3-Phosphoinositide-dependent Protein Kinase β (IKKB) Phosphorylation Activates NF-κB Signaling*

The NF-κB kinase (IKK)/NF-κB and phosphatidylinositol 3-oh-kinase/3-phosphoinositide-dependent protein kinase-1 (PDK1)/Akt pathways regulate various cellular functions, especially cell survival. These two pathways are often activated in many tumors and are thought to be associated with tumor progression. However, the cross-talk between them remains unclear. Here we show that PDK1 can activate IKK/NF-κB signaling in addition to Akt signaling to promote cell survival. Screening kinases that could modulate NF-κB activity revealed that expression of an upstream Akt kinase PDK1 up-regulates NF-κB transcriptional activity. We found that PDK1 directly phosphorylates IKKB at the Ser181 residue in the activation loop, leading to NF-κB nuclear translocation and NF-κB-dependent anti-apoptotic gene expression. IKKα is not required for PDK1-mediated NF-κB activation because NF-κB activation was observed in IKKα−/− mouse embryonic fibroblasts (MEF) cells as in wild type MEF cells. Akt, which was previously reported to activate IKKα, did not participate in the PDK1-dependent IKKβ or NF-κB activation. The siRNA-mediated PDK1 gene silencing attenuated NF-κB activity and increased TRAIL-mediated cytotoxicity. Moreover, expression of constitutively active IKKβ overcame the PDK1 siRNA-mediated susceptibility to TRAIL. These results indicate that PDK1 is a critical regulator of cell survival by modulating the IKK/NF-κB pathway in addition to the Akt pathway.

The transcription factor NF-κB plays a critical role in inflammation, cell proliferation, and apoptosis (reviewed in Refs. 1–3). NF-κB is amplified or rearranged in many hematopoietic and solid tumors. Persistent nuclear NF-κB activity is also described in several human cancers as a result of constitutive activation of upstream kinases (2). NF-κB is composed of dimers of the Rel protein family. Classical NF-κB complexes, mostly p65 and p50, are sequestered in the cytoplasm of resting cells by association with an IκB family protein (reviewed in Refs. 4–6). Once the cell is stimulated by agents such as tumor necrosis factor-α (TNF-α),2 interleukin 1β (IL-1β), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), UV irradiation, virus infection, or oxidative stress, the IκB is phosphorylated by IκB kinase (IKK) complex (7, 8). This phosphorylation targets IκB for degradation via the ubiquitin-proteasome pathway and allows nuclear translocation of NF-κB (9, 10).

The IKK complex, with its native molecular mass of 700–900 kDa, contains catalytic subunits IKKα and IKKβ and regulatory subunit NEMO (reviewed in Refs. 6 and 11). It has been well established that IKK activation requires phosphorylation of conserved two serine residues in the activation loops (Ser179 and Ser180 in IKKα and Ser177 and Ser181 in IKKβ) (8). Members of mitogen-activated protein kinase kinase kinase kinases 1–3, NF-κB-inducing kinase (NIK), NF-κB-activating kinase, and transforming growth factor-β-activated kinase, have been shown to activate IKK (reviewed in Ref. 4).

3-Phosphoinositide-dependent protein kinase-1 (PDK1) was originally identified as a protein-serine/threonine kinase to phosphorylate Akt at the Thr308 residue in its activation loop (12, 13). Later studies have shown that PDK1 is not only an Akt kinase but also a kinase that phosphorylates several members of the protein kinase A, G, and C (AGC) family, including p70 ribosomal protein S6 kinase (p70S6K), serum and glucocorticoid-inducible kinases (SGKs), protein kinase C (PKC) isoforms, and p90 ribosomal protein S6 kinases (RSKs) at the equivalent residues of Thr308 in Akt (reviewed in Ref. 14). Therefore, PDK1 functions as a pivotal molecule for the activation of a number of signaling pathways involved in proliferation and survival. All of the AGC kinases have amino acid sequences very similar to those surrounding the Thr308 residue in Akt. These consensus motifs in activation loop of the AGC kinases are (S/T)FCGTXXE, where X represents any amino acid (14). PDK1, which is also one of the AGC kinases, is autophosphorylated at the Ser241 residue within its activation loop to be active (15).

