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 (TβRI) and type II (TβRII) 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 TβRI or TβRII 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 TβRI and TβRII and inhibit TGF-β signaling (12). Moreover, STRAP was shown to stabilize the complex formation between Smad7, an inhibitory Smad, and activated TβRI-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 PKA, 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 P13K signaling can be activated by TGF-β, suggesting a functional link between P13K 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 P13K/PDK1 and TGF-β signaling pathways.
STRAP Interacts with PDK1

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-PDK1(CA) (5'-GGCAATTCCATGCCCCAGCTCCGCCG-3') contain an EcoRI site (underlined). The reverse primers for FLAG-PDK1(PH) (5'-GGCTTCGAGTCATCGACAGGCGGTC-3') and FLAG-PDK1(CA) (5'-GCCAATGGCTGATGGCTCAGAGGCTG-3') contain an XhoI and a SalI site (underlined). The amplified PCR products for deletion mutants were cut with EcoRI plus XhoI and cloned into pEhori plus XhoI and pSalI, and subcloned into pBluescript KS (Stratagene). Finally, the ClaI/NotI fragment was cloned into pEBG cut with ClaI and subcloned into pBluescript KS (Stratagene). 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 GST expression vector—GST-STRAP, a STRAP cDNA (pJG4-5-STRAP) was subcloned into pBluescript KS (Stratagene). Finally, the ClaI/NotI fragment was cloned into pEBG cut with ClaI and NotI, yielding pEBG-STRAP (GST-STRAP). On the other hand, to generate FLAG-STRAP, a FLAG cDNA (pG4-5-STRAP) was subcloned as an EcoRI/XhoI fragment into the EcoRI/Sall site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26). The porcine TGF-β1 cDNA was cloned as an EcoRI/XhoI fragment into the EcoRI/SalI site of pFLAG-PAG (26).

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). 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 immunoblots 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 fluorescein 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 × 10⁴ 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-counted with a hemocytometer at the indicated times as 100%.

**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. 2A, 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 cotransfection experiments using FLAG-STRAP-transfected 293T cell extract and endogenous PDK1 (Fig. 2B). Endogenous PDK1 was immunoprecipitated from cell lysates, and Western blot analysis showed that PDK1 was precipitated (Fig. 2B, 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. 2B, 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. 2C, 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),
and FLAG-PDK1(CA), harboring the catalytic domain (amino acids 67–359) as described in Fig. 3A, 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. 3B, 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. 4A, 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
cells transfected with plasmid vectors expressing GST-PDK1 and FLAG-STRAP. As shown in Fig. 4B (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 Interacts with PDK1**—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. 5A, 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. 5A, 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. 5B). 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. 5C, 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. 5C, 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. 6A) 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. 6A), PKB/Akt phosphorylation (Fig. 6B), and Bad phosphorylation (Fig. 6C). 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. 7A (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...
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. 7A (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. 7A, 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), 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. 7C) and cell counting kit-8 method (Fig. 7D). 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
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. 8A, 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. 4B), 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-14-3-3-3 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. 8B). 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. 9A). 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 PDK1KD mutant on TGF-β-induced transcription (where KD indicates kinase-dead). As shown in Fig. 9A, both wild-type (black bars) and catalytically inactive PDK1KD (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 PKD1 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. 9B). 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)0 MLP-Luc, containing multiple Smad3/Smad4-binding CAGA boxes (33). Similar results were also observed with the (CAGA)0 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 of apoptotic nuclei.
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-FLAG 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. 9C, 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. 9D, 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 1 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. 10A, compared with control cells expressing GST-TβR1(TD) and FLAG-Smad7, the coexpression of STRAP significantly stabilized the association between TβR1(TD) and Smad7 about 3-fold (Fig. 10A, 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. 10B, 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 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 pathay. 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-
lin, which can increase the physical association between PDK1 and STRAP (Fig. 4B).

