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Regulation of Transforming Growth Factor-β Signaling and PDK1 Kinase Activity by Physical Interaction between PDK1 and Serine-Threonine Kinase Receptor-associated Protein*

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To gain more insights about the biological roles of PDK1, we have used the yeast two-hybrid system and *in vivo* binding assay to identify interacting molecules that associate with PDK1. As a result, serine-threonine kinase receptor-associated protein (STRAP), a transforming growth factor-β (TGF-β) receptor-interacting protein, was identified as an interacting partner of PDK1. STRAP was found to form *in vivo* complexes with PDK1 in intact cells. Mapping analysis revealed that this binding was only mediated by the catalytic domain of PDK1 and not by the pleckstrin homology domain. Insulin enhanced a physical association between PDK1 and STRAP in intact cells, but this insulin-induced association was prevented by wortmannin, a phosphatidylinositol 3-kinase inhibitor. In addition, the association between PDK1 and STRAP was decreased by TGF-β treatment. Analysis of the activities of the interacting proteins showed that PDK1 kinase activity was significantly increased by coexpression of STRAP, probably through the inhibition of the binding of 14-3-3, a negative regulator, to PDK1. Consistently, knockdown of the endogenous STRAP by the transfection of the small interfering RNA resulted in the decrease of PDK1 kinase activity. PDK1 also exhibited an inhibition of TGF-β signaling with STRAP by contributing to the stable association between TGF-β receptor and Smad7. Moreover, confocal microscopic study and immunostaining results demonstrated that PDK1 prevented the nuclear translocation of Smad3 in response to TGF-β. Knockdown of endogenous PDK1 with small interfering RNA has an opposite effect. Taken together, these results suggested that STRAP acts as an intermediate signaling molecule linking between the phosphatidylinositol 3-kinase/PDK1 and the TGF-β signaling pathways.

Transforming growth factor-β (TGF-β)[2] is involved in the regulation of many cellular responses, including cell proliferation, differentiation, apoptosis, migration, extracellular matrix formation, tissue repair, and immune homeostasis (1–3). TGF-β signals through heteromeric complexes of transmembrane type I (TβR-I) and type II (TβR-II) serine-threonine kinase receptors (4–6). TGF-β receptors subsequently propagate signals downstream through direct interaction with cytoplasmic Smads, and possibly other proteins as well (7–9). Smad proteins are subdivided into three classes, the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads), and the inhibitory Smads (I-Smads). Once phosphorylation, R-Smads, including Smads 1, 2, 3, 5, and 8, dissociate from the type I TGF-β receptor and physically associate with Co-Smads such as Smad 4, and translocate into the nucleus and regulate the transcription from specific gene promoters (10). In addition to Smads, several proteins interacting with TβR-I or TβR-II have been identified (4, 5, 11). Among them, serine-threonine kinase receptor-associated protein (STRAP), a novel WD40 domain-containing protein, was shown to interact with both TβR-I and TβR-II and to inhibit TGF-β signaling (12). Moreover, STRAP was shown to stabilize the complex formation between Smad7, an inhibitory Smad, and activated TβR-I in the inhibition of TGF-β signaling, preventing Smad2 and Smad3 from access to the receptor (13).

The PDK1 (3-phosphoinositide-dependent protein kinase-1) has been demonstrated to phosphorylate and activate many members of the protein kinase A, G, and C subfamily of protein kinases that include PKB, p70 S6 kinase, protein kinase A, serum- and glucocorticoid-induced kinase (SGK), and a variety of protein kinase C isoforms (14). PDK1 is broadly expressed and has been described as constitutive (15–17). However, it was shown recently that PDK1 activity could be stimulated by phosphatidylinositol 3,4,5-trisphosphate in the presence of sphingosine (18) and modulated by several other PDK1-interacting partners (19–21). These observations strongly suggest that other regulatory mechanisms of the PDK1 activity may exist. In addition, recent studies have shown that insulin inhibits TGF-β-mediated apoptosis and that PI3K signaling can be activated by TGF-β, suggesting a functional link between PI3K and TGF-β signaling pathways (22–25). Therefore, it is necessary to investigate how these two signaling pathways are physically and functionally associated with each other.

In this study, we show that STRAP interacts with PDK1 and that this interaction is important for the modulation of PDK1 activity. Moreover, coexpression of PDK1 results in the enhancement of the STRAP-dependent TGF-β transcriptional inhibition. These findings provide insights into the cross-talk between PI3K/PDK1 and TGF-β signaling pathways.

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[2] The abbreviations used are: TGF-β, transforming growth factor-β; STRAP, serine-threonine kinase receptor-associated protein; SGK, serum/glucocorticoid-regulated kinase; PAG, proliferation-associated gene; PI3K, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; TβR, transforming growth factor-β receptor; GST, glutathione *S*-transferase; GFP, green fluorescent protein; PH, pleckstrin homology; siRNA, small interfering RNA; CMV, cytomegalovirus; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TNF, tumor necrosis factor; R-Smads, regulated Smad; Co-Smads, common Smads; I-Smads, inhibitory Smads.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Reagents—293T, HepG2, Hep3B, and SK-N-BE(2)C cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Invitrogen) as described (26). The eukaryotic glutathione *S*-transferase (GST) expression vector (pEBG) and pFLAG-CMV-2 vector with a FLAG epitope were obtained as described previously (27). The Myc-tagged human wild-type and kinase-dead PDK1 were kindly provided by Dr. B. Hemmings (Friedrich Miescher Institute, Basel, Switzerland). The FLAG-tagged Smad2, Smad3, Smad4, and Smad7 were a gift from Dr. R. Derynck (University of California, San Francisco). The p3TP-Lux reporter plasmid was a kind gift from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York). To generate two deletion constructs, FLAG-PDK1(PH) and FLAG-PDK1(CA), we performed a PCR using the full-length PDK1 cDNA as the template. The forward primers for FLAG-PDK1(PH) (5′-GCGAATTCAACATAGAGCAGTACATT-3′) and FLAG-PDK-1(CA) (5′-GCGAATTCCATG CCCAGCCTCCGCCG-3′) contain an EcoRI site (underlined). The reverse primers for FLAG-PDK1(PH) (5′-GCCTCGAGTCACTGCACAGCGGCGTC-3′) and FLAG-PDK-1(CA) (5′-GCCAGCTGGGTGAGCTTCGGAGGCGT-3′) contain an XhoI and a SalI site (underlined). The amplified PCR products for deletion mutants were cut with EcoRI plus XhoI and EcoRI plus SalI, and cloned into pFLAG-PAG (26), a PAG cDNA cloned into pFLAG-CMV-2 vector, to generate the FLAG-PDK1(PH) and FLAG-PDK1(CA) constructs, respectively. The identity of all PCR products was confirmed by nucleotide sequencing analysis on both strands. To generate GST-STRAP, we first cloned the EcoRI/XhoI fragment of STRAP cloned in pJG4-5 into pBacPAK9 (Clontech), digested with EcoRI plus NotI, and subcloned into pBluescript KS (Stratagene). Finally, the ClaI/NotI fragment of the resulting plasmid was cloned into pEBG cut with ClaI and NotI, yielding pEBG-STRAP (GST-STRAP). On the other hand, to generate FLAG-STRAP, a STRAP cDNA (pJG4-5-STRAP) was subcloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 was purchased from R & D Systems. The anti-GST antibody and anti-FLAG (M2) antibody were as described (27). Anti-PDK1 antibody used for immunoprecipitation and immunoblot was from Santa Cruz Biotechnology. The insulin, wortmannin, isopropyl β-D-thiogalactopyranoside, dithiothreitol, aprotinin, phenylmethylsulfonyl fluoride, and anti-β-actin antibody were purchased from Sigma. Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore Corp. (Bedford, MA). [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences.

Preparation of STRAP-specific Antiserum—The anti-STRAP polyclonal rabbit antiserum was raised against GST-tagged STRAP protein, produced in *Escherichia coli*. The recombinant STRAP protein was purified with glutathione-Sepharose 4B beads and used to immunize rabbits. The animals were boosted four times every week and bled from the ear vein 10 days after the last injection. A titer of the antiserum was measured by Western blotting and enzyme-linked immunosorbent assay method. Antiserum was removed by centrifugation after incubation at 37 °C for 3 h.

Yeast Two-hybrid Specificity Test—A fish plasmid, pJG4-5 harboring PDK1, was transformed back into EGY48 cells along with either the bait plasmid, pEG202 harboring STRAP, or other several bait plasmids available in our laboratory as described (28).

Transient Transfection and in Vivo Interaction Assay—Each plasmid DNA indicated under "Results" was transfected into 293T or HepG2 cells with a calcium phosphate precipitation method or Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions. After overnight incubation, the cells transfected were incubated in the presence or absence of TGF-β1 (100 pM) for 20 h. Cells were then washed and solubilized with lysis buffer containing 0.1% Nonidet P-40 as described (28). Detergent-insoluble materials were removed by centrifugation, and the cleared lysates were mixed with glutathione-Sepharose beads (Amersham Biosciences), and beads were washed three times with the lysis buffer. For immunoblotting, coprecipitates or whole cell extracts were resolved by SDS-PAGE and then transferred to PVDF membranes. The membranes were immunoblotted with the indicated antibodies and then developed using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Assay of PDK1 Kinase Activity—Cells transiently transfected were washed three times with ice-cold PBS and solubilized with 100 μl of lysis buffer (20 mM Hepes, pH 7.9, 10 mM EDTA, 0.1 M KCl, and 0.3 M NaCl). The cleared lysates were mixed with glutathione-Sepharose beads and rotated for 2 h at 4 °C. After washing the precipitate three times with lysis buffer and then twice with kinase buffer (50 mM Hepes, pH 7.4, 1 mM dithiothreitol, and 10 mM $MgCl_2$), the precipitate was incubated with 5 μCi of [γ-^{32}P]ATP at 37 °C for 15 min in the presence of kinase buffer containing 500 ng of recombinant SGK (Upstate), separated by 8% SDS-PAGE, transferred to PVDF membranes, and detected by autoradiography.

Small Interfering RNA (siRNA) Experiments—The PDK1 siRNA of the oligonucleotides (5′-GGACUCGAACUCCUUUGAATT-3′) targeting a coding region (amino acids 420–425) on the human PDK1 (GenBank™ accession number Y15056) and STRAP siRNA oligonucleotides (334, 5′-GGGUGCAACACUGAAUAAG-3′; 515, 5′-UUACGCAUAUAUGACUUGA-3′) corresponding to two coding regions (334, amino acids 64–69; 515, amino acids 124–129) of human STRAP (GenBank™ accession number BC000162) were synthesized by Samchully Pharm. Co. Ltd. (Seoul, Korea). The sense and antisense oligonucleotides for each siRNA were mixed and heated at 90 °C for 2 min, and the combined reaction was incubated at 30 °C for 1 h. 293T or HepG2 cells were plated in 6-well flat-bottomed microplates (Nunc) at a concentration of 2×10^5 cells per well the day before transfection. siRNA oligonucleotides (50 nM) were transfected into cells using the Lipofectamine Plus method. After 48 h transfection, reverse transcription-PCR or immunoblottings were carried out to confirm the down-regulation of target proteins.