During the screening of new kinases that could activate NF-κB signaling, we observed that PDK1 promoted NF-κB activation and its nuclear localization. We found that PDK1 directly bound to, phosphorylated, and activated IKK. The siRNA-mediated PDK1 gene silencing attenuated NF-κB activation and enhanced TRAIL-mediated cytotoxicity, suggesting that PDK1 regulates NF-κB signaling. These results indicate that PDK1 plays a critical role in cell survival promotion by activating IKK/NF-κB signaling in addition to Akt signaling.

**EXPERIMENTAL PROCEDURES**

Reagents, Cell Culture Conditions, and MTT Assay—The recombinant human soluble TNF-α and TRAIL were obtained from Genzyme-Teche (Minneapolis, MN). The recombinant human IKKβ and SGK proteins were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The PKC inhibitor Gö6983, GF 109203X, myristoylated PKCζ, 4-morpholinepropanesulfonic acid, PARP, poly(ADP-ribose) polymerase; GSK3, glycogen synthase kinase 3; FLIP, FLICE-inhibitory protein; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; KD, kinase-dead; EGFP, enhanced green fluorescent protein.
pseudosubstrate inhibitor, and myristoylated PKCθ pseudosubstrate inhibitor were obtained from Calbiochem (La Jolla, CA). Human embryonic kidney 293T, human fibrosarcoma HT1080, wild type (WT) mouse embryonic fibroblast (MEF), and IKKα−/− MEF (16) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human breast cancer MCF-7 and human lung cancer A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. To assess cell viability, the MTT assay was employed. In brief, the cells were incubated with MTT for 2.5 h. Formazan products were solubilized with Me2SO, and the optical density was measured at 525 nm, with a reference at 650 nm, using a microplate spectrophotometer (Benchmark Plus; Bio-Rad).

**Plasmid Construction**—The human WT-IKKα and WT-IKKβ cDNAs were generated by reverse transcription-PCR with 293T mRNA as the template and then subcloned into a pc5FLAG vector (17), a pHM6 vector (Roche Applied Science), or a pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA). Substitutions of Lys44 for Met (K44M), Ser177 for Ala (S177A), Ser181 for Ala (S181A), both Ser177 and Ser181 for Ala (S177A/S181A), or both Ser177 and Ser181 for Glu (S177/E S181E) in IKKβ cDNA were accomplished using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Myc-tagged human full-length PDK1 cDNA (WT-PDK1) in a pcCMV3 vector was kindly provided by Drs. P. Hawkins and K. Anderson (The Babraham Institute, Cambridge, UK) (18). The NH2-terminal deleted PDK1 cDNA (ΔN51-PDK1) and the kinase-dead forms (KD) of PDK1 cDNA (K111A/D223A or S241A) in a pcCMV3 vector or a pFLAG-CMV-2 vector (Sigma) were generated as described previously (19, 20). The human NIK cDNA in a pEV vector was kindly provided by Dr. W. C. Greene (University of California, San Francisco, CA). The murine NH2-terminal myristoylated (Myr) PDK1 p110α cDNA, human constitutively active form (CA) of integrin-linked kinase cDNA (S343D), mouse/chicken chimera CA-Src cDNA (Y529F), rat CA-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 cDNA (S32A/S36A), and murine Myr-akt1 cDNA in a pUSEamp vector were purchased from Upstate Biotechnology, Inc. The active form of human v-Raf-1 cDNA containing the membrane-targeting CAAX motif (CAAX-Raf-1) in a pcCMV vector was purchased from BD Biosciences Clontech. The human CA-akt1 cDNA (E40K or T308D/S473D) in a pFLAG-CMV-2 vector or a pEGFP-C1 vector was established in our laboratory (20, 21). The human WT-PKCα cDNA was generated by reverse transcription-PCR with 293T mRNA as the template and then subcloned into a pcDNA3 vector (Invitrogen). The DN-PKCα cDNA (K274W) in a pcDNA3 vector was generated by a QuikChange site-directed mutagenesis kit. The WT- and DN-PKCIζ cDNAs in a pcDNA3 vector were kindly provided by Dr. J. Moscat (Universidad Autónoma, Madrid, Spain) (22).

**Transient Transfection, Immunoprecipitation, and Western Blot Analysis**—The cells were transfected with the appropriate plasmids using the SuperFect transfection reagent (Qiagen) or Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. The PDK1–2 and -4 siRNAs were designed as described previously (19). The coding strands of the siRNAs were: UGGUGAGGACCGA-GAGUG (PDK1–2; directed to nucleotides 83–101) and GAGAC-CUCUGGAGAAACU (PKCδ–4; directed to nucleotides 929–947). Non silencing control siRNA was purchased from Qiagen. The oligonucleotides had 3′-dT-dT overhangs. The sequence of siRNA targeted to both akt1 and akt2 (Aktc) was reported previously (23). The cells were transfected with siRNAs using the Lipofectamine 2000 reagent, according to the manufacturer’s instructions.

