3-Phosphoinositide-dependent PDK1 Negatively Regulates Transforming Growth Factor-β-induced Signaling in a Kinase-dependent Manner through Physical Interaction with Smad Proteins*

Received for publication, October 2, 2006, and in revised form, February 27, 2007. Published, JBC Papers in Press, February 27, 2007, DOI 10.1074/jbc.M609279200

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We have reported previously that PDK1 physically interacts with STRAP, a transforming growth factor-β (TGF-β) receptor-interacting protein, and enhances STRAP-induced inhibition of TGF-β signaling. In this study we show that PDK1 coimmunoprecipitates with Smad proteins, including Smad2, Smad3, Smad4, and Smad7, and that this association is mediated by the pleckstrin homology domain of PDK1. The association between PDK1 and Smad proteins is increased by insulin treatment but decreased by TGF-β treatment. Analysis of the interacting proteins shows that Smad proteins enhance PDK1 kinase activity by removing 14-3-3, a negative regulator of PDK1, from the PDK1-14-3-3 complex. Knockdown of endogenous Smad proteins, including Smad3 and Smad7, by transfection with small interfering RNA produced the opposite trend and decreased PDK1 activity, protein kinase B/Akt phosphorylation, and Bad phosphorylation. Moreover, coexpression of Smad proteins and wild-type PDK1 inhibits TGF-β-induced transcription, as well as TGF-β-mediated biological functions, such as apoptosis and cell growth arrest. Inhibition was dose-dependent on PDK1, but no inhibition was observed in the presence of an inactive kinase-dead PDK1 mutant. In addition, confocal microscopy showed that wild-type PDK1 prevents translocation of Smad3 and Smad4 from the cytoplasm to the nucleus, as well as the redistribution of Smad7 from the nucleus to the cytoplasm in response to TGF-β. Taken together, our results suggest that PDK1 negatively regulates TGF-β-mediated signaling in a PDK1 kinase-dependent manner via a direct physical interaction with Smad proteins and that Smad proteins can act as potential positive regulators of PDK1.

* This work was supported by the Molecular and Cellular BioDiscovery Research Program Grant 2004-01593 from the Korea Science and Engineering Foundation funded by the Korea Government (Ministry of Science and Technology), Korean Health 21 R&D Project, Ministry of Health & Welfare Grant A060378, a Chungbuk National University Grant in 2005, in part by a Korea Research Foundation Grant KRF-2004-015-C00410, and the Research Center for Bioresources and Health and Industrial Technology Evaluation and Planning and Ministry of Commerce Industry and Energy, Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TGF-β, transforming growth factor-β; Smad, Sma and Mad-related protein; TβR, transforming growth factor-β receptor; STRAP, serine-threonine kinase receptor-associated protein; SGK, serum/glucocorticoid regulated kinase; PI3K, phosphatidylinositol 3-kinase; PKB/Akt, protein kinase B; GST, glutathione S-transferase; PAI-1, plasminogen activator inhibitor-1; GFP, green fluorescent protein; PtdIns, phosphatidylinositol; WT, wild type; KD, kinase dead; siRNA, small interfering RNA; CMV, cytomegalovirus; PH, pleckstrin homology; re.Smad, recombinant Smad.

Transforming growth factor-β (TGF-β) plays a critical role in the modulation of a wide variety of biological and developmental processes (1, 2). The diverse cellular responses elicited by TGF-β are triggered by activation of TGF-β receptors (type I and II), which are serine/threonine kinases. TGF-β receptors subsequently propagate signals through phosphorylation of intracellular signaling mediators referred to as Smads (3–5). There are three functional classes of Smad proteins (6), the receptor-regulated Smads (R-Smads), the common Smads (Co-Smads), and the inhibitory Smads (I-Smads). The R-Smads are directly phosphorylated and activated by the type I TGF-β receptor and undergo homotrimerization and heterodimerization with a Co-Smad (Smad 4). The activated heteromeric Smad complexes are translocated into the nucleus and cooperate with other nuclear cofactors to regulate the transcription of target genes (7). Smad-mediated signaling may be simple but it is under the control of a number of Smad-interacting proteins. Several lines of evidence have demonstrated the existence of cellular Smad regulators that interact with Smads to control the subcellular localization and the rate of R-Smad association with the TGF-β receptor and subsequent phosphorylation at the plasma membrane or in the cytoplasm or nucleus. Several of these Smad regulators have been identified, including the FYVE domain protein SARA (8), microtubules (9), Daxx (10), the truncated receptor-like molecule BAMBI (11), the ubiquitin ligase Smurf1 (12), the integral inner nuclear membrane protein MAN1 (13), and I-Smads (14–16), Smad6 and Smad7. Thus, identification and characterization of additional Smad-interacting molecules should provide greater insight into the regulation of Smad-mediated signaling. In addition, growth factor- and insulin-mediated signaling pathways modulate TGF-β signaling through a physical interaction between PKB/Akt and Smad3 (17), suggesting a possible cross-talk between TGF-β- and PI3K/PDK1-mediated signaling pathways.
The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a member of the protein kinase A, G, and C subfamily of protein kinases with a PH domain that binds phosphoinositides such as PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, for its activity and phosphorylates Thr-308 of PKB/Akt. Phosphorylation on both Thr-308 and Ser-473 is required for maximal activation of PKB/Akt (18–20). Furthermore, these residues are independently phosphorylated by PDK1 (for Thr-308) and PDK2 (for Ser-473). Emerging evidence indicates that PDK1 kinase activity is controlled by several cellular proteins that interact with PDK1, including Hsp90 (21), 14-3-3 (22), protein kinase C-related kinase 2 (23), and STRAP (24). These observations strongly suggest that the PDK1-interacting proteins can regulate PDK1 activity. In this study, we show that there are direct physical and functional interactions between PDK1 and Smad proteins (Smad2, -3, -4, and -7) and that these interactions may play an important role in the regulation of both the PDK1 and Smad activities involved in PI3K/PDK1- and TGF-β-mediated signaling.

**MATERIALS AND METHODS**

**Cell Culture and Plasmids**—293T, HepG2, Hep3B, HaCaT, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). The Myc-tagged human wild-type and kinase-dead PDK1 plasmids were obtained as described previously (24). FLAG-tagged Smad2, Smad3, Smad4, and Smad7 were a kind gift from Dr. R. Derynck (University of California, San Francisco). The B42-Smad3 constructs, B42-MH1(L), B42-MH1, B42-MH2(L), and B42-MH2, and the p21-Luc reporter plasmid were kindly provided by Dr. H-S. Choi (Chonnam National University, Kwangju, Korea). The p3TP-Lux reporter plasmid was a kind gift from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York). Two deletion constructs, FLAG-PDK1(PH) and FLAG-PDK1(CA), were generated by PCR using the full-length PDK1 cDNA as the template as described previously (24). To generate four Smad3 deletion constructs, FLAG-MH1(L), FLAG-MH1, FLAG-MH2(L), and FLAG-MH2, the four B42-Smad3 plasmids were digested with EcoRI and XhoI, and the EcoRI/XhoI fragments were cloned into pFLAG-PAG (25), a proliferation-associated gene cDNA cloned into the pFLAG-CMV-2 vector, cut with EcoRI and Sall.