Luciferase Reporter Assay—HepG2 cells were transfected according to the Lipofectamine Plus method with the p3TP-Lux reporter plasmid, along with each expression vector as indicated. After 48 h, the cells were harvested, and luciferase activity was monitored with a luciferase assay kit (Promega) following the manufacturer's instructions. Light emission was determined with a Berthold luminometer (Microlumat LB96P). The cell extracts containing equal amounts of PDK1 and STRAP, determined by Western blot analysis, were used for luciferase assay, and the total DNA concentration was constantly kept by supplementing with empty vector DNAs. The values were adjusted with respect to expression levels of a cotransfected β-galactosidase reporter control, and experiments were repeated at least three times.

Assays for Cell Death and Cell Survival—293T cells undergoing apoptosis, after treatment with TNF-α (20 ng/ml) and cycloheximide (10 μg/ml), were quantitated by staining with the fluorescein isothiocyanate-conjugated annexin V and the fluorescent dye propidium iodide according to the manufacturer's instructions (Roche Applied Science). Cells in 6-cm dishes treated with TNF-α and cycloheximide for 14 h were harvested and incubated with annexin V- and propidium iodide-containing buffer for 10 min at room temperature and then washed with PBS as described (28). 10,000 events were analyzed per sample using a FACSCalibur-S system (BD Biosciences). For a cell death experiment

using the GFP system, 293T cells grown on sterile coverslips were transfected with pEGFP, an expression vector encoding GFP, together with expression vectors as indicated. After 24 h of transfection, the cells were treated with TNF-α and cycloheximide. The cells were fixed with ice-cold 100% methanol, washed three times with PBS, and then stained with bisbenzimide (Hoechst 33258). The coverslips were washed with PBS, then mounted on glass slides, using Gelvatol, and visualized under a fluorescence microscope as described previously (28). The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells. For a cell survival assay, 293T cells transfected with a STRAP-specific siRNA (515) for 12 h were seeded in 24-well plates at a concentration of $4–5 \times 10^4$ cells per well and allowed to grow in Dulbecco's modified Eagle's medium supplemented with 1% FBS. The cell number was counted with a hemocytometer at the indicated times. By using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay-based cell counting kit-8 (Dojindo), the percentage of cell survival was determined by estimating the value of parental cells at the indicated times as 100%.

Immunofluorescene and Confocal Microscopy—Hep3B cells were plated and transfected with FLAG-Smad3 and/or MYC-tagged PDK1 constructs on sterile coverslips, placed on ice, and washed three times with ice-cold PBS prior to fixation with 4% paraformaldehyde for 10 min at room temperature. Cells were then washed with PBS, treated with 0.2% Triton X-100, and rewashed with PBS. The mouse anti-FLAG (M2) antibody diluted 1000-fold in PBS or rabbit anti-MYC antibody diluted 200-fold in PBS was applied for 2 h at 37 °C. The cells were then washed three times with PBS and incubated with 1000-fold diluted Alexa Fluor-594 anti-mouse secondary antibody or Alexa Fluor-488 anti-rabbit secondary antibody (Molecular Probes) at 37 °C for 1 h. The coverslips were washed three times with PBS and then mounted on glass slides, using Gelvatol. Confocal laser scanning microscopy observations were done on a Bio-Rad MRC 1024 15-milliwatt argon-krypton laser as described (28).

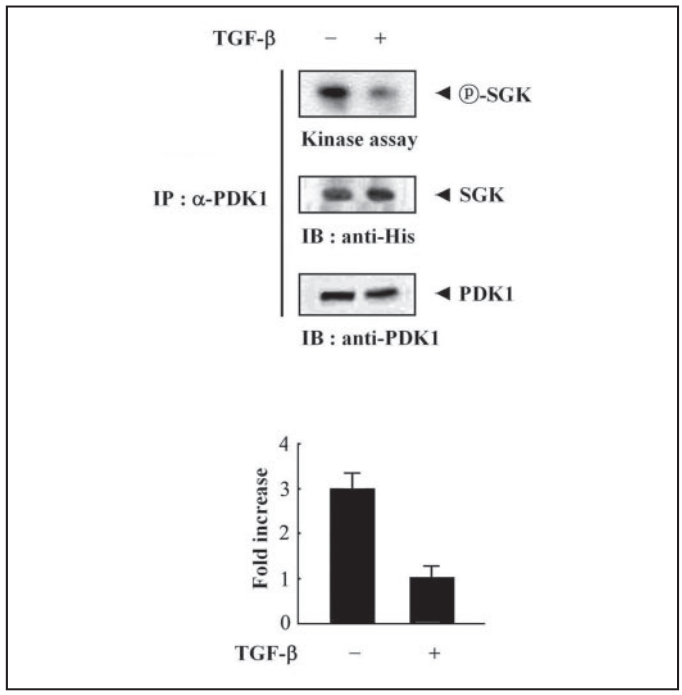

FIGURE 1. **Regulation of PDK1 activity by TGF-β.** 293T cells were incubated for 20 h in the presence (+) or absence (−) of TGF-β1 (100 pM). Endogenous PDK1 was immunoprecipitated (*IP*) with an anti-PDK1 antibody, and the immunoprecipitates were subjected to *in vitro* kinase assay using SGK as a substrate, followed by SDS-PAGE and autoradiography (*top panel*). The *circled P*-SGK indicates the position of the phosphorylated SGK. The amounts of SGK used in the assay and immunoprecipitated PDK1 were analyzed with anti-His and anti-PDK1 antibodies using the same blot (*2nd* and *3rd panels*). Band intensities were quantitated using NIH Image software, and the fold increase relative to TGF-β1-treated samples was calculated (*bottom panel*). The data shown are the mean ± S.D. of duplicate assays and are representative of at least three independent experiments. *IB*, immunoblot.

RESULTS

PDK1 Interacts with STRAP—To explore the mechanism by which PDK1 activity is regulated, we employed a yeast two-hybrid specificity test to search PDK1-interacting proteins using more than 30 different baits available in our laboratory, together with the fish plasmid, pJG4-5 harboring a full-length of PDK1 cDNA, as described previously (28). From this random screening, we found that PDK1 cDNA interacted with a tested bait plasmid, pEG202 harboring STRAP (results not shown). Based on this, to investigate a possible cross-talk between PDK1 and TGF-β signaling pathways, we first performed coimmunoprecipitation experiments and *in vitro* kinase assay using the human embryonic kidney carcinoma cell line 293T. Endogenous PDK1 immunoprecipitate from cell lysates treated with TGF-β significantly reduced PDK1 activity about 3.0-fold compared with the control PDK1 immunoprecipitate untreated with TGF-β (Fig. 1), suggesting that a functional link between PDK1 and TGF-β signaling pathways may exist.

We then speculated that PDK1 might interact with STRAP in intact cells, and we performed cotransfection experiments using GST- and FLAG-tagged eukaryotic expression vectors. In these experiments, the wild-type PDK1 and STRAP were coexpressed as a GST fusion protein and a FLAG-tagged protein in cells, respectively. The interactions of FLAG-tagged STRAP proteins to the GST-PDK1 fusion proteins were analyzed by immunoblotting with an anti-FLAG antibody. As shown in Fig. 2*A*, the STRAP was detected in the coprecipitate only when coexpressed with the GST-PDK1 but not with the control GST alone, in addition to the failure of the binding of the control vector alone (CMV) to GST-PDK1, demonstrating that PDK1 physically interacts with STRAP. In order to confirm the interaction between PDK1 and STRAP, we next carried out coimmunoprecipitation experiments using FLAG-STRAP-transfected 293T cell extract and endogenous PDK1 (Fig. 2*B*). Endogenous PDK1 was immunoprecipitated from cell lysates, and Western blot analysis showed that PDK1 was precipitated (Fig. 2*B, lower panel*). The binding of STRAP was subsequently analyzed by immunoblotting with an anti-FLAG antibody. STRAP was only present in the PDK1 immunoprecipitate when coexpressed with the FLAG-STRAP (Fig. 2*B, upper panel*) but not in the control transfected the vector alone (CMV). In addition, to examine the interaction between the two endogenous proteins, we produced antiserum specific to STRAP as described under "Materials and Methods." The specificity of the antiserum was confirmed by immunoblotting using cell extracts from 293T cells transfected with FLAG-STRAP (results not shown). As shown in Fig. 2*C*, immunoprecipitation of endogenous PDK1 by anti-PDK1 antibody and then immunoblotting with STRAP-specific antiserum showed the interaction of these two endogenous proteins, regardless of cell types used. This interaction was also confirmed by reciprocal coimmunoprecipitation experiments in which STRAP-specific antiserum, instead of anti-PDK1 antibody, was used for immunoprecipitation (results not shown). Taken together, our results demonstrate that PDK1 associates with STRAP *in vivo*.

Catalytic Domain of PDK1 Specifically Binds to STRAP in Vivo—To determine which region of PDK1 was necessary for the association with STRAP, we generated two PDK1 deletion constructs FLAG-PDK1(PH), comprising the carboxyl-terminal PH domain (amino acids 411–556),