The cells were harvested and solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1.5 mM magnesium chloride, 137 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 12 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, and 1 mM aprotinin) (17). In some experiments, nuclear and cytoplasmic fractions were separated using the NE-PER extraction kit (Pierce), according to the manufacturer’s instructions. Tagged proteins were immunoprecipitated with an anti-FLAG agrose (clone M2, Sigma) or an anti-Myc agrose (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) (17). In some experiments, the cell lysates were incubated with protein A-Sepharose (Zymed Laboratories, South San Francisco, CA) that had been conjugated with a normal rabbit IgG or an anti-IKKβ (S447O7, Santa Cruz Biotechnology) or an anti-NEMO antibody (FL-419)- conjugated or an anti-PDK1 antibody (E-3)-conjugated agrose (Santa Cruz Biotechnology). Then the immunoprecipitated proteins or the cell lysates were electrophoresed and blotted onto a nitrocellulose membrane. The membranes were incubated with antibodies to Akt, IKKα, IKKβ, Ser(P)180/181-IKKα/β, Ser(P)27-IκBα, NF-κB p65, or cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), PDK1 (BD Transduction Laboratories, Lexington, KY), glycogen synthase kinase 3 (GSK3) or Ser(P)21-GSK3α (Upstate Biotechnology), FLAG tag (clone M2; Sigma), Ser(P)176/177-IKKα/β, Thr(P)256-SGK, Myc tag (clone 9E10), IκBα (C-21), or actin (C-2) (Santa Cruz Biotechnology), His tag or X-linked inhibitor of apoptosis (XIAP) (clone 2F1) (MBL, Nagoya, Japan), FLICE-inhibitory protein (FLIP) (clone NF-6; Apotech, Epalinges, Switzerland), or PARP or topoisomerase IIβ (BD Biosciences Pharmingen, San Diego, CA). Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. After washing, the membranes were developed with an ECL system, according to the manufacturer’s instructions (Amersham Biosciences). The blots were scanned using an EPSON ES-2200 scanner supported by Adobe Photoshop 5.5 and were quantified using NIH Image 1.62 software.

**Immunostaining and Apoptosis Assay**—For immunostaining, MCF-7 cells were transfected with pc5FLAG-WT-IKKβ together with a pcCMV3 vector encoding none or WT-PDK1. Four hours after transfection, the cells were plated onto glass-bottomed dishes coated with poly-d-lysine (MatTek Corporation, Ashland, MA). After incubation for a further 20 h, the cells were washed twice with phosphate-buffered saline (PBS). The cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton X-100 in PBS for 10 min. After incubation for 1 h in PBS supplemented with 10% bovine serum albumin, the labeling was carried out by incubation for 1 h with a mouse monoclonal anti-FLAG tag antibody (clone M2, Sigma) and a rabbit polyclonal anti-NF-κB p65 antibody (C-20; Santa Cruz Biotechnology), followed by a 1-h incubation with an Alexa Fluor 488-conjugated goat anti-mouse IgG and an Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Subsequently, the cells were incubated with a TRITC-conjugated anti-Myc tag antibody (clone 9E10; Santa Cruz Biotechnology) and Hoechst 33342 (Molecular Probes) for 30 min. For visualizing apoptotic cells showing nuclear condensation and fragmentation, HT1080 cells were transfected with nonsilencing control siRNA or PDK1–2 siRNA. After transfection for 24 h, the cells were also transfected with a pEGFP-C1 vector encoding none, S177E/S181E-IKKβ (CA-IKKβ) or T308D/S473D-Akt (CA-Akt). After further incubation for 24 h, the cells were treated with or without 100 ng/ml of TRAIL for 6 h. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and stained with Hoechst 33342. After washing the cells, we visualized with...
them using a fluorescence microscope (Olympus IX-70; Olympus, Tokyo, Japan) equipped with a CCD camera.

**Pull-down Assay**—FLAG-tagged WT-PDK1 protein was in vitro translated with pc5FLAG-WT-PDK1 using the TnT quick coupled transcription/translation system (Promega, Madison, WI), according to the manufacturer’s instructions. As a control experiment, a pc5FLAG vector encoding none was used. The FLAG-tagged proteins were immunopurified with an anti-FLAG agarose and incubated with recombinant IKKβ that co-precipitated with FLAG-tagged PDK1 was detected by immunoblot analysis.