**Reagents**—Porcine TGF-β1 and anti-Smad7 antibody were purchased from R&D Systems (Minneapolis, MN). The anti-GST and anti-FLAG (M2) antibodies have been described previously (26). The anti-PDK1, anti-Smad4, anti-14-3-3 θ, anti-histone H2B, anti-CDK4, and anti-cyclin D1 antibodies used for immunoprecipitation and immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Smad3 was obtained from Upstate Inc. (Charlottesville, VA). Anti-phospho-Ser/Thr was purchased from Abcam plc (Cambridge, UK). The anti-Smad2, anti-Akt, anti-Bad, anti-phospho-Akt(Thr-308), and anti-phospho-Bad (Ser-136) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Insulin, Wortmannin, isopropyl β-D-thiogalactopyranoside, dithiothreitol, aprotinin, phenylmethylsulfonyl fluoride, hydroxyurea, propidium iodide, RNase A, and the anti-β-actin antibody were purchased from Sigma. Polyvinylidene difluoride membrane was obtained from Millipore Corp. (Bedford, MA). The Alexa Fluor-594 anti-mouse and Alexa Fluor-488 anti-rabbit secondary antibodies were obtained from Molecular Probes. [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

**Transient Transfection, in Vivo Interaction Assay, and Western Blot Analysis**—Cells were transfected with appropriate plasmids using WelFect-Ex™ Plus (WelGENE, Daegu, Korea), according to the manufacturer’s instructions. After culturing overnight, the transfected cells 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 (27). Detergent-insoluble materials were removed by centrifugation, and the cleared lysates were incubated with glutathione-Sepharose beads (Amersham Biosciences) and then washed three times with the lysis buffer. For Western blotting, coprecipitates or whole cell extracts were resolved by SDS-PAGE. For immunoprecipitations, cell lysates were incubated with protein A-Sepharose that had been conjugated to the appropriate antibodies (anti-Myc, anti-PDK1, anti-Smad3, anti-Smad4, anti-Akt, and anti-Bad). The immunoprecipitated proteins were electrophoresed and blotted onto polyvinylidene difluoride membranes. The membranes were immunoblotted with the indicated antibodies and then developed using an ECL detection system according to the manufacturer’s instructions (Amersham Biosciences).

**PDK1 Kinase Assay**—To estimate PDK1-dependent serum glucocorticoid-regulated kinase (SGK) phosphorylation in vitro, 293T cells transiently transfected with the appropriate plasmids were washed three times with ice-cold phosphate-buffered saline (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 MgCl₂), the precipitate was incubated with 5 µCi of [γ-32P]ATP at 37 °C for 15 min in the presence of kinase buffer containing 500 ng of recombinant SGK (Upstate). The reactions were separated by electrophoresis and visualized by autoradiography.

**Small Interfering RNA (siRNA) Treatment**—siRNAs and their complementary RNA strands were synthesized by SamChull Pharm. Ltd. (Seoul, Korea). The sequences used were as follows: PDK1 siRNA(a) targeting a coding region (amino acids 420–425) of the human PDK1 (24); PDK1 siRNA(b) (5’-GAGACCUCGUUGGAGAAACU-3’), corresponding to a coding region (amino acids 310–316) of the human PDK1; Smad2 siRNA (5’-GCAGACUAUCUCCUACATT-3’), corresponding to a coding region (amino acids 247–253) of the human Smad2 (GenBank™ accession number AF027964); Smad3 siRNA (5’-GCAGACUAUCUCCUACATT-3’), corresponding to a coding region (amino acids 109–115); b, 5’-ACC-UUCCCCGAAUCGGUG-3’), corresponding to coding regions (a, amino acids 206–212) of the human Smad3 (GenBank™ accession number BC050743); Smad7 siRNA (a, 5’-GCCUAUUGCCUUGGACAAAAGT-3’; b, 5’-GUUCUCCUAAGGCUUUUTT-3’), corresponding to coding regions (a, amino acids 363–369) of the human Smad7 (GenBank™ accession number NM005904); and a nonspecific control siRNA (28) (5’-GCGC-
GGGCGACGUUGGUGUTT-3’). The sense and antisense oligonucleotides for each siRNA were mixed and heated at 90 °C for 2 min, annealed at 30 °C for 1 h, and transfected into 293T, Hep3B, HaCaT, or HeLa cells using the WelFect-Ex™ Plus method. Cell lysates were collected 48 h post-transfection and analyzed by immunoblotting to confirm the down-regulation of target proteins.

Luciferase Reporter Assay—HepG2 cells were transfected using WelFect-Ex™ Plus with the p3TP-Lux or p21-Luc reporter plasmids, along with each expression vector as indicated. The cells were harvested 48 h post-transfection, and luciferase activity was measured using the Promega dual luciferase assay kit according to the manufacturer’s instructions. Light emission was determined with a VICTOR™ luminometer (1420 luminescence counter, PerkinElmer Life Sciences). The total DNA concentration was kept constant by supplementing with empty vector DNA. The data were normalized to the expression levels of a cotransfected β-galactosidase reporter control, and experiments were repeated at least four times.

Cell Death Assay—The number of HeLa or HaCaT cells undergoing apoptosis after treatment with TGF-β1 (HeLa, 10 ng/ml for 20 h; HaCaT, 2 ng/ml for 20 h) was quantified using the GFP system, as described previously (24). Cells grown on sterile coverslips were transfected with pEGFP, an expression vector encoding GFP, together with the indicated expression vectors. The cells were treated with TGF-β1, at 24 h post-transfection. The cells were fixed with ice-cold 100% methanol, washed three times with PBS, and then stained with a bisbenzimide (Hoechst 33258). The coverslips were washed with PBS, then mounted on glass slides using Gelvatol, and visualized using a fluorescence microscope (Leica DM IRB, Germany). 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.

Preparation of Recombinant Proteins—Recombinant glutathione S-transferase (GST) fusion vectors containing Smad3 and Smad4 were constructed by subcloning the cDNA fragments of Smad3 and Smad4 into pGEX4T-1 (Amersham Biosciences) and purified by affinity chromatography on glutathione-Sepharose 4B columns (Amersham Biosciences) as described previously (25).

FACS Analysis—HaCaT cells (2 × 10^5/60-mm dish) transfected with the indicated combinations of plasmid vectors (empty vector, PDK1, Smad3, and Smad7) and siRNA duplexes (Smad3, Smad7, PDK1, and control siRNAs) were washed with ice-cold PBS and then synchronized in G0/G1 by treating with hydroxyurea (2 mm) for 20 h. The fraction of cells in each stage of the cell cycle was analyzed after 10% serum treatment for 24 h in the presence or absence of TGF-β1 (2 ng/ml). Trypsinized cells were washed twice with ice-cold PBS and incubated at 37 °C for 30 min with a solution (1 mm Tris-HCl, pH 7.5) containing 50 μg/ml propidium iodide and 1 mg/ml RNase A. The cells in each phase of the cell cycle were identified using the ModFitLT version 3.0 (PMac) program. Flow cytometry analysis was performed using FACSCalibur-S system (BD Biosciences).

Indirect Immunofluorescence—Hep3B cells were plated and transfected with FLAG-Smads (Smad3, Smad4, and Smad7) and/or Myc-tagged wild-type and kinase-dead 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 cells were incubated with mouse anti-FLAG (M2), diluted 1:1000 in PBS, or rabbit anti-Myc, diluted 1:200 in PBS, for 2 h at 37 °C. The cells were then washed three times with PBS and incubated with Alexa Fluor-594 anti-mouse or Alexa Fluor-488 anti-rabbit secondary antibodies, diluted 1:1000 in PBS, at 37 °C for 1 h. The coverslips were washed three times with PBS and then mounted on glass slides using Gelvatol. Proteins were visualized using a Leica Dmlre2 confocal microscopy (Germany).