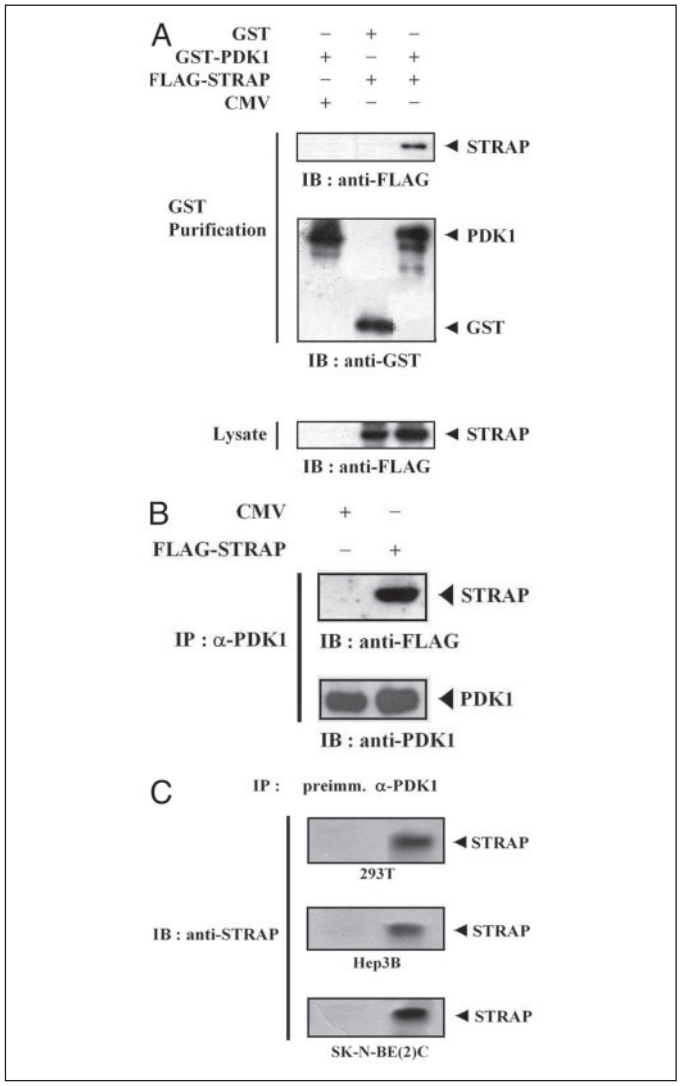

FIGURE 2. **Association of PDK1 with STRAP *in vivo*.** *A,* pEBG vector alone (GST), as a negative control, and pEBG-PDK1 (GST-PDK1) were cotransfected with either FLAG-tagged STRAP (FLAG-STRAP) containing the full-length human STRAP cDNA or pFLAG-CMV2 vector (*CMV*) into 293T cells. GST fusion proteins were purified on glutathione-Sepharose beads (*GST purification*), and the complex formation (*top panel*) and the amount of FLAG-tagged STRAP used for an *in vivo* binding assay (*bottom panel, Lysate*) were determined by anti-FLAG antibody immunoblot (*IB*). The same blot was stripped and re-probed with an anti-GST antibody (*middle panel*) to confirm the expression of GST-PDK1 and a GST control (*GST*). *B*, 293T cells were transiently transfected with the vector alone (*CMV*), as a negative control, or FLAG-STRAP, and lysed and immunoprecipitated (*IP*) with an anti-PDK1 antibody. The PDK1 immunoprecipitate was analyzed for the presence of STRAP by immunoblot analysis using anti-FLAG antibody (*upper panel*). The amount of immunoprecipitated PDK1 was analyzed using anti-PDK1 antibody (*lower panel*). *C*, cell lysates from the parental 293T, Hep3B, and SK-N-BE(2)C cells were immunoprecipitated with rabbit preimmune serum (*preimm.*) or rabbit anti-PDK1 antibody (α-*PDK1*). The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with STRAP-specific antiserum. These experiments were performed in duplicate at least four times with similar results.

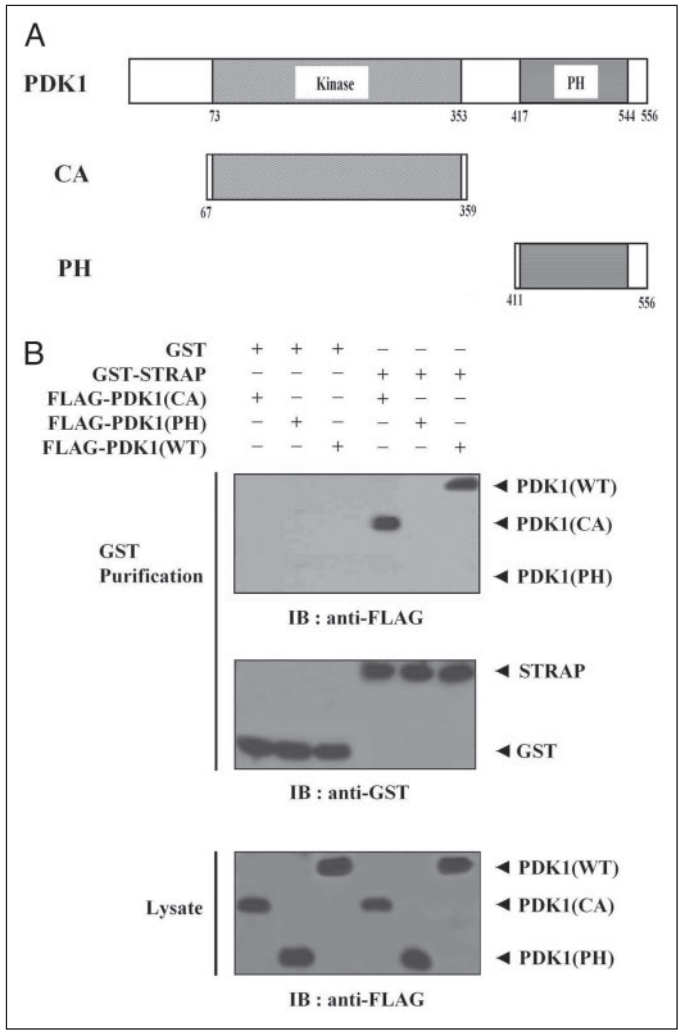

FIGURE 3. **Domains of PDK1 involved in the PDK1-STRAP association.** *A*, the schematic structures of PDK1 wild-type and deletion constructs. *Kinase* and *PH* indicate the catalytic kinase domain and PH domain of PDK1, respectively. The *numbers* indicate amino acid residues, and amino acid number of domain boundaries is indicated. *B*, 293T cells were cotransfected with a GST alone or GST-STRAP, together with FLAG-PDK1 (*WT*, wild-type PDK1), FLAG-PDK1(CA) containing only the catalytic kinase domain of PDK1 (amino acids 67–359), and FLAG-PDK1(PH) harboring only the PH domain of PDK1 (amino acids 411–556), and precipitated with glutathione-Sepharose beads (*GST purification*). A complex formation was determined by immunoblot (*IB*) analysis using anti-FLAG antibody (*top panel*). The same blot was re-probed with an anti-GST antibody to confirm the expression of GST fusion proteins in the coprecipitates (*middle panel*), and the expression level of FLAG-tagged proteins in total cell lysates (*Lysate*) was analyzed by immunoblot analysis using anti-FLAG antibody (*bottom panel*). These experiments were performed in duplicate at least five times with similar results.

and FLAG-PDK1(CA), harboring the catalytic domain (amino acids 67–359) as described in Fig. 3*A*, and we examined the ability of the constructs to form a complex with STRAP. GST-STRAP construct was expressed in 293T cells and used for *in vivo* binding assay with FLAG-PDK1(PH), FLAG-PDK1(CA), and wild-type FLAG-PDK1. As shown in Fig. 3*B*, the binding of wild-type FLAG-PDK1 and FLAG-PDK1(CA) with GST-STRAP was detectable (*top panel, 4th* and *6th lanes*), whereas the interaction could not be observed between FLAG-PDK1(PH) and GST-STRAP (*top panel, 5th lane*). These results clearly defined the amino-terminal catalytic kinase domain of PDK1 as essential for STRAP binding *in vivo*.

TGF-β and Insulin Modulate PDK1-STRAP Complex Formation—It has been demonstrated previously that the STRAP phosphorylation is slightly modulated when cells are stimulated by TGF-β and that TGF-β signaling pathway is regulated by insulin stimulation (13, 25). Therefore, we assessed whether TGF-β and insulin can influence the PDK1-STRAP complex formation in cells following TGF-β and insulin treatment. Twenty four hours after transfection, cells were incubated in media with or without 100 pM TGF-β1 for 20 h. PDK1 was precipitated, and the coprecipitation of STRAP was determined by anti-FLAG immunoblot assay. As illustrated in Fig. 4*A*, upon TGF-β treatment, the association between PDK1 and STRAP was significantly decreased ~2.5-fold (*top panel, 1st* and *2nd lanes*). We next examined the effect of insulin on the physical interaction between PDK1 and STRAP in 293T

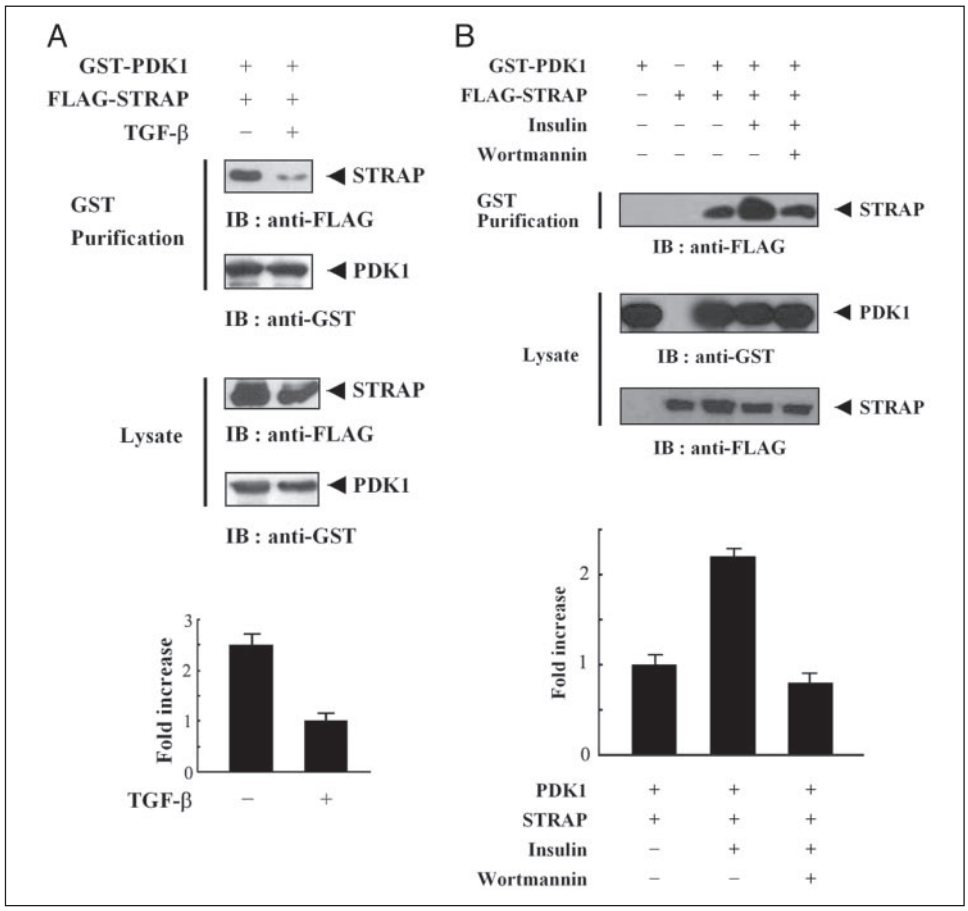

FIGURE 4. **Regulation of the PDK1-STRAP complex formation by TGF-β and insulin.** *A*, decrease of the association between PDK1 and STRAP by TGF-β. 293T cells transfected with the indicated expression vectors were incubated with Dulbecco's modified Eagle's medium containing 0.2% FBS in the presence (+) or absence (−) of TGF-β1 (100 pM) for 20 h. Cell lysates were purified on glutathione-Sepharose beads (*GST purification*) and immunoblotted (*IB*) with an anti-FLAG antibody (*top panel*). The amount of precipitated PDK1 (*GST purification*) and the expression level of GST-tagged PDK1 proteins in total cell lysates (*Lysate*) were analyzed using anti-GST antibody, respectively (*2nd* and *4th panels*). The level of PDK1-STRAP complex formation was shown as a *bar graph*, after densitometric reading of the autoradiograms and normalization for PDK1 and STRAP protein levels, and fold increase relative to TGF-β1-treated samples was calculated (*bottom panel*). *B*, increase of the association between PDK1 and STRAP by insulin. 293T cells were transfected with expression vectors encoding GST-PDK1 and FLAG-STRAP as indicated. After 48 h of transfection, the cells were incubated for 30 min with or without 100 nM wortmannin and then treated with 100 nM insulin for 20 min. The cell lysates were subjected to precipitations with glutathione-Sepharose beads (*GST purification*). The resulting precipitates were examined by immunoblot analysis with an anti-FLAG antibody (*top panel*). The equivalent amounts of GST-PDK1 and FLAG-STRAP were assessed by immunoblot analysis of total cell lysates (*2nd* and *3rd panels*, *Lysate*). The relative level of PDK1-STRAP complex formation was quantitated by densitometric analyses, and fold increase relative to untreated samples was calculated (*bottom panel*). The data shown are the mean ± S.D. of duplicate assays and are representative of at least five independent experiments.

cells transfected with plasmid vectors expressing GST-PDK1 and FLAG-STRAP. As shown in Fig. 4*B* (*top panel, 3rd* to *5th lanes*), exposure of the cells to insulin resulted in an increase in the PDK1-STRAP complex formation (about 2.2-fold), but this effect was inhibited by wortmannin, a PI3K inhibitor, implying that STRAP can be involved in the PI3K/PDK1 signaling pathway.