**In Vitro Kinase Assay**—In advance of the in vitro kinase assay, FLAG-tagged K44M-IKKβ was immunoprecipitated from 293T cells and incubated with λ-protein phosphatase (New England BioLabs, Beverly, MA), according to the manufacturer’s instructions. After washing, the FLAG-tagged K44M-IKKβ was incubated with immunopurified, FLAG-tagged ΔN51-PDK1 or Myc-tagged WT- or S241A-PDK1 in kinase reaction buffer for PDK1 (50 mM Tris-HCl, pH 7.5, 4 mM MOPS, pH 7.2, 5 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 1.1 mM EGTA, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 25 μM protein kinase A inhibitor, 1 μM microcystin-LR, 10 mM magnesium acetate, 25 μM ATP, 1 μM phosphocreatine, and 10 μM creatine kinase). After incubation at 30 °C for 30 min, the reactions were stopped with SDS-PAGE sample buffer and subjected to SDS-PAGE and immunoblotting with an anti-phospho-IKKβ antibody.

**In Vitro Mobility Shift Assay**—NFκB DNA binding activity was determined by electrophoretic mobility shift assay using unlabeled competitor or a specific antibody to p65. We obtained similar result using MCF-7 cells. In the mobility shift assay, nuclear extracts were observed in positive samples with WT- or mutant-oligo (upper panels). Luciferase activities were calculated (bottom panel). The error bars represent the standard deviations of triplicate transfection experiments.

FIGURE 1. PDK1 promotes NFκB transactivation. A, HT1080 cells were transfected with a pc5FLAG vector encoding WT-IKKβ and an NFκB-Luc plasmid together with a plasmid encoding none (Mock), WT-NIK, Myc-P32, ΔN51-PDK1, CA-Akt, CA-integrin-linked kinase (ILK), CA-Src, CAAX-Raf-1, or CA mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MEK1). After transfection for 24 h, luciferase activities were calculated with the dual luciferase reporter assay system. The error bars represent the standard deviations of triplicate transfection experiments. B, MCF-7 cells were transfected with a pcFLAG-CMV-2 vector encoding none (Mock) or ΔN51-PDK1 (+) together with a pc5FLAG vector encoding none (−) or WT-IKKβ (+). After transfection for 24 h, the nuclear extracts were analyzed for NFκB DNA binding activity by electrophoretic mobility shift assay with WT- or mutant-oligo (top panel). Several samples were preincubated with an unlabeled competitor or an antibody to p65, as indicated. The cell lysates were electrophoresed and immuno-blotted with antibodies to FLAG tag or actin (lower panels). C, MCF-7 cells were transfected with a pcCMV3 vector encoding none (right panel) or WT-PDK1 (left panel) together with a pc5FLAG-WT-IKKβ plasmid. After transfection for 24 h, the cells were fixed and stained. The FLAG-tagged IKKβ- or Myc-tagged PDK1-transfected cells were observed in green or red by staining with antibodies to FLAG or Myc tag, respectively (top and second panels). The endogenous p65 proteins were observed in red by staining with an anti-p65 antibody (third panels). The nuclei were observed in blue by staining with Hoechst 33342 (fourth panels). Merge represents an overlay of p65 in red and nuclei in blue (bottom panels). D, an average of p65 localization in the nucleus or cytoplasm was determined by counting more than 100 FLAG-positive (IKKβ) or both FLAG- and Myc-positive (IKKβ) cells in C. E, MCF-7 cells were transfected with a pcFLAG-CMV-2 vector encoding none (−) or ΔN51-PDK1 (+) together with a pc5FLAG vector encoding none (−) or WT-IKKβ (+). After transfection for 24 h, the cytoplasmic and nuclear fractions were separated, electrophoresed, and immunoblotted with antibodies to p65, Ser(P)32-IκBα, IKκα/β, FLAG tag, or topoisomerase Topo IIβ, p65, Western blotting.