RESULTS
Identification of PDK1 as a Smad-interacting Protein—We have found previously that STRAP, a TGF-β receptor interacting protein, physically interacts with PDK1 in mammalian cells (24). In addition, STRAP inhibits TGF-β signaling by stabilizing the TGF-β-receptor-Smad7 complex, and STRAP itself binds to Smad proteins such as Smad2, Smad3, and Smad7 (29). Based on these data, we reasoned that PDK1 might interact with Smad proteins, as well as with STRAP, in intact cells. To examine whether PDK1 directly binds to Smad proteins, we performed in vivo binding assays and coimmunoprecipitation experiments using overexpressed or endogenous proteins in 293T cells. The interaction of FLAG-tagged Smad proteins with a Myc-PDK1 fusion protein was analyzed by immunoprecipitation with an anti-Myc antibody, followed by immunoblotting with an anti-FLAG antibody. Smad2, -3, -4, and -7 were detected in the immunoprecipitate when coexpressed with Myc-PDK1 (Fig. 1A), indicating that PDK1 physically interacts with Smad proteins in cells. To confirm the interaction of PDK1 with Smad proteins in vivo, we next performed coimmunoprecipitation experiments with endogenous PDK1 and exogenous FLAG-tagged Smad proteins (Fig. 1B). Endogenous PDK1 was immunoprecipitated with an anti-PDK1 antibody from cell lysates, and the binding of Smad proteins was subsequently analyzed by immunoblotting with an anti-FLAG antibody. Smad proteins were present in the PDK1 immunoprecipitate (upper panel), but not in immunoprecipitates from control lysates of cells transfected with empty vector alone (CMV). Moreover, to examine the interaction between the two endogenous proteins, immunoprecipitation of endogenous PDK1 using an anti-PDK1 antibody was performed, and the binding of the endogenous Smad proteins (Smad2,-3, and -7) was subsequently analyzed by immunoblotting with the indicated anti-Smad antibodies. As shown in Fig. 1C, endogenous PDK1 physically interacted with the endogenous Smad proteins used in 293T cells. We have further analyzed this association using other cell lines, including Hep3B cells and SK-N-BE2C cells (27), a human neuroblastoma line, and we confirmed that this association could occur in vivo (data not shown). To determine whether the Smad proteins can be substrates for PDK1 in vitro, recombinant Smad proteins (re.Smad3 and -4) were expressed in Escherichia coli and purified and then used as substrates in a PDK1 kinase assay. Extracts from 293T cells expressing GST-
PDK1 and FLAG-STRAP were purified with glutathione-Sepharose beads, and incubated with [γ-³²P]ATP to allow phosphorylation of the recombinant Smad proteins. As shown in Fig. 1D, the Smad proteins were phosphorylated by PDK1 when the in vitro kinase assays were performed using re.Smad3 and -4 as substrates. However, phosphorylation of the recombinant Smad proteins was not detected in the absence of PDK1 (data not shown). In addition, we observed that the coexpression of STRAP, a potential positive regulator of PDK1 (24), significantly increased the phosphorylation of Smad proteins by PDK1 (Fig. 1D, 2nd versus 3rd lane and 4th versus 5th lane). Similar results showing that the Smad proteins can be phosphorylated by PDK1 were also observed in vivo using cells coexpressing PDK1 and Smad2, -3, -4, or -7 instead of the recombinant Smad proteins (data not shown). To further confirm that the phosphorylation of Smad proteins by PDK1 occurs in vivo,
we compared PDK1-mediated phosphorylation of Smad3/4 in cells expressing wild-type (WT) PDK1 or a kinase-dead (KD) PDK1 mutant or in the presence of a PDK1 siRNA. Expression of wild-type PDK1 produced a higher level of Smad3/4 phosphorylation, compared with cells expressing either kinase-dead PDK1 or a PDK1-specific siRNA (Fig. 1E). Taken together, our results indicate that PDK1 directly interacts with Smad proteins in vivo and that Smad proteins can be substrates for PDK1.

Mapping of the PDK1 and Smad Protein Domains Involved in the PDK1-Smad Complex Formation—To establish which regions of PDK1 are necessary for association with the Smad proteins, we generated two PDK1 deletion constructs FLAG-PDK1(PH), comprising the carboxyl-terminal pleckstrin homology (PH) domain (amino acids 411–556), and FLAG-PDK1(CA), harboring the catalytic domain (amino acids 67–359), as described previously (24), and we examined whether these constructs were able to interact with Smad proteins in vivo. Wild-type FLAG-PDK1 and FLAG-PDK1(PH), which lacks the catalytic domain of PDK1, interacted with Smad2, -3, -4, and -7 when the proteins were coexpressed in 293T cells (Fig. 2A). However, FLAG-PDK1(CA), which contains only the catalytic domain, was unable to do so (Fig. 2A, top panel), indicating that the interaction with Smad proteins is mediated via the carboxy-terminal PH domain of PDK1. Next, to examine which region of Smad3 was required for binding of PDK1 in vivo, we generated four FLAG-tagged Smad3 deletion constructs (Fig. 2B, upper panel). The FLAG-MH1 (amino acids 1–136), FLAG-MH1(L) (amino acids 1–231), FLAG-MH2, and FLAG-MH2(L), and cell lysates were purified with glutathione-Sepharose beads (GST purification). The complex formation between PDK1 and Smad3 deletion constructs was determined by immunoblotting with the anti-FLAG antibody (lower, top panel).

Expression levels of FLAG-Smad3 deletion constructs were confirmed by Western blot analysis of total cell extracts using the anti-FLAG antibody (lower, bottom panel). These experiments were independently performed at least four times with similar results.