STRAP Is a Positive Regulator of PDK1 Kinase Activity—In order to establish the physiological role for the PDK1-STRAP association, we first examined the effect of STRAP on PDK1 kinase activity. PDK1 was precipitated from the transfected cells, and its activity was measured by *in vitro* kinase assay using SGK as a substrate (29). As shown in Fig. 5*A*, coexpression of PDK1 with STRAP resulted in an increase of PDK1 activity ~4-fold (*top panel, 2nd* and *4th lanes*). As a control, expression levels of the transiently expressed PDK1 protein were analyzed in GST pull-down precipitates, and the amount of PDK1 in all lanes was similar (Fig. 5*A*, *4th panel*), indicating that the observed differences in phosphorylated SGK were not because of differences in PDK1 expression levels. We next tested whether STRAP could enhance PDK1 autophosphorylation. PDK1 was immunoprecipitated from the cell extracts expressing PDK1 alone or PDK1, together with STRAP, and the effect of STRAP on PDK1 activity in the immunocomplex was determined by autophosphorylation assays. Results show that PDK1 is more autophosphorylated in the presence of STRAP than in the absence of STRAP (Fig. 5*B*). These findings suggest that STRAP may be a positive regulator of PDK1 activity. PKB/Akt has been implicated in contributing to the sequestration of Bad away from the pro-apoptotic signaling pathway by Bad phosphorylation (30). To examine whether the downstream targets of PDK1 are affected by the overexpression of STRAP, we performed cotransfection experiments with plasmid vectors expressing GST-Bad, MYC-PDK1, and FLAG-STRAP, and cell lysates were precipitated with glutathione-Sepharose beads and immunoblotted with an anti-phospho-Bad antibody. As shown in Fig. 5*C*, coexpression of STRAP with PDK1 significantly induced the Bad phosphorylation compared with that of the PDK1 expression alone (*top panel, 2nd* and *3rd lanes*). In addition, as expected, the PDK1-induced Akt phosphorylation was also increased by STRAP coexpression (Fig. 5*C*, *bottom panel*). In order to clearly confirm the physiological role of STRAP in the regulation of the PI3K/PDK1 signaling pathway, the *in vitro* kinase assay (Fig. 6*A*) or immunoblot analyses (Fig. 6, *B* and *C*) were performed in 293T cells transfected with STRAP-specific siRNAs using SGK as a substrate or anti-phospho-antibodies as indicated, respectively. As a result, reducing the amount of endogenous STRAP in cells with sequence-specific siRNAs resulted in a significant decrease of PDK1 kinase activity (Fig. 6*A*), PKB/Akt phosphorylation (Fig. 6*B*), and Bad phosphorylation (Fig. 6*C*). Thus, it is evident from these experiments that STRAP physically interacts with PDK1 and positively modulates PDK1 as well as its downstream targets such as PKB/Akt and Bad *in vivo*.

Interaction between PDK1 and STRAP Attenuates TNF-α-induced Apoptosis—Because STRAP associates with PDK1 and enhances PDK1 kinase activity (see Fig. 5), we next analyzed the effect of STRAP on TNF-α-induced apoptosis. To this end, we chose 293T cells, which are susceptible to TNF-α-induced apoptosis (28, 31), and we performed dual annexin V/propidium iodide staining as described under "Materials and Methods." As illustrated in Fig. 7*A* (*white bars*), the transfection of 293T cells with a vector encoding PDK1 resulted in a slight decrease in apoptotic cell death as expected, and this suppression was potentiated when PDK1 was coexpressed with STRAP, indicating that STRAP plays

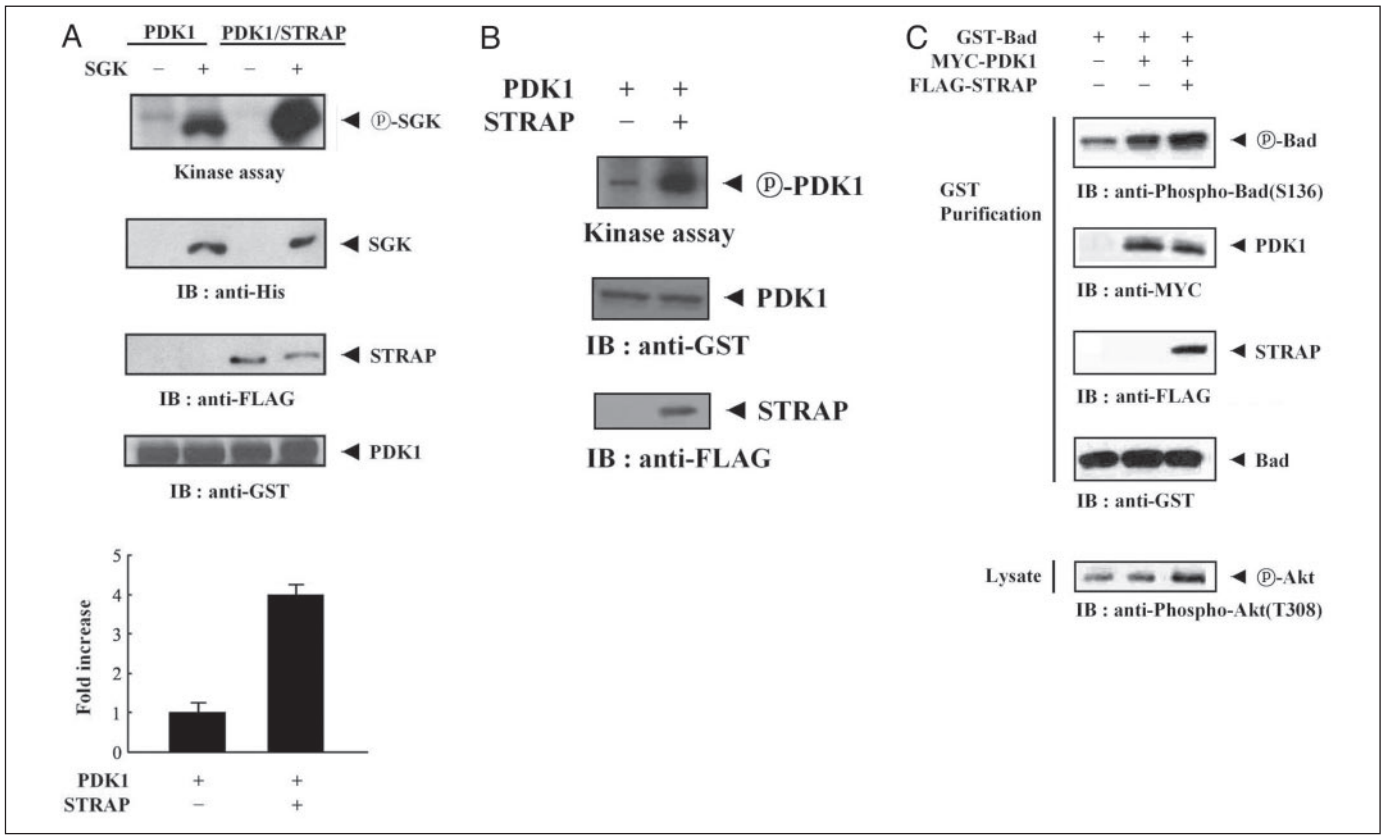

FIGURE 5. **Up-regulation of PDK1 activity by STRAP.** *A*, stimulation of PDK1 activity by STRAP coexpression. 293T cells were transiently transfected with GST-PDK1 together with an empty vector (*CMV*) or FLAG-STRAP. Cell lysates were subjected to precipitation with glutathione-Sepharose beads, and then the precipitates were analyzed for PDK1 activity by *in vitro* kinase assay using SGK as a substrate. The *circled P*-SGK indicates the position of the phosphorylated SGK. The same blot was stripped and re-probed with the indicated antibodies to determine the equivalent amount of substrate used for *in vitro* kinase assay (*2nd panel*), a complex formation between PDK1 and STRAP (*3rd panel*), and the expression level of precipitated PDK1 (*4th panel*). The PDK1 activity was quantified and shown as a *bar graph,* and fold increase relative to control samples expressing only PDK1 was calculated (*bottom panel*). The data shown are the mean ± S.D. of duplicate assays and are representative of at least five independent experiments. *B*, autophosphorylation of PDK1. After 48 h of transfection with the indicated expression vectors, cell lysates were subjected to precipitation with glutathione-Sepharose beads and then analyzed by *in vitro* kinase assay without a substrate. The *circled P*-PDK1 indicates the position of the autophosphorylated PDK1 (*top panel*). The expression level of PDK1 and STRAP proteins in the precipitates was analyzed using anti-GST and anti-FLAG antibodies, respectively (*middle* and *bottom panels*). These experiments were performed in duplicate at least three times with similar results. *C*, PDK1-mediated phosphorylation of Bad and Akt. GST-Bad was transiently cotransfected with MYC-PDK1 in the presence or absence of FLAG-STRAP. As a negative control, 293T cells were transfected with GST-Bad alone. Transfected cells were precipitated with glutathione-Sepharose beads (*GST purification*), and the level of GST-Bad phosphorylation was measured by immunoblot (*IB*) analysis using anti-phosphoserine 136-specific Bad antibody (*top panel*). Anti-MYC immunoblot for PDK1 (*2nd panel*), anti-FLAG immunoblot for STRAP (*3rd panel*), and anti-GST immunoblot for Bad (*4th panel*) were prepared from the same blot. The PDK1-mediated Akt phosphorylation was determined from the same lysates used for Bad phosphorylation by immunoblot analysis using anti-phosphothreonine 308-specific Akt antibody (*bottom panel*, *Lysate*). These experiments were performed in duplicate at least three times with similar results.

an important role in the modulation of PDK1-mediated survival signaling pathway by direct binding with PDK1.

