 β antibody (I and J), and the ILK1 kinase assay was performed using ILK1 substrate peptide (FITC-^{336}SMADVKFSFQ^{345}) (A and B). The activity of wild-type ILK1 (A) was inhibited, according to the increase of both SGK1 activity (C) and 14-3-3 association (I). However, ILK1 S246A (B) was constantly active, regardless of the increase of SGK1 activity (D). ILK1 S246A did not form a protein complex with 14-3-3 (J). The bead treated with the untransfected cell lysate was used as the negative control of SGK1 or ILK1 assay. The relative protein kinase activity was indicated below. The phosphorylated peptide product (◀) and the unphosphorylated peptide substrate (◁) are marked. Figures represent three or more independent experiments.

sphorylation sites. Recently, MEKK3 was also found to be inhibited by SGK1 (Chun et al., 2003). Because both ASK1/2 and MEKK3 are involved in SEK1-SPAK1/2 and SGK1/MKK6-SAPK signaling cascades, it seems that SGK1/Akt1 functions on ASKs/MEKK3 antagonistically. We found here that ILK1 is one of the SGK1 substrate proteins with the consensus motif (RxRxxS/TX; X is a hydrophobic amino acid) (Fig. 1) and that both Thr^{181} and Ser^{246} of ILK1 were phosphorylated by SGK1 (Fig. 2). Because of the substrate protein similarity between Akt and SGK1, Akt kinase may also phosphorylate both Thr^{181} and Ser^{246} of ILK1 and contribute to the binding between ILK1 and 14-3-3. Whether Akt phosphorylates both Thr^{181} and Ser^{246} of ILK1 remains to be characterized.

We found that ILK1 forms protein complexes with 14-3-3 ε, ζ, or θ isotypes in COS-1 cells (Fig. 3), and the phosphorylation of ILK1 Ser^{246}, but not Thr^{181}, by SGK1 enhances the association between 14-3-3 and ILK1 (Fig. 3C, 4-6). Furthermore, we demonstrated that auto-phosphorylation activity of ILK1 on Ser^{343} is inhibited by phosphorylation by SGK1, followed by interaction between ILK1 and 14-3-3 (Fig. 5 and 6). Thus SGK1 seems to inhibit ILK1 signaling negatively in cooperation with 14-3-3 (Fig. 7).

However, it is presently unclear whether the phosphorylation of ILK1 Thr^{181} residue by SGK1 also contributes to the association with 14-3-3 or control of ILK1 kinase activity. Because Thr^{181} residue is close to PH domain of ILK1, the phosphorylation seems to regulate ILK1 subcellular localization.

It was also reported that ILK1 overexpression results in translocation of β-catenin to the nucleus, resulting in activation of LEF-1 transcription factor (Novak et al., 1998). ILK1 directly phosphorylates GSK-3 and PKB/Akt, whereas a kinase-deficient form of ILK1 severely inhibits PKB Ser^{473} phosphorylation (Persad et al., 2001). Thus, it seems that ILK1 as one of receptor-proximal effectors of PI3K signaling regulates the kinase activity of PKB through its Ser^{473} (equivalent to SGK1 Ser^{422}) phosphorylation, suggesting that ILK1 is one of the upstream components in SGK1 signal transduction (Fig. 6). In our preliminary results using recombinant SGK1 protein and peptide substrates, we also observed that ILK1 phosphorylates SGK1 on Ser^{422} (data not shown). Several other data, however, are in disagrement with the notion that ILK1 functions as the putative PDK2 to activate PKB through phosphorylation on Ser^{473} of PKB (Hill et al., 2002; Hodgkinson et al., 2002; Hresko et al., 2003). It was also