FIGURE 2. Mapping of the binding site involved in PDK1-Smad complex formation. A, mapping of PDK1 domains involved in Smads binding. The structure of the WT PDK1 is depicted with the relative locations of its catalytic domain (CA) and PH domain. The numbers indicate amino acid residues, and the amino acid numbers of the domain boundaries are indicated (upper panel). 293T cells were cotransfected with GST alone or GST-Smads (Smad2, -3, -4, and -7), together with FLAG-PDK1(WT), FLAG-PDK1(CA), and FLAG-PDK1(PH), and purified with glutathione-Sepharose beads (GST purification). The amount of PDK1(PH) and PDK1(WT) bound to Smad proteins was determined by Western blot (WB) analysis using an anti-FLAG antibody (lower, top panel). The same stripped blot was re-probed with an anti-GST antibody to determine the expression of GST fusion proteins in the co precipitates (lower, middle panel), and the expression of FLAG-tagged PDK1 protein in total cell lysates was analyzed by Western analysis using the anti-FLAG antibody (lower, bottom panel, Lysate). B, mapping of Smad3 domains involved in PDK1 binding. 293T cells were transiently transfected with vector alone (GST), or GST-PDK1, in combination with the indicated FLAG-Smad3 deletion constructs, FLAG-MH1, FLAG-MH1(L), FLAG-MH2, and FLAG-MH2(L), and cell lysates were purified with glutathione-Sepharose beads (GST purification). The complex formation between PDK1 and Smad3 deletion constructs was determined by immunoblotting with the anti-FLAG antibody (lower, top panel). Expression levels of FLAG-Smad3 deletion constructs were confirmed by Western blot analysis of total cell extracts using the anti-FLAG antibody (lower, bottom panel). These experiments were independently performed at least four times with similar results.
FIGURE 3. Regulation of PDK1-Smad association by TGF-β and insulin. A, decrease in the association between PDK1 and Smad proteins in response to TGF-β. 293T cells transfected with the indicated expression vectors were incubated with Dulbecco’s modified Eagle’s medium containing 0.2% fetal bovine serum in the presence (+) or absence (−) of TGF-β (100 pM) for 20 h. Cell lysates were purified on glutathione-Sepharose beads (GST purification) and immunoblotted with an anti-FLAG antibody (top panel). The amount of precipitated GST and GST-tagged PDK1 was analyzed using an anti-GST antibody (middle panel). Expression levels of FLAG-tagged Smads (Smad2, -3, -4, and -7) were confirmed by Western blot (WB) analysis of total cell extracts using the anti-FLAG antibody (bottom panel). B, increase in the association between PDK1 and Smad proteins in response to insulin. 293T cells were transfected with an expression vector encoding GST-PDK1 and the indicated FLAG-tagged Smads (Smad2, -3, -4, and -7). At 48 h post-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 precipitation with glutathione-Sepharose beads (GST purification). The resulting precipitates were analyzed by immunoblot analysis with an anti-FLAG antibody to identify complex formation between PDK1 and Smad proteins (top panel). Equivalent amounts of GST-PDK1 were precipitated, as assessed by immunoblot analysis with the indicated Smad antibodies (bottom panel). C, decrease in Smad3/4 phosphorylation in response to TGF-β (24). Cell lysates were analyzed by immunoblot analysis with an anti-phospho-Ser/Thr antibody (top panel). Equivalent amounts of PDK1 were precipitated, as assessed by immunoblot analysis with an anti-PDK1 antibody (middle panel). Expression levels of PDK1 and β-actin were confirmed by Western blot analysis of total cell lysates using the indicated antibodies (bottom panel).
PDK1 Interacts with Smad Proteins

**FIGURE 4. Smad proteins enhance PDK1 activity.** A, 293T cells were transiently transfected with GST-PDK1 in combination with the indicated FLAG-tagged Smads (Smad2, -3, and -7) or with the positive control STRAP (24). Cell lysates were subjected to precipitation with glutathione-Sepharose beads and then the precipitates were analyzed for PDK1 kinase activity using an in vitro kinase assay with SGK as a substrate (top panel). The same blot was stripped and re-probed with the indicated antibodies to determine the expression level of precipitated PDK1 (2nd panel) and to show that equivalent amounts of substrate (His-SGK) were used in the kinase assays (3rd panel). The presence of Smad proteins and STRAP in total cell lysates was analyzed by Western blot (WB) analysis using the anti-FLAG antibody (bottom panel, Lysate). The circled p-SGK indicates the position of the phosphorylated SGK. B and C, PDK1-mediated phosphorylation of PKB/Akt and Bad. GST-Akt or GST-Bad was transiently cotransfected with Myc-PDK1 in the presence or absence of FLAG-tagged Smads (Smad2, -3, and -7). As a negative control, 293T cells were transfected with GST-Akt or GST-Bad alone. Transfected cells were precipitated with glutathione-Sepharose beads (GST purification), and the level of GST-Akt or GST-Bad phosphorylation was measured by immunoblot analysis using an anti-phospho-Thr 308-specific Akt antibody or an anti-phospho-Ser-136-specific Bad antibody (top panels). The anti-GST immunoblot for Akt or Bad (2nd panels) was prepared from the same blot. The expression levels of PDK1 and Smads in total cell lysates were analyzed by Western blot analysis using anti-Myc and anti-FLAG antibodies, respectively (3rd and bottom panels). D, effect of Smad siRNA duplexes on PDK1 kinase activity, PKB/Akt phosphorylation, and Bad phosphorylation. 293T cells were transfected with Smad siRNA duplexes (Smad3 siRNAs (a and b) and Smad7 siRNAs (a and b)). Total cell lysates were immunoprecipitated (IP) with anti-PDK1 antibody. The PDK1 immunoprecipitate was analyzed for PDK1 activity using an in vitro kinase assay with SGK as a substrate (top panel). The amounts of immunoprecipitated PDK1 and SGK in the assay were analyzed with anti-PDK1 and anti-His antibodies, respectively (2nd and 3rd panels). Total cell lysates transfected with Smad siRNA duplexes were immunoprecipitated with the indicated antibodies (IP: α-PDK1, IP: α-Bad). The PKB/Akt and Bad immunoprecipitates were analyzed for Akt and Bad phosphorylation by immunoblot analysis using anti-phospho-Thr308-specific Akt and anti-phospho-Ser136-specific Bad antibodies, respectively (4th and 6th panels). The amounts of immunoprecipitated Akt and Bad were analyzed with anti-Akt and anti-Bad antibodies using the same blot (5th and 7th panels). As a control, the expression levels of endogenous Smad3 and Smad7 in the total cell lysates were analyzed by Western blot analyses using the indicated antibodies (8th panel). The expression level of endogenous β-actin was determined by anti-β-actin immunoblotting (bottom panel). The circled p-SGK, circled p-Akt, and circled p-Bad indicate the position of the phosphorylated SGK, Akt, and Bad. These experiments were independently performed at least four times with similar results.
PDK1 contributes to the ability of Smad proteins to modulate PDK1 kinase activity. First, PDK1 was precipitated from transfected 293T cells using FLAG-tagged Smad proteins (Smad2, -3, and -7) and STRAP (24) as a positive control, and PDK1 kinase activity was monitored by an in vitro kinase assay using SGK as a substrate, as described previously (30). Coexpression of Smad proteins with PDK1 resulted in a significant increase in PDK1 kinase activity (Fig. 4A, top panel, 1st lane versus 2nd to 5th lanes). As a control, the expression level of the precipitated PDK1 was analyzed in GST pulldown precipitates, and the amount of PDK1 in all lanes was similar (Fig. 4A, 2nd panel), indicating that the observed differences in phosphorylated SGK were not because of differences in the PDK1 expression levels in the cells. PKB/Akt, a downstream target of PDK1, has been implicated in contributing to the sequestration of Bad from the pro-apoptotic signaling pathway by Bad phosphorylation (31). To examine whether downstream targets of PDK1, such as PKB/Akt and Bad, are also affected by coexpression of Smad2, -3, and -7, we monitored PKB/Akt phosphorylation and Bad phosphorylation in cells expressing PDK1 and Smad proteins, or PDK1 alone, using immunoblotting. Coexpression of Smad proteins with PDK1 significantly induced PKB/Akt phosphorylation compared with PDK1 expression alone (Fig. 4B, top panel, 2nd lane versus 3rd to 5th lanes). In addition, the PKB/Akt activation induced by Smad proteins also increased Bad phosphorylation (Fig. 4C, top panel, 2nd lane versus 3rd to 5th lanes). To confirm the physiological role of Smad proteins in regulation of the PI3K/PDK1 signaling pathway, PDK1 kinase activity, PKB/Akt phosphorylation, and Bad phosphorylation were determined in 293T cells transfected with Smad-specific siRNAs (Smad3 and Smad7 siRNAs) using SGK as a substrate or anti-phospho-antibodies, as indicated. Reducing the amount of endogenous Smad proteins in cells with sequence-specific siRNAs resulted in a dose-dependent decrease in PDK1 kinase activity (Fig. 4D, top panel), PKB/Akt phosphorylation (Fig. 4D, 4th panel), and Bad phosphorylation (Fig. 4D, 6th panel). As a control, the down-regulation of endogenous Smad proteins (Smad3 and -7) and β-actin was monitored by immunoblotting (Fig. 4D, 8th and bottom panels). Taken together, these findings suggest that Smad proteins positively regulate the PI3K/PDK1 signaling pathway through direct interaction with PDK1.