To confirm further the involvement of STRAP in the suppression of TNF-α-induced apoptosis, 293T cells were transiently transfected with GFP alone and with GFP and PDK1. In addition, cells were cotransfected with PDK1 and STRAP, together with GFP. Apoptotic cells were scored by a change in nuclear morphology among GFP-positive cells after inducing apoptosis by TNF-α treatment as described under "Materials and Methods." As shown in Fig. 7*A* (*black bars*), ~28% of 293T cells expressing PDK1 were apoptotic following TNF-α treatment. Cells cotransfected with PDK1 and STRAP expression plasmids showed higher apoptotic suppression (about 25% inhibition) than cells transfected with the PDK1 expression plasmid alone. However, this inhibitory effect was not because of STRAP itself, because the amount of STRAP used for these apoptotic analyses could not influence a change in apoptosis (see Fig. 7*A*, *4th bar*). The effect of STRAP on TNF-α-induced apoptosis was further assessed by the small interfering RNA experiments using a STRAP-specific siRNA (515) because this siRNA showed a little stronger effect on the down-regulation of endogenous STRAP compared with the another STRAP-specific siRNA (334). As shown in Fig. 7*B*, the apoptotic cell death was increased by the coexpression of STRAP-specific siRNA (515), depending on the amount of STRAP-specific siRNA. Next, to provide more evidence that STRAP is directly involved in the modulation of PI3K/PDK1 signaling crucial for cellular responses such as cell survival, cell growth, and protein synthesis, we examined the effect of STRAP on cell growth under the down-regulated conditions of endogenous STRAP using trypan blue exclusion (Fig. 7*C*) and cell counting kit-8 method (Fig. 7*D*). As a result, the property of STRAP to enhance the cell survival was confirmed in a dose-dependent manner from both experiments. To examine further the roles of STRAP in the cell growth, a human neuroblastoma stable cell line expressing STRAP (Strap) was established and used for the proliferative assays. As shown in Fig. 7, *E* and *F*, compared with parental SK-N-BE(2)C cells (*Control*) and pcDNA3-His vector transfectants (*Vector*), as negative controls, the growth rate under normal serum conditions was increased by the ectopic expression of STRAP. Taken together, these data clearly indicate that STRAP positively regulates PI3K/PDK1-mediated protection against TNF-α-induced apoptosis and cell survival.

STRAP Regulates PDK1 Kinase Activity by 14-3-3 Dissociation—Recently, it was reported that the binding of 14-3-3 to PDK1 suppresses PDK1 kinase activity (20). Based on this, we speculated that STRAP, a

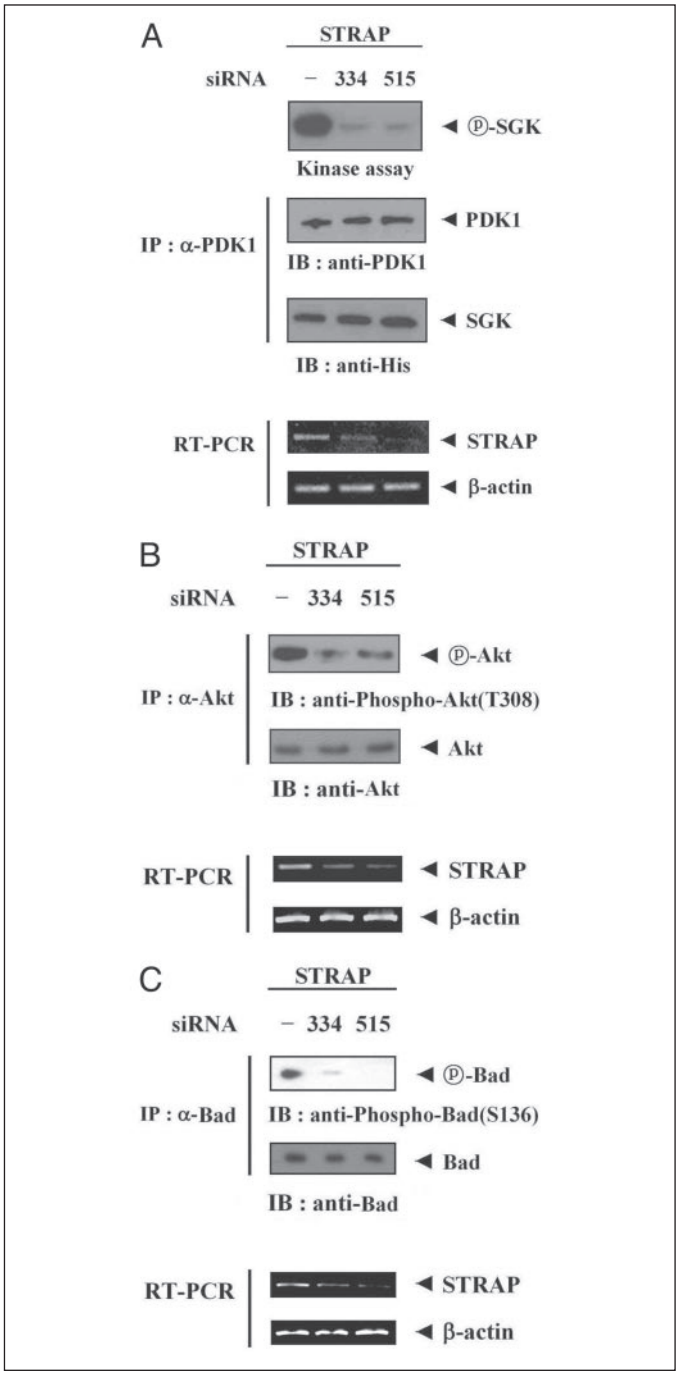

FIGURE 6. **Effect of STRAP siRNA duplexes on PI3K/PDK1 signaling pathway.** 293T cells were cultured in 6-well plates as described under "Materials and Methods" and transfected with two kinds of STRAP siRNA duplexes (334 and 515). Total cell lysates were immunoprecipitated (*IP*) with the indicated antibodies. The PDK1 immunoprecipitate was analyzed for PDK1 activity by *in vitro* kinase assay using SGK as a substrate (*A*, *top panel*). The amounts of immunoprecipitated PDK1 and SGK used in the assay were analyzed with anti-PDK1 and anti-His antibodies using the same blot (*A*, *2nd* and *3rd panels*). The PKB/Akt and Bad immunoprecipitates were also analyzed for Akt activity and Bad phosphorylation by immunoblot (*IB*) analyses using anti-phospho-Akt(Thr-308) and anti-phospho-Bad(Ser-136) antibodies (*B* and *C*, *top panels*). The amounts of immunoprecipitated Akt and Bad used in this assay were analyzed with anti-Akt and anti-Bad antibodies using the same blot, respectively (*B* and *C*, *middle panels*). The *circled P*-SGK, *circled P*-Akt, and *circled P*-Bad indicate the position of the phosphorylated SGK, Akt, and Bad, respectively. Expression levels of endogenous STRAP and β-actin mRNA in each sample were monitored by reverse transcription (*RT*)-PCR using STRAP-specific (forward, 5′-AACCATCAATTCTGCATCTC-3′; reverse, 5′-AAGGAAAGATGCAATCTGAA-3′) and β-actin-specific primers (forward, 5′-CAAGAGATGGCCACGGCTGCT-3′; reverse, 5′-TCCTTCT-GCATCCTGTCGGCA-3′). These experiments were performed in duplicate at least four times with similar results.

potential positive regulator of PDK1, might modulate PDK1-14-3-3 complex formation. To determine whether the mechanism of the stimulation of PDK1 activity by STRAP is correlated with the dissociation of 14-3-3 from PDK1-14-3-3 complex, we examined the effect of STRAP on PDK1-14-3-3 association by using *in vivo* binding assay as described under "Materials and Methods." Transfected cells were precipitated with glutathione-Sepharose beads, and the binding of 14-3-3 to PDK1 was estimated by immunoblot analyses using anti-FLAG antibody. As shown in Fig. 8*A*, coexpression of STRAP significantly decreased PDK1-14-3-3 association with ~54% compared with the control (*top panel, 3rd* and *4th lanes*). This result indicates that STRAP may enhance PDK1 kinase activity by stimulation of the dissociation of 14-3-3, a potential negative regulator of PDK1, from PDK1-14-3-3 complex.

Because insulin increased the association between PDK1 and STRAP, and this effect was blocked by wortmannin treatment (see Fig. 4*B*), we next investigated whether insulin has a similar effect on PDK1-14-3-3 complex formation in intact cells. 293T cells were transfected with FLAG-14-3-3 or a vector alone (CMV). Immunoblot analysis using anti-FLAG antibody of the PDK1 immunoprecipitates revealed that, as expected, the interaction between PDK1 and 14-3-3 was significantly decreased with about 50% in the cells treated with insulin compared with the control without insulin treatment (Fig. 8*B*). Taken together, these results suggest that STRAP, like 14-3-3, can be also a modulator of downstream targets of the PI3K signaling pathway activated by insulin, in addition to its original role as an intracellular signal mediator in TGF-β signal transduction.

PDK1 Negatively Regulates TGF-β-mediated Transcription—STRAP was known to negatively regulate the TGF-β-induced transcription in a dose-dependent manner and existed as a homo- or hetero-oligomer (12, 13). To determine whether PDK1 also regulates STRAP activity using the same approach, we cotransfected the p3TP-Lux reporter plasmid, containing elements from the plasminogen activator inhibitor-1 promoter (32), with expression vectors encoding for PDK1 and/or STRAP into HepG2 cells, which are highly responsive to TGF-β. We first examined the effect of increasing amounts of PDK1 on TGF-β-induced transcription. Overexpression of PDK1, like STRAP, suppressed the TGF-β-induced increase in luciferase activity in a dose-dependent manner (Fig. 9*A*). In order to further establish whether the activity of PDK1 is involved in the suppression of TGF-β-induced transcription, we next analyzed the effect of the catalytically inactive $PDK1^{KD}$ mutant on TGF-β-induced transcription (where KD indicates kinase-dead). As shown in Fig. 9*A*, both wild-type (*black bars*) and catalytically inactive $PDK1^{KD}$ (*white bars*) decreased TGF-β-induced transcription to a similar extent, suggesting that the suppression of TGF-β-induced transcription by PDK1 is independent of its kinase activity. We then attempted to determine whether the interaction of PDK1 with STRAP can influence TGF-β-induced transcription. Results showed that the addition of PDK1 to STRAP led to an enhancement of the inhibitory effect of STRAP in TGF-β signaling in a dose-dependent manner (Fig. 9*B*). Once again, these results suggest that PDK1 activity seems not to be required for the STRAP-induced suppression of TGF-β signaling. In addition, we examined whether PDK1 has a similar inhibitory effect on another TGF-β-responsive reporter $(CAGA)_9$ MLP-Luc, containing multiple Smad3/Smad4-binding CAGA boxes (33). Similar results were also observed with the $(CAGA)_9$ MLP-Luc reporter in HepG2 cells (results not shown). To confirm further the inhibitory effect of PDK1 in the TGF-β signaling, we also used a constitutively active form of type 1 TGF-β receptor, instead of TGF-β, to alternatively initiate TGF-β signaling. The results obtained in these experiments were almost identical to those of TGF-β treatment (results not shown). Finally, we determined