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-
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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REFERENCES

1. Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. (1994) Biochim. Biophys. Acta 1222, 71–80
2. Derynck, R., and Feng, X. H. (1997) Biochim. Biophys. Acta 1333, 105–150
3. Massague, J., Attisano, L., and Wrana, J. L. (1994) Trends Cell Biol. 4, 172–178
4. Engel, M. E., Datta, P. K., and Moses, H. L. (1998) J. Cell. Biochem. 30, (suppl.) 111–122
5. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 389, 465–471
6. Massague, J. (1998) Annu. Rev. Biochem. 67, 753–791
7. Piek, E., Heldin, C. H., and ten Dijke, P. (1999) FASEB J. 13, 2105–2124
8. Attisano, L., and Wrana, J. L. (2000)Curr. Opin. Cell Biol. 12, 235–243
9. Massague, J., and Wotton, D. (2000)EMBO J. 19, 1745–1754
10. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–740
11. Kawabata, M., Imamura, T., Miyazono, K., Engel, M. E., and Moses, H. L. (1995) J. Biol. Chem. 270, 29628–29631
12. Datta, P. K., Chytil, A., Gorska, A. E., and Moses, H. L. (1998) J. Biol. Chem. 273, 34671–34674
13. Datta, P. K., and Moses, H. L. (2000) Mol. Cell. Biol. 20, 3157–3167
14. Vanhaezebroeck, B., and Alessi, D. R. (2000)Biochem. J. 346, 561–576
15. Casamayor, A., Morrice, N., and Alessi, D. R. (1999)Biochem. J. 342, 287–292
16. Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbisson, D., Ashworth, A., and Bownes, M. (1997)Curr. Biol. 7, 776–789
17. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998)Science 279, 707–710
18. King, C.-C., Zenke, F. T., Dawson, P. E., Dutl, E. M., Newton, A. C., Hemmings, B. A., and Bokoch, G. M. (2000) J. Biol. Chem. 275, 18108–18113
19. Fujita, N., Sato, S., Ishida, A., and Tsuruo, T. (2001) J. Biol. Chem. 277, 10336–10353
20. Sato, S., Fujita, N., and Tsuruo, T. (2002) J. Biol. Chem. 277, 39360–39367
21. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999)Curr. Biol. 9, 393–404
22. Tanaka, S., and Wands, J. R. (1996)Cancer Res. 56, 3391–3394
23. Buchemann, C. L., Willy, C., Buchmann, A., Schmiechen, A., and Schwarz, M. (2000)Carcinogenesis 21, 47–52
24. Chen, R. H., Su, Y. H., Chuang, R. L., and Chang, T. Y. (1998)Oncogene 17, 1959–1968
25. Higaki, M., and Shimokado, K. (1999)Arterioscler. Thromb. Vasc. Biol. 19, 2127–2132
26. Jung, H., Kim, T., Chae, H.-Z., Kim, K.-T., and Ha, H. (2001) J. Biol. Chem. 276, 15594–15510
27. Kim, T., Jung, H., Min, S., Kim, K.-T., and Ha, H. (1999) FEBS Lett. 460, 363–368
28. Seong, H.-A., Kim, K.-T., and Ha, H. (2003)J. Biol. Chem. 278, 9655–9662
29. Prasad, N., Topping, R. S., Zhou, D., and Decker, S. J. (2000)Biochemistry 39, 6929–6935
30. Del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997)Science 278, 687–689
31. Koh, H., Lee, K. H., Kim, D., Kim, S. W., and Chung, J. (2000)J. Biol. Chem. 275, 34451–34458
32. Wieser, R., Wrana, J. L., and Massague, J. (1995)EMBO J. 14, 2199–2208
33. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huett, S., and Gauthier, J.-M. (1998)EMBO J. 17, 3091–3100
34. Nakao, A., Akrafkite, M., Moreau, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., and ten Dijke, P. (1997) Nature 389, 631–635
35. Frodin, M., Jensen, C. J., Merienne, K., and Gammeltoft, S. (2000)EMBO J. 19, 2924–2934
36. Park, J., Hill, M. M., Hess, D., Brazil, D. P., Hofsteenge, J., and Hemmings, B. A. (2001) J. Biol. Chem. 276, 37459–37471
37. Grillo, S., Greneaux, T., Casamayor, A., Alessi, D. R., Le Marchand-Brustel, Y., and Tanti, J. F. (2000)EMBO J. 19, 6642–6649
38. Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., and Arteaga, C. L. (2000) J. Biol. Chem. 275, 36803–36810
39. Runyan, C. E., Schnaper, H. W., and Poncelet, A.-C. (2004) J. Biol. Chem. 279, 2632–2639

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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-β. 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.

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