Smad-induced Stimulation of PDK1 Activity Is Mediated by 14-3-3 Dissociation—The binding of 14-3-3 to PDK1 suppresses PDK1 kinase activity (22), and STRAP, a positive regulator of PDK1, stimulates the dissociation of 14-3-3 from the PDK1-14-3-3 complex and enhances PDK1 kinase activity (24). Therefore, we tested whether the Smad proteins that act as potential positive regulators of PDK1 can modulate PDK1-14-3-3 complex formation. We examined the effect of Smad proteins on PDK1-14-3-3 association using in vivo binding assays, as described under “Materials and Methods.” GST-PDK1 and FLAG-14-3-3 were transiently transfected into 293T cells in the presence of Smad2, -3, -4, and -7. At 48 h post-transfection, complex formation between PDK1 and 14-3-3 (top panel) and the amounts of FLAG-Smads and FLAG-14-3-3 in the total cell lysates (3rd and bottom panels, Lysate) were determined by an anti-FLAG antibody immunoblot. The abundance of GST alone and GST-PDK1 in GST precipitates was determined using an anti-GST antibody immunoblot (2nd panel). 

FIGURE 5. Smad proteins stimulate the dissociation of 14-3-3 from PDK1-14-3-3 complexes. A, 293T cells were transfected with the indicated combinations of plasmid vectors expressing a vector alone (GST), GST-PDK1, FLAG-14-3-3, and FLAG-Smads (Smad2, -3, -4, and -7). At 48 h post-transfection, complex formation between PDK1 and 14-3-3 (top panel) and the amounts of FLAG-Smads and FLAG-14-3-3 in the total cell lysates (3rd and bottom panels, Lysate) were determined by an anti-FLAG antibody immunoblot. The abundance of GST alone and GST-PDK1 in GST precipitates was determined using an anti-GST antibody immunoblot (2nd panel). B, effect of Smad-specific siRNAs on the association between endogenous PDK1 and 14-3-3 proteins. 293T cells were transfected with the indicated Smad-specific siRNA duplexes (Smad2, -3, -4, and -7), and the complex formation between PDK1 and 14-3-3 (top panel) was determined by immunoblotting with an anti-14-3-3 antibody. As a control, expression levels of endogenous Smad2, Smad3, Smad4, and Smad7 in total cell lysates were analyzed by Western blot (WB) analysis using antibodies specific for Smad proteins (bottom panel). Con. indicates a nonspecific control siRNA. These experiments were independently performed at least four times with similar results.
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presence or absence of Smad2, -3, -4, and -7. Cell lysates were precipitated with glutathione-Sepharose beads, and the binding of 14-3-3 to PDK1 was monitored by immunoblot analysis using the anti-FLAG antibody. Coexpression of Smad proteins significantly increased the dissociation of 14-3-3 from the PDK1-14-3-3 complex, compared with control cells that were not transfected with Smad proteins (Fig. 5A, top panel, 2nd lane versus 3rd to 6th lanes). As a control, the expression levels of PDK1 and 14-3-3 were determined, and the amount of these proteins in all lanes was similar (Fig. 5A, 2nd and bottom panels), indicating that the change in binding between PDK1 and 14-3-3 was not because of differences in PDK1 and 14-3-3 expression. We then performed RNA interference to determine the physiological role of Smad proteins in the regulation of PDK1-14-3-3 complex formation. 293T cells were transfected with control or Smad siRNAs, and the immunoprecipitation of endogenous PDK1 using an anti-PDK1 antibody was performed, and the binding of endogenous 14-3-3 to PDK1 was analyzed by immunoblotting with the anti-14-3-3 antibody. The interaction between endogenous PDK1 and 14-3-3 was significantly enhanced by Smad siRNAs compared with the control siRNA (Fig. 5B, top panel, 1st lane versus 2nd to 5th lanes). As a control, the down-regulation of endogenous Smad2, -3, -4, and -7 was monitored by immunoblotting (Fig. 5B, bottom panel). These results suggest that Smad proteins enhance PDK1 kinase activity by stimulating the dissociation of 14-3-3, a known negative regulator of PDK1, from the PDK1-14-3-3 complex.

Smad Proteins Enhance PDK1-mediated Stimulation of Cell Growth—

We have shown that the coexpression of Smad2, -3, and -7 stimulates aspects of PI3K/PDK1 signaling, including PDK1 kinase activation, PKB/Akt phosphorylation, and Bad phosphorylation (see Fig. 4). We therefore extended our analysis to investigate whether Smad proteins can stimulate serum-induced cell growth, which is one of the most important biological functions of PI3K/PDK1 signaling. We used flow cytometry analysis using HaCaT cells to monitor the percentage of cells in S phase of the cell cycle (33). As shown in Fig. 6A, HaCaT cells coexpressing PDK1 and Smad3 (or Smad7) function.

FIGURE 6. Smad proteins stimulate PDK1-mediated cell cycle progression. HaCaT cells (2 x 10^5/dish) transfected with the indicated combinations of plasmid vectors (A, vector alone, PDK1, Smad3, and Smad7) or siRNA duplexes (B and C, PDK1 siRNA, Smad3 siRNA, Smad7 siRNA, and control siRNA) were synchronized in G0/G1 by hydroxyurea treatment (2 mM) for 20 h. Cells were collected before (0 h starvation) or after 10% serum treatment for 24 h (24 h serum stimulation) to determine the cell numbers in the G1, S, and G2/M phases by flow cytometry. Each experiment was repeated at least four times.
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significantly increased the percentage of cells in S phase compared with control cells expressing PDK1 alone (70 versus 54%). However, Smad3 proteins alone did not change the accumulation of S phase cells (~42%, Smad3 and Smad7) compared with controls (~45%, parental HaCaT cells (~) and vector), indicating that the increase in S phase cells when PDK1 and Smad proteins were coexpressed (+ PDK1, Smad3 and Smad7) is because of PDK1 activation by Smad proteins, probably through a direct interaction. To further confirm the involvement of Smad proteins in the stimulation of PDK1-mediated cell growth, flow cytometry analysis of HaCaT cells transfected with Smad3- or Smad7-specific siRNAs was performed. Reducing the amount of endogenous Smad3 or Smad7 by sequence-specific siRNAs in the presence of PDK1 resulted in a considerable decrease in the percentage of S phase cells compared with control cells expressing PDK1 alone (Fig. 6B, 49–52% versus 42%). However, we did not observe a reduction in the percentage of S phase cells by the knockdown of endogenous Smad3 or Smad7 when endogenous PDK1 was also knocked down by siRNA treatment (Fig. 6C). Together, these results suggest that Smad proteins, such as Smad3 and Smad7, that directly interact with PDK1 play an important role in the modulation of PDK1-mediated cell growth.