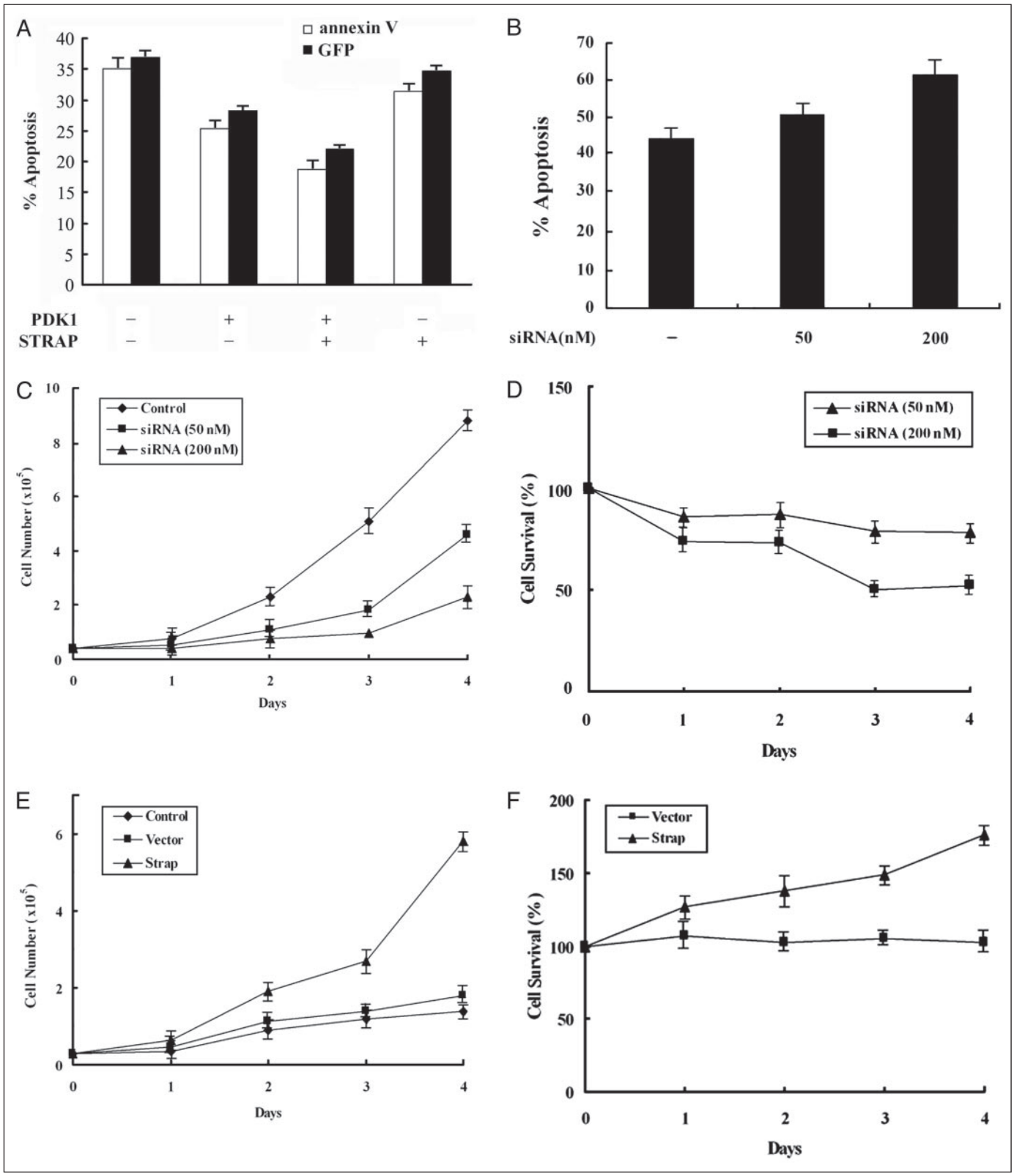

FIGURE 7. **STRAP enhances PDK1-mediated inhibition of apoptosis.** *A*, effect of STRAP on TNF-α-induced apoptosis. 293T cells were transiently transfected with an expression vector encoding PDK1 (2 μg) as indicated in the presence or absence of an expression vector encoding STRAP (2 μg). Transfected or parental cells were incubated for 24 h and treated with TNF-α (20 ng/ml) and cycloheximide (10 μg/ml) for 14 h to induce apoptosis. Apoptotic cell death was determined by flow cytometry for annexin V and propidium iodide (annexin V). Results shown are average of duplicate samples and are representative of three independent experiments. For a cell death experiment using the GFP system, 293T cells were transiently transfected with expression vectors encoding PDK1 (3 μg) and STRAP (3 μg) along with an expression vector encoding GFP (3 μg) as indicated. To induce apoptosis, transfected cells were treated with TNF-α and cycloheximide as described above. GFP-positive cells were analyzed for the presence of apoptotic nuclei with a fluorescence microscope (GFP). The data shown are the means ± S.D. of triplicate assays and are representative of at least three independent experiments. *B*, effect of STRAP-specific siRNA on TNF-α-induced apoptosis. 293T cells were transiently transfected with a STRAP-siRNA duplex (515) along with an expression vector encoding GFP (3 μg), and the transfected or parental untransfected cells (−) were incubated for 24 h and treated with TNF-α as described above to induce apoptosis. A fluorescence microscope was used to analyze the presence

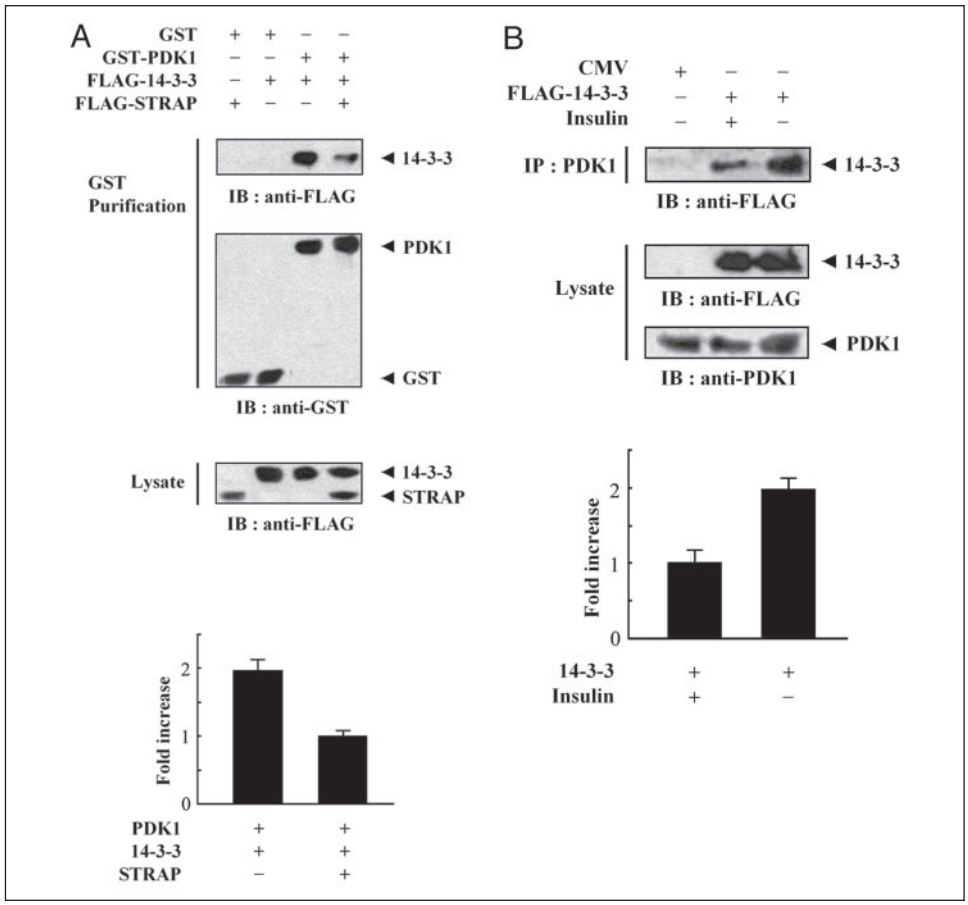

FIGURE 8. **Down-regulation of PDK1-14-3-3 complex formation by STRAP and insulin.** *A*, STRAP stimulates the dissociation of 14-3-3 from the PDK1-14-3-3 complex. 293T cells were transfected for 48 h with the indicated combinations of plasmid vectors expressing pEBG vector alone (*GST*), GST-PDK1, FLAG-14-3-3, and FLAG-STRAP; and the complex formations between PDK1 and 14-3-3 (*top panel*) and the amounts of FLAG-STRAP and FLAG-14-3-3 used for *in vivo* binding assay in total cell lysates (*3rd panel, Lysate*) were determined by anti-FLAG antibody immunoblot (*IB*). The abundance of GST-tagged proteins in GST precipitates was determined by anti-GST antibody immunoblot (*2nd panel*). *B*, insulin effect on PDK1-14-3-3 complex formation. After 48 h of transfection with or without FLAG-14-3-3, the cells were incubated for 20 min in the presence (+) or absence (−) of 100 nM insulin. The cells were immunoprecipitated (*IP*) with an anti-PDK1 antibody and then immunoblotted with an anti-FLAG antibody to determine the complex formation between PDK1 and 14-3-3 (*top panel*). As a negative control, 293T cells were transfected with the vector alone (*CMV*). Expression levels of transfected FLAG-14-3-3 (*2nd panel*) and endogenous PDK1 (*3rd panel*) were confirmed by immunoblot analyses with the indicated antibodies using total cell lysates (*Lysate*). The level of the complex formation between PDK1 and 14-3-3 was quantified and shown as a *bar graph* using densitometric analyses as described, and fold increase relative to STRAP-transfected and insulin-treated samples was calculated (*A* and *B*, *bottom panels*). These experiments were performed in duplicate at least four times with similar results.

the roles of PDK1 in TGF-β-induced transcription using the PDK1 knockdown system. As shown in Fig. 9*C*, the transfection of PDK1 siRNA resulted in a significant increase of TGF-β-induced transcription that was proportional to the amount of PDK1 siRNA transfected. As a control, the cells transfected with PDK1 siRNA showed a significant reduction of endogenous PDK1 compared with the nontransfected cells (Fig. 9*D*, *upper panel*), indicating that the PDK1 siRNA used in this experiment is an effective siRNA duplex for the suppression of endogenous PDK1 expression. Taken together, these findings strongly suggest that PDK1 associates with STRAP and enhances the STRAP-induced inhibition of TGF-β signaling.