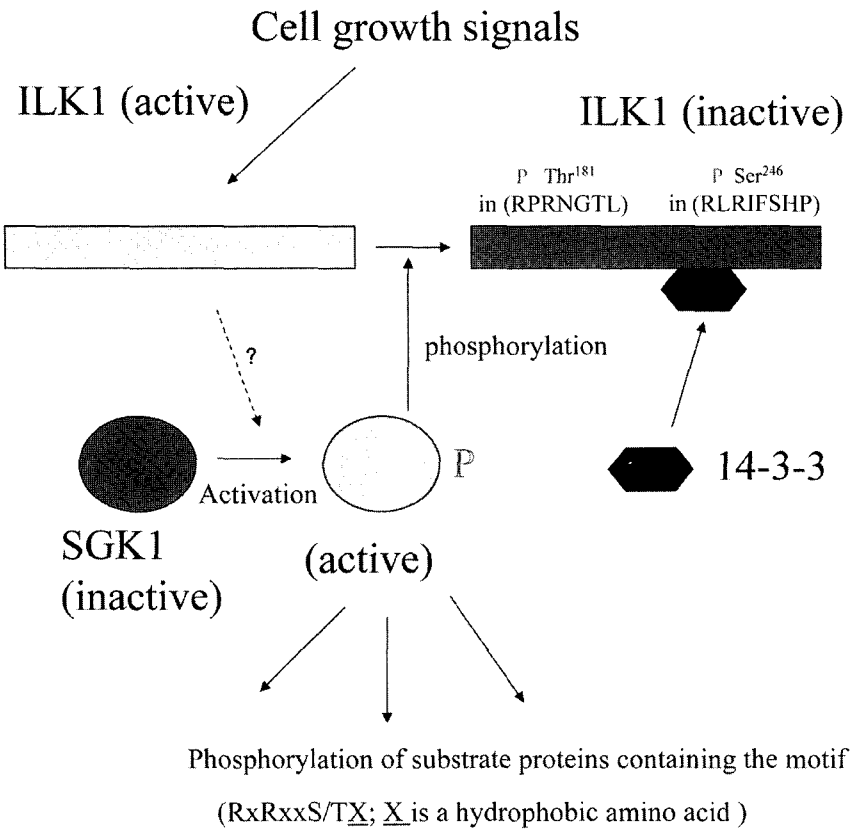

Fig. 7. Schematic diagram for the interaction among ILK1, SGK1 and 14-3-3. The kinase activity of ILK1 is stimulated by cell signals. SGK1 which is a potent substrate of ILK1 propagates signals to downstream through the phosphorylation on Ser/Thr residue of the motif (RxRxxS/TX; X is a hydrophobic amino acid). SGK1 phosphorylates both Thr^{181} and Ser^{246} residues of ILK1. The phosphorylation on Ser^{246} residue of ILK1 results in the enhancement of 14-3-3 binding which, in turn, inhibits its kinase activity.

reported that ILK1 functions primarily as an adaptor protein within the integrin adhesion complexes in *C. elegans* (Lin et al., 2003; Mackinnon et al., 2002). Therefore, it is still unclear whether ILK1 participates in the PI3K/Akt signal transducion pathway.

If ILK1 is an upstream component of SGK1 signal transduction pathway (Persad et al., 2001), both the phosphorylation of ILK1 by SGK1 and the binding of ILK1 by 14-3-3 are likely to make up a feedback regulation mechanism (Fig. 7). Otherwise, ILK1, as an adaptor protein within integrin adhesion complexes, may be regulated by SGK1 phosphorylation. Although our data showed that self-phosphorylation activity of ILK1 is inhibited through SGK1 phosphorylation (Fig. 4-7), whether ILK1 functions as adaptor protein within integrin adhesion complexes in the downstream of SGK1 remains to be characterized. Furthermore, it is presently unclear whether the self-phosphorylation activity of ILK1 is related with the regulation of its protein kinase activity or protein-protein interaction. We are currently pursuing the function of ILK1 self-phosphorylation.

Here, we present evidence that 14-3-3 is also an ILK1-interacting protein, but whether 14-3-3 binding to ILK1 affects the assembly of cell adhesion complex is not fully understood. There are eight known 14-3-3 isotypes (β, γ, ε, η, σ, θ, τ, and ζ), which form a homo or hetero dimer. Among the isotypes, we found that 14-3-3θ, ε and ζ isotypes form a complex with ILK1 in COS-1 cell. However, we cannot rule out the possibility that these differences represent differential expression of 14-3-3 isotypes in COS-1 cells rather than binding affinity of each isotype to ILK1.

In conclusion, we found that SGK1 phosphorylates Thr^{181} and Ser^{246} of ILK1 using recombinant ILK1 fusion proteins and fluorescein-labeled ILK1 peptides. We also observed that the phosphorylation of Ser^{246}, but not Thr^{181} enhances 14-3-3 binding and inhibits the auto-phosphorylation activity of ILK1. Thus, these results suggest that SGK1 inhibits ILK1 in a feedback mechanism in cooperation with 14-3-3 (Fig. 7).

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