PDK1 Inhibits TGF-β-induced Transcription—Because PKB/Akt was shown to form a complex with Smad3 and inhibit TGF-β-induced transcription (17, 34), we next tested whether PDK1, an upstream target of PKB/Akt, can also regulate TGF-β-induced transcription. To examine the effect of increasing amounts of PDK1 on TGF-β-induced transcription, we cotransfected HepG2 cells with PDK1 and Smad3 (or Smad7), together with the p3TP-Lux reporter plasmid containing elements from the PAI-1 promoter (35) or with the p21-Luc reporter plasmid, in the presence or absence of TGF-β. The addition of PDK1 negatively regulated the Smad3- or Smad7-induced transcription in a dose-dependent manner, suggesting that PDK1, like PKB/Akt, inhibits the TGF-β-induced transcriptional activation (Fig. 7, A and B). We also tested whether the activity of PDK1 affects Smad3- or Smad7-induced transcription. HepG2 cells were cotransfected with wild-type PDK1 or the catalytically inactive kinase-dead PDK1 mutant, together with the p3TP-Lux reporter (Fig. 7, A and B, left panels) or the p21-Luc reporter (right panels), and luciferase assays were carried out as indicated. The wild-type PDK1 negatively regulated Smad3- and Smad7-induced transcription, in a dose-dependent manner, whereas coexpression of catalytically inactive kinase-dead PDK1 had little effect on Smad3- or Smad7-induced transcription (Fig. 7, A and B, black bars versus white bars). These data indicate that the negative regulation of Smad3- or Smad7-induced transcription by PDK1 is dependent on its kinase activity. To examine the possibility that the effect of the inactive kinase-dead PDK1 was because of the lack of a direct physical interaction between the kinase-dead PDK1 and the Smad proteins, we carried out cotransfection experiments using GST-Smad proteins (Smad2, -3, -4, and -7) and Myc-PDK1 constructs (WT and KD). Both wild-type and kinase-dead PDK1 proteins associate with Smad proteins to a similar extent (Fig. 7C), suggesting that, in addition to a direct physical interaction with Smad proteins, the kinase activity of PDK1 is necessary for the negative regulation of Smad-induced transcription. To further analyze the negative role of PDK1 in TGF-β signaling, we examined the effect of PDK1 siRNAs on TGF-β-mediated gene responses in HeLa and HaCaT cells. The transfection of PDK1 siRNAs (a and b) resulted in up-regulation of TGF-β targets, including plasminogen activator inhibitor-1 (PAI-1), a cyclin-dependent kinase inhibitor p21(amp), and Smad7, as well as down-regulation of CDK4 and cyclin D1, which are involved in TGF-β-induced G1 arrest (Fig. 7D). Taken together, these findings clearly suggest that PDK1 physically associates with Smad proteins and negatively regulates Smad-induced transcription.

PDK1 Modulates the Association between the Type I TGF-β Receptor and Smad Proteins—To explore how PDK1 cooperates with Smad proteins in the negative regulation of TGF-β-induced transcription, we examined the effect of PDK1 on the association between TβR1(TD), an activated type I TGF-β receptor, and Smad3 and -7, because we reasoned that PDK1 could modulate TGF-β-induced transcription by altering the association between TβR1(TD) and the Smad proteins. FLAG-Smad proteins (Smad3 and -7) were cotransfected with GST-TβR1(TD) into 293T cells in the presence or absence of wild-type and kinase-dead PDK1 constructs. Compared with the control cells expressing GST-TβR1(TD) and FLAG-Smads (Fig. 8A, top left panel, 4th and 9th lanes), the coexpression of wild-type PDK1 significantly decreased the association between TβR1(TD) and Smad3 (~57% decrease; Fig. 8A, top left panel, 4th versus 5th lanes) or increased the association between TβR1(TD) and Smad7 (~37% increase; Fig. 8B, top left panel 4th versus 5th lane), whereas the KD PDK1 had no effect on association of the proteins (Fig. 8, A and B, top left panels, 9th versus 10th lane). To further confirm whether the association

FIGURE 7. Modulation of Smad-dependent transcriptional activity by PDK1. A, effect of PDK1 on Smad3-dependent transcription. HepG2 cells were transfected as described under "Materials and Methods" with increasing amounts of PDK1 (WT and KD), as indicated, and 0.2 μg of Smad3, together with an empty pFLAG-CMV2 vector and 0.3 μg of p3TP-Lux (left panel) or 0.3 μg of p21-Luc reporter plasmid (right panel), and incubated in the presence (+) or absence (−) of 100 pg of TGF-β1. Luciferase activity was measured 48 h after transfection. B, effect of PDK1 on Smad7-dependent transcription. HepG2 cells were transiently transfected with 0.3 μg of p3TP-Lux reporter (left panel) or p21-Luc reporter (right panel), 0.05 μg of Smad7, 0.1 μg of β-galactosidase internal control, and increasing amounts of PDK1 (WT and KD), as indicated, and incubated in the presence (+) or absence (−) of TGF-β1. Luciferase assays were performed as described under "Materials and Methods." Luciferase expression from triplicate samples is normalized to the lacZ expression and the standard deviations are less than 5%. C, association of Smad proteins with KD PDK1. The association was determined by coprecipitation with glutathione-Sepharose beads (GST purification) followed by Western blot with an anti-Myc antibody (top panel). GST-Smads (Smad2, -3, -4, and -7) in the precipitates were determined by Western blot with (W8) or anti-GST antibody (middle panel). Expression levels of Myc-tagged PDK1 proteins (WT or KD) were confirmed by Western blot analysis of total cell extracts using an anti-Myc antibody (bottom panel, Lysate). D, regulation of endogenous TGF-β1 targets by PDK1 siRNAs. HeLa or HaCaT cells were transfected with 200 ng each of control siRNA (Con.) or PDK1 siRNAs (PDK1(a) and PDK1(b)) and treated with TGF-β1 (HeLa cells, 10 ng/ml for 20 h; HaCaT cells, 2 ng/ml for 20 h). Expression of TGF-β1 targets (PAI-1, p21, Smad7, CDK4, and Cyclin D1) was determined by Western blot with antibodies specific to each protein. As a control, expression levels of endogenous PDK1 (6th panel) and β-actin (bottom panel) were determined by anti-PDK1 and anti-β-actin immunoblotting. These experiments were independently performed in duplicate at least four times with similar results.
between TβR1(TD) and the Smad proteins is dependent on PDK1, TβR1(TD)-Smad protein complex formation was determined in 293T cells using a PDK1-specific siRNA to knockdown PDK1 expression. Knockdown of endogenous PDK1 had an opposite effect on the association (Fig. 8, A and B, top right panels, 3rd versus 4th lanes). These data provide evidence that PDK1 negatively regulates TGF-β signaling through modulation of the direct interaction between the TGF-β receptor and Smad3 and -7.