PDK1 Potentiates the Association between Activated Type I TGF-β Receptor and Smad7—To explore how PDK1 negatively regulates TGF-β-mediated transcription, we examined the effect of PDK1 on the association between TβR1(TD), an activated type 1 TGF-β receptor, and Smad7 because STRAP was known to inhibit TGF-β signaling by stabilizing the complex formation between TβR1(TD) and Smad7 (13, 34). 293T cells were transiently transfected with GST-TβR1(TD), FLAG-Smad7, FLAG-STRAP, and MYC-PDK1. As shown in Fig. 10*A*, compared with control cells expressing GST-TβR1(TD) and FLAG-Smad7, the coexpression of STRAP significantly stimulated the association between TβR1(TD) and Smad7 about 3-fold (Fig. 10*A*, *top panel, 3rd* and *4th lanes*), consistent with the previous observations obtained by Datta and Moses (13). Furthermore, the addition of PDK1, together with STRAP, caused a stronger association between TβR1(TD) and Smad7 (about 4-fold increase), indicating that PDK1, like STRAP, plays a key role in the stabilization of TβR1(TD)-Smad7 complex and contributes to the inhibition of TGF-β signaling (*top panel, 4th* and *5th lanes*). We then examined whether the coexpression of PDK1 affects the association between STRAP and Smad7 because STRAP contributes to the stable association between TβR1(TD) and Smad7 for inhibiting TGF-β signaling (13). As a result, as shown in Fig. 10*B*, the interaction of Smad7 with STRAP was increased about 2-fold in the transfected cells expressing PDK1 compared with the control cells without PDK1 (*top panel, 2nd* and *3rd lanes*), indicating that PDK1 may enhance the inhibitory TGF-β signaling by assisting STRAP to recruit Smad7 to TGF-β receptor and to stabilize the association between Smad7 and TGF-β receptor. Collectively, these results suggest that PDK1, together with STRAP, might stabilize the complex formation of TβR-Smad7 for blocking TGF-β signaling.

PDK1 Prevents the Nuclear Translocation of Smad3—Because Smad3 is able to strongly reverse the synergistic inhibition of TGF-β-dependent transcription by STRAP and Smad7 (13), we next examined whether PDK1 could modify Smad3 movement. The cells were transfected with MYC-PDK1 or FLAG-Smad3 in the presence of Smad3 or PDK1, respectively. As shown in Fig. 11, cells expressing Smad3 in the absence

of apoptotic nuclei among GFP-positive cells. *C* and *D*, effect of STRAP-specific siRNA on cell proliferation. 293T cells transfected with a STRAP-siRNA duplex (515) at the indicated concentrations were seeded in 24-well plates at a concentration of 4×10^4 cells per well. The cell number of viable cells at the indicated times was counted with a hemocytometer, determined by trypan blue exclusion (*C*), or a cell counting kit-8 (*D*). Control (◆) indicates the parental 293T cells untransfected with a STRAP-siRNA duplex (515). The percentage of cell survival was determined by estimating the value of parental untransfected cells at the indicated times as 100%. Each point indicates the means ± S.E. of two separate experiments carried out in duplicate. *E* and *F*, effect of STRAP overexpression on cell proliferation. Cells (*Control*, parental SK-N-BE(2)C cells; *Vector*, pcDNA3-His empty vector transfectants; *Strap*, STRAP stable transfectants) were seeded in 24-well plates at a concentration of 3×10^4 cell per well and then counted to determine the cell number of viable cells at the indicated times by trypan blue exclusion (*E*) or cell counting kit-8 method (*F*). The percentage of cell survival was determined by estimating the value of parental SK-N-BE(2)C cells at the indicated times as 100%. Each point indicates the means ± S.E. of two separate experiments carried out in triplicate.

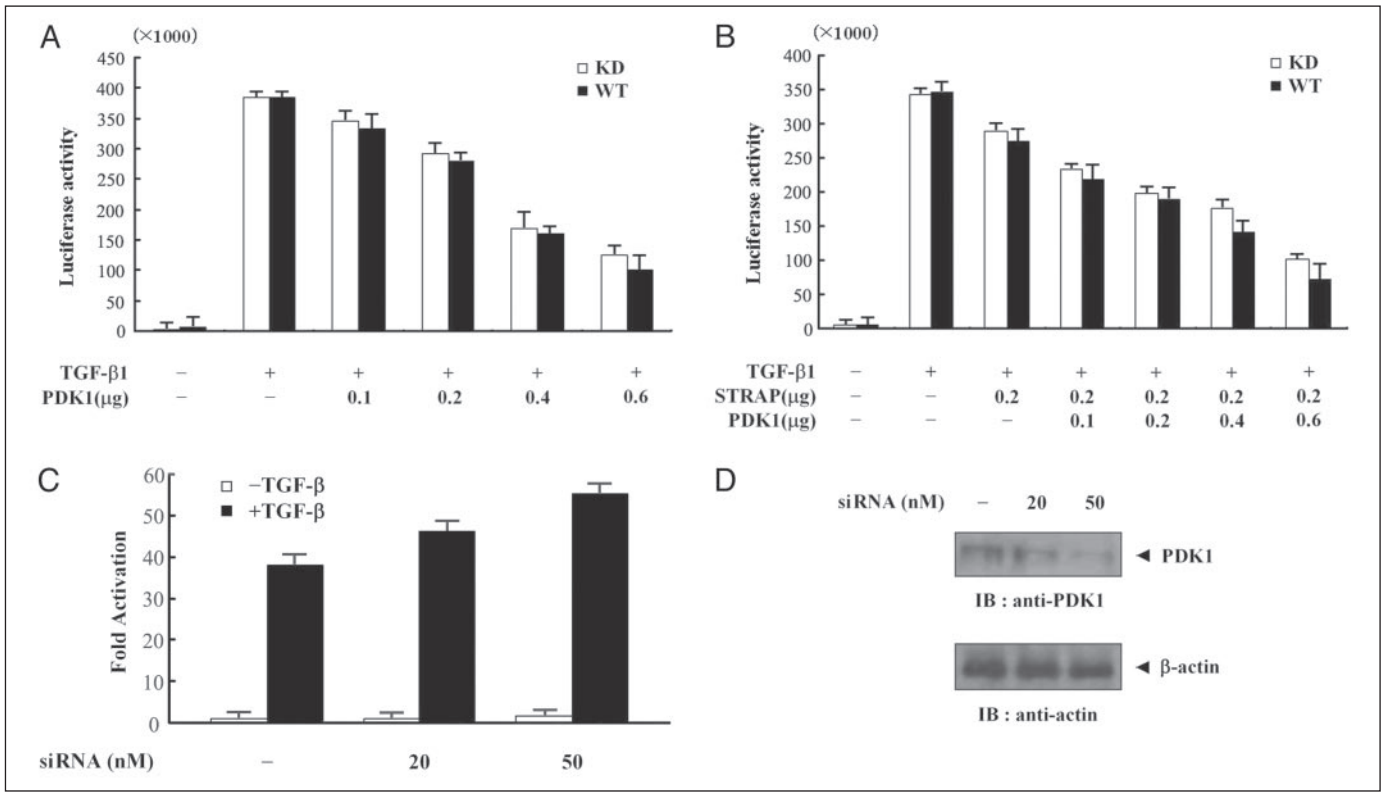

FIGURE 9. **Effect of PDK1 on the negative regulation of TGF-β signaling induced by STRAP.** *A*, inhibition of TGF-β-induced transcription by PDK1. HepG2 cells were transfected as described under "Materials and Methods" with increasing amounts of PDK1 (*WT*, a wild-type form; *KD*, a kinase-dead form) as indicated in the presence or absence of 100 pM TGF-β1, together with an empty pFLAG-CMV2 vector and 0.3 μg of p3TP-Lux reporter. Luciferase activity was measured 48 h after transfection. *B*, effect of increasing amounts of PDK1 (*WT*, a wild-type form; *KD*, a kinase-dead form) on STRAP-induced suppression of transcription. HepG2 cells were transiently transfected with 0.3 μg of p3TP-Lux reporter, 0.1 μg of β-galactosidase internal control, and increasing amount of PDK1 as indicated in the presence or absence of STRAP (0.2 μg). Luciferase assays were performed as described under "Materials and Methods." Normalized luciferase expression from triplicate samples is presented relative to the LacZ expressions, and the standard deviations are less than 5% (*A* and *B*). *C* and *D*, enhancement of TGF-β-induced transcription by PDK1 siRNA. HepG2 cells were transfected with 0.3 μg of p3TP-Lux reporter and with increasing amount of PDK1 siRNA as indicated in the presence (*black bar*) or absence (*white bar*) of 100 pM TGF-β1, and luciferase activity was measured 48 h after transfection (*C*). Expression levels of endogenous PDK1 (*upper panel*) and β-actin (*lower panel*) were determined by anti-PDK1 and anti-actin immunoblotting (*IB*), respectively. (*D*). The relative luciferase activity was normalized against β-galactosidase activity, and fold activation relative to TGF-β-untreated samples without PDK1 siRNA was calculated. The data are representative of at least four independent experiments.

of TGF-β exhibit only cytosolic staining, but TGF-β treatment increased its nuclear translocation, as expected (*upper panel, 1st* and *2nd lanes*). However, upon TGF-β treatment the coexpression of PDK1 inhibited the nuclear translocation of Smad3 (*upper panel, 2nd* and *4th lanes*), whereas the coexpression could not influence the Smad3 movement in untreated cells (*upper panel, 1st* and *3rd lanes*). On the other hand, the change of PDK1 localization by Smad3 was not observed in the presence or absence of TGF-β (*lower panel*). Taken together, these results suggest that PDK1 coexpression prevents the normal translocation of Smad3 from the cytoplasm to the nucleus in response to TGF-β.

DISCUSSION

Several reports suggest the involvement of cellular proteins for controlling PDK1 kinase activity. RSK2 has been shown to interact with PDK1 and to potentiate PDK1 activity (35). In addition, PDK1 activity was controlled by PDK1-interacting proteins such as Hsp90 (19), 14-3-3 (20), and protein kinase C-related kinase 2 (21). Moreover, recent studies have shown that Src kinases regulate PDK1 activity by PDK1 phosphorylation at tyrosine residues (36, 37). These observations raise the possibility that additional proteins may be involved in the regulation of PDK1 activity, even though PDK1 activity has been thought to be constitutively active in cells (15, 17). To address this question, we sought to identify cellular proteins that directly associate with PDK1. Here we report an isolation of STRAP as a PDK1-interacting protein.