**PDK1 Modulates the Subcellular Localization of Smad Proteins**—Because coexpression of wild-type PDK1 negatively regulated Smad3- and Smad7-dependent transcription in a dose-dependent manner (Fig. 7), we hypothesized that PDK1 modifies the intracellular localization of Smad3, -4, and -7, which are essential to TGF-β signaling. Therefore, we performed immunofluorescence microscopy analysis using Hep3B cells transfected with Smad proteins alone or together with either wild-type PDK1 or the kinase-dead mutant in the presence or absence of TGF-β. In the absence of TGF-β, Smad3 and Smad4 predominantly exhibited a cytoplasmic distribution, whereas Smad7 was mainly detected in the nucleus. However, TGF-β treatment significantly increased the nuclear localization of Smad3 and Smad4 (Fig. 9, A and B, upper panels, 1st versus 2nd lane). In contrast, the translocation of Smad7 from the nucleus to the cytoplasm was stimulated by TGF-β treatment (Fig. 9C, upper panel, 1st versus 2nd lane). Coexpression of wild-type PDK1 inhibited the nuclear translocation of Smad3 and Smad4 (Fig. 9, A and B, upper panels, 2nd versus 4th lane), as well as the translocation of Smad7 from the nucleus into the cytoplasm (Fig. 9C, upper panel, 2nd versus 4th lane) in response to TGF-β. However, the coexpression of kinase-dead PDK1 had no effect on the intracellular localization of Smad3, -4, or -7 (Fig. 9, A–C, upper panels, 2nd versus 6th lane), consistent with the reporter

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**FIGURE 8. Modulation of activated type I TGF-β receptor and Smad protein binding by PDK1.** 293T cells were transfected with the indicated combinations of plasmid vectors expressing TβR1(TD), an activated type I TGF-β receptor, FLAG-Smad3, FLAG-Smad7, and Myc-wild-type PDK1 (WT) or Myc-KD PDK1, and the cell lysates were subjected to precipitation with glutathione-Sepharose beads (GST purification). Complex formation between TβR1(TD) and Smad3 (A, left, top panel) or TβR1(TD) and Smad7 (B, left, top panel) was determined by anti-FLAG antibody immunoblot. The amounts of PDK1 and Smads (Smad3 and -7) in the total cell lysates were determined by immunoblot analysis with anti-Myc and anti-FLAG antibodies, respectively (A and B, left, 3rd and 4th panels, Lysate). Expression level of GST-TβR1(TD) in GST precipitates was determined by anti-GST antibody immunoblot (A and B, left, GST purification, 2nd panels). Quantification of the blots was done by band densitometry. The relative level of the complex formation was quantified by densitometric analysis, and fold increase relative to control samples cotransfected with GST-TβR1(TD) and Smad3 (or Smad7) was calculated (A and B, left, bottom panels). 293T cells were transfected with the indicated siRNA duplexes (PDK1 siRNA and control siRNA), together with plasmid vectors expressing TβR1(TD), FLAG-Smad3, or FLAG-Smad7, and complex formation between TβR1(TD) and Smad3 (A, right, top panel) or TβR1(TD) and Smad7 (B, right, top panel) was determined by anti-FLAG antibody immunoblot. The expression level of endogenous PDK1 was determined by anti-PDK1 immunoblotting (A and B, right, bottom panels). These experiments were performed in duplicate at least three times with similar results. WB, Western blot.
assay data (Fig. 7). To provide further evidence that wild-type PDK1 is physiologically responsible for modulation of the intracellular localization of Smad proteins, we performed siRNA experiments using a PDK1-specific siRNA. Reducing the amount of endogenous PDK1 in cells showed a stronger effect on the translocation of Smad proteins compared with positive control cells treated with TGF-β in the absence of PDK1 (Fig. 9, A–C, upper panels, 2nd versus 7th lane). To verify whether the knockdown of endogenous PDK1 could alter the subcellular localization of Smad proteins, Hep3B cells transfected with wild-type or kinase-dead PDK1, together with a nonspecific control siRNA or a PDK1-specific siRNA, were treated with TGF-β and separated into cytoplasmic and nuclear fractions. Each fraction was analyzed by Western blot analysis. The accumulation of Smad3 and Smad4 in the nuclear fraction was significantly increased in PDK1-knockdown Hep3B cells compared with the control cells expressing a nonspecific control siRNA (Fig. 9, A and B, lower left panels, 3rd versus 4th lanes), whereas the cytoplasmic accumulation of Smad3 and Smad4 was markedly decreased (Fig. 9, A and B, lower right panels, 3rd versus 4th lanes). In contrast, a decrease in nuclear Smad3 and Smad4 was observed in cells expressing wild-type PDK1 (Fig. 9, A and B, lower left panels, 1st versus 2nd lanes), consistent with the confocal microscopy data (Fig. 9, A and B, upper panels). In addition, the opposite trend was observed for translocation of Smad7 in response to TGF-β under the same conditions (Fig. 9 C). As a control, expression levels of exogenous and endogenous PDK1 were determined by Western blot analysis. Hep3B cells displayed a significant decrease in the amount of endogenous PDK1 after transfection of PDK1-specific siRNA (Fig. 9D, right panel). These results indicate that wild-type PDK1 prevents the normal translocation of Smad proteins in response to TGF-β.

PDK1 Interacts with Smad Proteins
FIGURE 9 — continued

PDK1 Interacts with Smad Proteins

**D**

| Condition | TGF-β1 | TGF-β1 |
|-----------|--------|--------|
| PDK1(WT)  | -      | +      |
| PDK1(KD)  | +      | -      |
| PDK1 siRNA| -      | -      |
| Con. siRNA| -      | +      |

Nucleus:
- WB: anti-Smad4
- WB: anti-Histone
- WB: anti-β-actin

Cytoplasm:
- WB: anti-Smad4
- WB: anti-Histone
- WB: anti-β-actin

Graphs:
- Fold increase vs. control

**E**

Smad7

Merge

| Condition | TGF-β1 | PDK1(WT) | PDK1(KD) | PDK1 siRNA |
|-----------|--------|----------|----------|------------|
| -         | +      | -        | +        | -          |

Graphs:
- Fold increase vs. control

**F**

| Condition | TGF-β1 | TGF-β1 |
|-----------|--------|--------|
| PDK1(WT)  | -      | +      |
| PDK1(KD)  | +      | -      |
| PDK1 siRNA| -      | -      |
| Con. siRNA| -      | +      |

Nucleus:
- WB: anti-Smad7
- WB: anti-Histone
- WB: anti-β-actin

Cytoplasm:
- WB: anti-Smad7
- WB: anti-Histone
- WB: anti-β-actin

Graphs:
- Fold increase vs. control

**G**

| Condition | PDK1(WT) | PDK1(KD) |
|-----------|----------|----------|
| -         | +        | -        |

Graphs:
- Fold increase vs. control

**FIGURE 9 — continued**
kinase-dead PDK1 on TGF-β-induced apoptosis using a GFP assay system (24). Apoptotic cells were scored by changes in nuclear morphology among GFP-positive cells after inducing apoptosis by TGF-β treatment, as described under “Materials and Methods.” HeLa cells were transfected with an expression plasmid encoding GFP, together with wild-type or kinase-dead PDK1, and incubated in the presence or absence of TGF-β. Approximately 48% of the HeLa cells were apoptotic following TGF-β treatment (Fig. 10A). Cells transfected with wild-type PDK1 showed higher apoptotic suppression (about 33% inhibition) than cells treated with TGF-β alone, and the effect was dose-dependent (Fig. 10A, left panel, 2nd versus 3rd and 4th lanes). However, the inhibitory effect on TGF-β-induced apoptosis was not observed in cells transfected with kinase-dead PDK1 (Fig. 10A, left panel, lane 2 versus lanes 5 and 6). These data are consistent with the results obtained from luciferase assays (Fig. 7) and indirect immunofluorescence studies (Fig. 9).

Furthermore, the knockdown of PDK1 with a PDK1-specific siRNA resulted in a significant and dose-dependent increase in TGF-β-induced apoptosis, whereas a control siRNA had no effect (Fig. 10A, right panel). We extended our analysis to examine whether TGF-β-induced apoptosis is also affected by modulating the amount of endogenous PDK1 in other TGF-β-responsive cells, such as HaCaT and Hep3B cells. Under the conditions described above, a similar PDK1-mediated, dose-dependent inhibition of TGF-β-induced apoptosis was observed in both HaCaT and Hep3B cells (Fig. 10B and data not shown). To examine whether TGF-β-induced cell cycle arrest was affected by knockdown of endogenous PDK1, which might act as a negative regulator of TGF-β signaling, we performed a flow cytometry analysis using HaCaT cells expressing a vector control, control siRNA (Fig. 10, Con. (si)), PDK1 siRNA (PDK1(si)), Smad3, Smad3/control siRNA, and Smad3/PDK1 siRNA. As shown in Fig. 10C, ~30% of cells expressing Smad3...
or Smad3/control siRNA accumulated in S phase after 24 h of serum stimulation in the presence of TGF-β, whereas ~39% of cells accumulated in S phase after 24 h of serum stimulation in the absence of TGF-β. However, a lower number of cells (~18%) were found to be in S phase in the presence of PDK1 siRNA, compared with ~30% in the absence of PDK1 siRNA. In addition, this inhibitory effect was not because of the presence of the PDK1 siRNA itself, because in the absence of Smad3, the PDK1 siRNA could not effect a significant change in the percentage of cells present in S phase, compared with the control cells expressing vector or control siRNA (~38 versus ~34%). These results indicate that PDK1 inhibits TGF-β-induced growth arrest and suggest that TGF-β-mediated biological functions, such as apoptosis and cell cycle arrest, are negatively regulated by PDK1.

**DISCUSSION**

We have recently shown that PDK1 interacts with STRAP, a TGF-β receptor-interacting protein, and that this interaction is involved in the activation of PDK1 activity (24). Furthermore, we have found that PDK1 could enhance the STRAP-dependent TGF-β transcriptional inhibition through a direct interaction, suggesting a possible cross-talk between the PI3K/PDK1 and TGF-β signaling pathways. Recently, cross-talk between PI3K/PDK1 and TGF-β signalings has been suggested in several systems (17, 34–39). TGF-β participated in PI3K/PDK1 activation and Akt phosphorylation in Swiss 3T3 cells and human mesangial cells (40), and LY294002, a PI3K inhibitor, blocked the Smad2 phosphorylation induced by TGF-β (41). Moreover, recent reports have shown that Akt, a downstream target of PDK1, physically interacts with Smad3 and suppresses TGF-β signaling (17, 34). Based on these observations, we hypothesized that the direct interaction between PDK1 and Smads occurs in vivo.

In this study, we have shown that PDK1 physically interacts with Smad proteins (Smad2, -3, -4, and -7) and functionally suppresses TGF-β-induced transcription. These results are analogous to our previous observations that a PDK1-STRAP association enhanced STRAP-induced inhibition of TGF-β signaling (24). In this context, these results suggest that PDK1 functions as a negative regulator of the TGF-β signaling pathway. Moreover, our data indicate that the kinase activity of PDK1 is necessary for its ability to suppress TGF-β-induced transcription (Fig. 7) and TGF-β-mediated biological functions such as apoptosis (Fig. 10), similar to a recent study showing that Akt, a downstream target of PDK1, associates with Smad2, -3, -4, and -7 and inhibits TGF-β signaling by an Akt-kinase-dependent mechanism (42). As shown in this study, wild-type PDK1 modulates Smad3-induced stimulation of TGF-β signaling, as well as Smad7-induced inhibition of TGF-β signaling, but the kinase-dead form of PDK1 did not (Fig. 7). These data indicate that the suppression of TGF-β signaling by PDK1 occurs via a PDK1 kinase-dependent pathway. In addition, wild-type PDK1 modified the normal movement of the Smad proteins, but the kinase-dead PDK1 did not (Fig. 9). These data
again support the hypothesis that PDK1 is a negative regulator of TGF-β signaling, and this modulation is dependent on the kinase activity of PDK1. Our present results do not support the possibility that suppression of TGF-β signaling by PDK1 is due only to the direct physical interaction between PDK1 and Smad proteins, because the kinase-dead PDK1 was shown to associate with Smad proteins at levels similar to wild-type PDK1, although it did not contribute to the negative regulation of TGF-β signaling (Fig. 7C).

In our previous report (24), we showed that PDK1 enhances the STRAP-induced inhibition of TGF-β signaling by stabilizing the association between TGF-β receptor and Smad7. Thus, it seems likely that the mechanism by which PDK1 suppresses the TGF-β-induced transcriptional activation is by modulating the association between the TGF-β receptor and Smad proteins. To test this hypothesis, we examined the effects of wild-type PDK1 and kinase-dead PDK1 on Smad3 and Smad7 binding to the TGF-β receptor (Fig. 8). Our results show that the ability of wild-type PDK1 to suppress the TGF-β-induced transcriptional activation correlates with PDK1-induced modulation of Smad3 and Smad7 binding to the TGF-β receptor. Wild-type PDK1 decreased the association between the TGF-β receptor and Smad3 and increased the association between the TGF-β receptor and Smad7, leading to the inhibition of the TGF-β-induced transcriptional activation. In contrast, no difference was found in the presence of the kinase-dead PDK1. These data indicate that the kinase activity of PDK1 is also important for modulation of complex formation between the TGF-β receptor and Smad proteins.

Smad3 plays a key role in the TGF-β signaling pathway and, upon TGF-β treatment, is phosphorylated by the TGF-β type I receptor at the SSXS motif in its carboxyl terminus and forms a complex with Smad4, a Co-Smad. The heterodimer accumulates in the nucleus to regulate transcription of target genes such as PAI-1, p21Cip1, Smad7, CDK4, CDK2, and cyclin D1. Recent studies have shown that cyclin-dependent protein kinases (CDK4 and CDK2) phosphorylate Smad3 and inhibit its activity (43, 44). The phosphorylation sites of CDK4 and CDK2 were mapped to Thr-8, Thr-178, and Ser-212 in the Smad3 linker region. They suggest that CDK-induced Smad3 phosphorylation stimulates the G1 to S transition. However, the underlying mechanism by which Smad3 phosphorylation inhibits the transcriptional activity of the protein remains to be determined. In addition to CDK4 and CDK2, the Smad3 linker region possesses phosphorylation sites for other protein kinases, including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase, p38, and calmodulin-dependent kinase II (32, 45–48). The phosphorylation of Smad3 and Smad4 by PDK1, illustrated in Fig. 1, D and E, reveals one aspect of the negative regulation of TGF-β-induced transcription, similar to the previous results (43, 44) showing that Smad3 phosphorylation is important for inhibition of TGF-β-induced transcriptional activation. However, further studies, including the identification of the PDK1 phosphorylation sites in Smad3 (or Smad4) and elucidation of the detailed mechanism by which Smad3 (or Smad4) phosphorylation inhibits TGF-β signaling, are required to completely elucidate the mechanism of inhibition.

Overall, our present study provides evidence that PDK1, like PKB/Akt (17, 34, 42), is correlated, either directly or indirectly, with the negative regulation of TGF-β signaling through direct interactions with Smad proteins and that Smad proteins, similar to STRAP (24), may function as adaptors coupling TGF-β signaling to PI3K/PDK1 signaling.

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