STRAP is known to be a WD40 domain containing protein, which interacts with TβR-I and TβR-II and negatively regulates TGF-β signaling (12). In addition, STRAP was found to interact with Smad7 for the synergistic effect on the inhibition of TGF-β signaling (13). Recent studies have shown that the PI3K pathway may be associated with the TGF-β signaling pathway. For example, TGF-β potentiated PI3K activation and Akt phosphorylation in Swiss 3T3 cells (25), and LY294002, a PI3K inhibitor, blocked the Smad2 phosphorylation induced by TGF-β (38). Runyan *et al.* (39) also demonstrated that TGF-β could activate PDK1 in human mesangial cells, resulting in the enhancement of Smad3-mediated collagen I expression. In this respect, the association of PDK1 with STRAP provides an interesting aspect to the regulation of PDK1 activity and TGF-β-induced transcription. In the case of the regulation of PDK1 activity, as shown in Fig. 5, a significant increase was observed in the PDK1 activity by direct binding of STRAP. This, together with the previous observations that PDK1 activity is controlled by its interacting proteins (19–21, 35), strongly suggests that the physical association of PDK1 and STRAP also plays an important role in the modulation of PI3K/PDK1 signaling pathway. In addition, as shown in Fig. 8, our present results demonstrate that STRAP coexpression significantly reduces the association of PDK1 with 14-3-3, a known negative regulator of PDK1 (20). Thus, it seems that the possible likely mechanism by which STRAP stimulates PDK1 activity would be through the removal of 14-3-3 from PDK1-14-3-3 complex, probably by competing with 14-3-3 under cellular stimulations. This notion was further supported by the fact that the binding affinity of 14-3-3 to the endogenous PDK1 was decreased by insu-

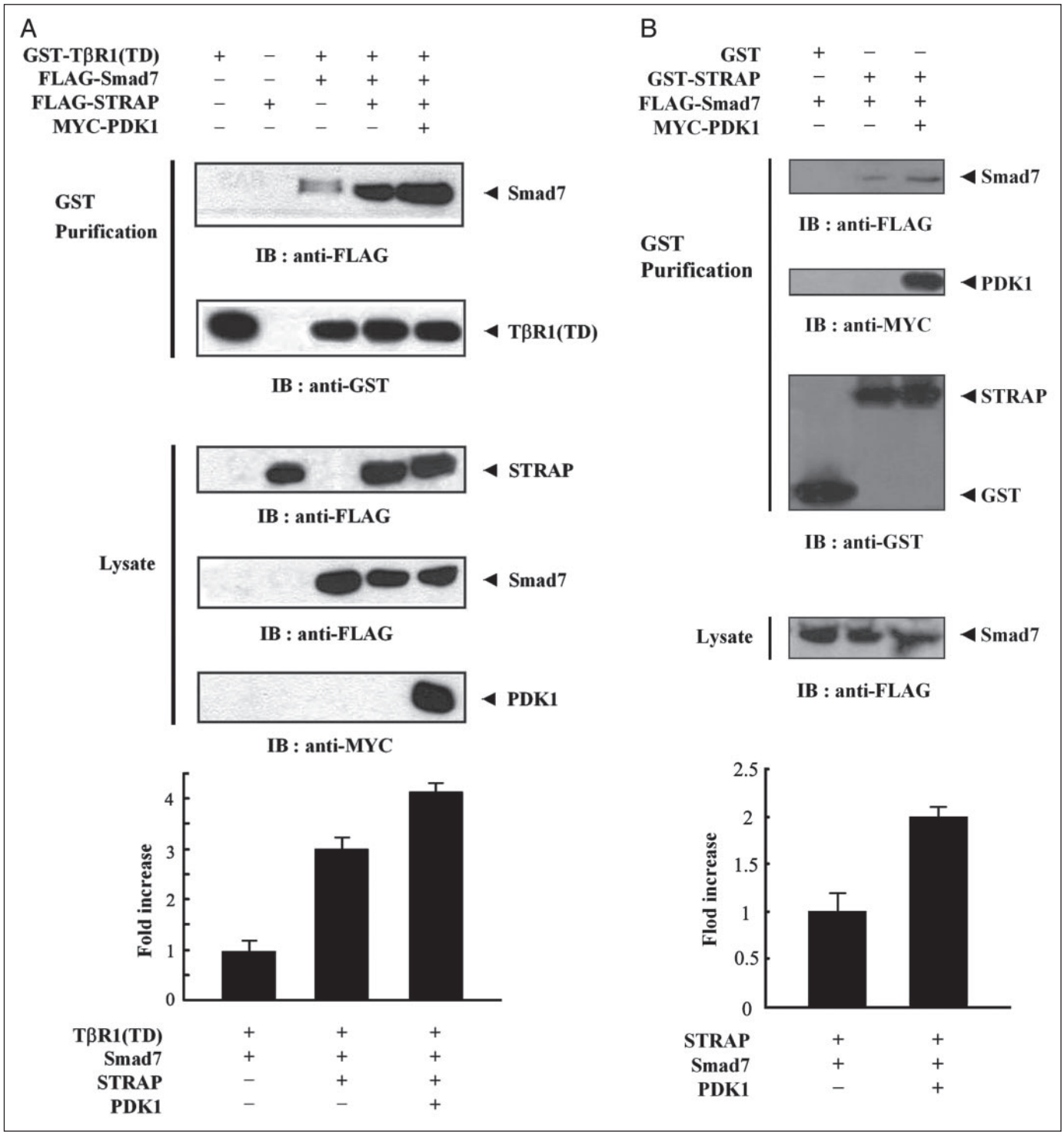

FIGURE 10. **Effect of PDK1 on the association between activated type I TGF-β receptor and Smad7.** *A*, 293T cells were transfected with the indicated combinations of plasmid vectors expressing TβR1(TD), an activated type I TGF-β receptor, FLAG-Smad7, FLAG-STRAP, and MYC-PDK1, and the cell lysates were subjected to precipitation with glutathione-Sepharose beads (*GST purification*). The complex formation between TβR1(TD) and Smad7 was determined by anti-FLAG antibody immunoblot (*IB*) (*top panel*). The amounts of FLAG-STRAP (*3rd panel*), FLAG-Smad7 (*4th panel*), and MYC-PDK1 (*5th panel*) used for *in vivo* binding assay in total cell lysates were determined by immunoblot analyses with the indicated antibodies (*Lysate*). Expression level of GST-TβR1(TD) in GST precipitates was determined by anti-GST antibody immunoblot (*2nd panel*). The relative amount of the complex formation between TβR1(TD) and Smad7 was quantified by densitometric analysis as described above, and fold increase relative to control samples transfected without PDK1 and STRAP was calculated (*bottom panel*). *B*, 293T cells transfected with the indicated plasmid vectors were precipitated with glutathione-Sepharose beads (*GST purification*), and the cell lysates were immunoblotted with the indicated antibodies (*top panel* for anti-FLAG, *2nd panel* for anti-MYC, and *3rd panel* for anti-GST). Expression level of FLAG-Smad7 in total cell lysates was confirmed by immunoblot analysis with an anti-FLAG antibody (*4th panel*, *Lysate*). The relative amount of the complex formation between STRAP and Smad7 was quantified and shown as a *bar graph* using densitometric analysis as described, and fold increase relative to control samples transfected without PDK1 was calculated (*bottom panel*). These experiments were performed in duplicate at least five times with similar results.

lin, which can increase the physical association between PDK1 and STRAP (Fig. 4*B*).

In this study, to see whether PDK1-STRAP complex formation can influence a biological function of STRAP, we analyzed the effect of PDK1 on the inhibitory TGF-β signaling induced by STRAP. Coexpression of PDK1 apparently potentiated the TGF-β-mediated transcrip-

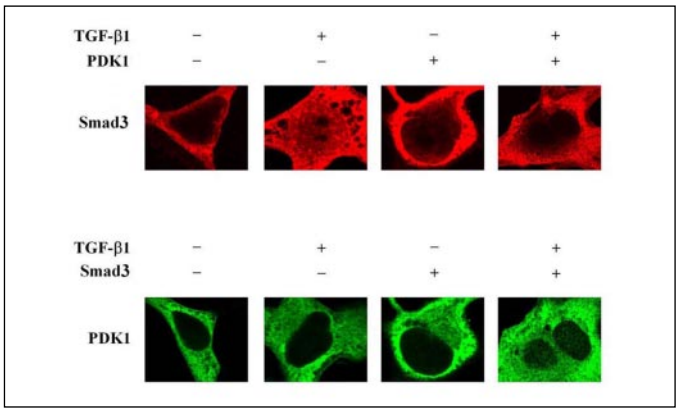

FIGURE 11. **Modulation of Smad3 localization by PDK1 coexpression.** Hep3B cells were transiently transfected with either FLAG-Smad3 or MYC-PDK1, together with MYC-PDK1 or FLAG-Smad3, in the presence or absence of 100 pM TGF-β1. Cells were immunostained with the anti-FLAG(M2) or anti-MYC antibody, followed by Alexa Fluor-594 anti-mouse secondary antibody (for *red*, Smad3) or Alexa Fluor-488 anti-rabbit secondary antibody (for *green*, PDK1), and analyzed by confocal microscopy. These experiments were independently performed at least five times with similar results.

tional inhibition induced by STRAP (Fig. 9). This would be analogous to the previous observation in which STRAP and Smad7 synergized the inhibition of TGF-β signaling by direct binding (13). In order to define the detailed mechanism of PDK1 function in the inhibition of TGF-β signaling, we further analyzed the relative strength of binding of Smad7 and the activated type I TGF-β receptor in 293T cells where these proteins were coexpressed with STRAP in the presence or absence of PDK1 because the association between Smad7 and the type I TGF-β receptor was shown previously to play a critical role in the inhibition of TGF-β signaling (34). As shown in Fig. 10*A*, a significant increase in the physical association between Smad7 and the activated type I TGF-β receptor was observed in the transfected cells expressing PDK1 compared with the control cells without PDK1, suggesting that PDK1, like STRAP, could contribute to the stabilization of the physical association between Smad7 and the type I TGF-β receptor for the inhibitory TGF-β signaling. This effect of PDK1 can be explained either by a direct physical binding of PDK1 to STRAP or by an enzymatic function of PDK1 on an interacting partner such as STRAP. However, our observed results that the kinase-dead mutant of PDK1 could not influence the formation of the PDK1-STRAP complex, compared with the control containing the wild-type PDK1, do not favor the second model describing the importance of the catalytic function of PDK1, probably through the phosphorylation of STRAP, in the modulation of the stable association between Smad7 and the type I TGF-β receptor. In support of this notion, we could not observe a direct phosphorylation of STRAP by PDK1 using *in vitro* kinase assay when cells were cotransfected with PDK1 and STRAP or the recombinant STRAP was used as a substrate (results not shown). However, we do not rule out the possibility that PDK1 itself, not through STRAP by physical interaction, directly functions to recruit Smad7 to the type I TGF-β receptor because PDK1 also interacts with Smad7 (results not shown). In summary, as shown in this report, our findings now demonstrate that STRAP may be a positive regulator of the PI3K/PDK1 signaling pathway in addition to its negative role in the TGF-β-mediated signaling pathway. Furthermore, our results suggest that PDK1 potentiates the inhibition of TGF-β signaling induced by STRAP through the stronger stabilization of the physical association between Smad7 and TGF-β receptor and the prevention of the nuclear translocation of Smad3. Based on our observed results, at this moment we imagine that the relative levels of association between PDK1 and STRAP, simply controlled by stimuli such as insulin, growth factors, and TGF-β, may be an important factor for determining whether STRAP functions as a regulator in the modulation of PI3K/PDK1 signaling pathway or TGF-β signaling pathway. In this regard, the more detailed mechanism of the cross-regulation between PI3K/PDK1 and TGF-β signaling pathways will be the interest of future study.

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