FIGURE 2. PDK1-mediated NFκB activation through IκB phosphorylation and degradation. A, HT1080 cells were transfected with a pcFLAG-CMV-2 vector encoding none (Mock), WT-IKKβ (−) or ΔN51-PDK1 (+) together with a pc5FLAG vector encoding none (−) or WT-IKKβ (+). After transfection for 24 h, the cell lysates were electrophoresed and immunoblotted with antibodies to IκBα, Ser(P)32-IκBα, or actin. We obtained similar result using MCF-7 cells. B, HT1080 cells were transfected with a pcFLAG-CMV-2 vector encoding none (Mock) or ΔN51-PDK1 (PDK1) together with a plasmid encoding none (Mock) or ΔN51-PDK1 (PDK1) and an NFκB-Luc plasmid. After transfection for 24 h, the cell lysates were electrophoresed and immunoblotted with antibodies to IκBα or FLAG tag (upper panels). Luciferase activities were calculated (bottom panel). The error bars represent the standard deviations of triplicate transfection experiments.
mm magnesium chloride, and 200 μM ATP) for 1.5 h at 30 °C. In an experiment, recombinant SGK was incubated with immunoprecipi-
tated PDK1 in the kinase reaction buffer for PDK1 for 30 min at 30 °C. The reactions were electrophoresed and immunoblotted.

**Reporter Assay**—The cells were transfected with various plasmids and a pNFκB-Luc plasmid (BD Biosciences Clontech) together with a pRL-TK or a phRL-TK plasmid (Promega) as internal control. 24 or 48 h later, the cell extracts were assayed for luciferase activity using a dual luciferase reporter assay system (Promega), according to the manufacturer's instructions. Luciferase activity was measured with LB 960 Microplate Luminometer Centro (Berthold, Bad Wildbad, Germany).

**Electrophoretic Mobility Shift Assay**—MCF-7 cells were transfected with a pc5FLAG vector encoding none (Mock) or WT-IKKβ together with a pNFκB-Luc plasmid and the indicated amount of a pFLAG-CMV-2 vector encoding ΔN51-PDK1. After transfection for 24 h, luciferase activities were calculated. The error bars represent the standard deviations of triplicate transfection experiments. We obtained similar result using 293T cells. E, WT or IKKβ-α MEF cells were transfected with a pc5FLAG vector encoding none (Mock) or WT-IKKβ together with an NFκB-Luc plasmid and the indicated amount of a pFLAG-CMV-2 vector encoding ΔN51-PDK1. After transfection for 24 h, luciferase activities were calculated. F, HT1080 cells were transfected with a pc5FLAG vector encoding WT-IKKβ together with a pFLAG-CMV-2 vector encoding ΔN51-PDK1 or E40K-Akt (CA-Akt) or pUSEamp vector encoding Myr-Akt. After transfection for 24 h, the cell lysates were electrophoresed and immunoblotted with antibodies to Ser(P)180/181-IKKβ or FLAG tag. We obtained similar result using 293T cells. G, HT1080 cells were transfected with a pc5FLAG vector encoding KD-IKK (K44M) together with a pFLAG-CMV-2 vector encoding none (−) or ΔN51-PDK1 (+). After transfection for 24 h, the cell lysates were electrophoresed and immunoblotted with antibodies to Ser(P)180/181-IKKβ or FLAG tag. We obtained similar result using 293T cells. H, HT1080 cells were transfected with a pc5FLAG vector encoding K44M-IKKγ (K44M) together with a pFLAG-CMV-2 vector encoding none (−) or ΔN51-PDK1 (+). After transfection for 24 h, the cell lysates were electrophoresed and immunoblotted with antibodies to Ser(P)180/181-IKKβ or FLAG tag. We obtained similar result using 293T cells.

**RESULTS**

**Induction of NF-κB Transcriptional Activity and Nuclear Localization by PDK1**—To identify new kinases associated with the NF-κB signal transduction pathway, we screened kinases using the NF-κB reporter construct. The cells were transfected with IKKβ and NF-κB reporter plasmids together with plasmids encoding kinases that mainly belong to the PI3K/PDK1/Akt or Raf/MEK pathway. We observed the increase in NF-κB activity in cells that had been transfected with PDK1 like that in NIK transfectants (Fig. 1A). In our experiments, we mainly used ΔN51-PDK1 for transfection experiments because it was reported to possess almost the same activity as full-length WT-PDK1 (24). In some experiments (Figs. 1C, 5B, 6A, and 6B), we used full-length WT-PDK1 and obtained similar results. Electrophoretic mobility shift assay revealed that PDK1 also enhanced NF-κB DNA binding activity (Fig. 1B). The specificity of the DNA binding activity was confirmed by experiments with an antibody to p65, the most studied subunit among.
the NF-κB family proteins and mutant oligonucleotide. To examine how PDK1 enhances NF-κB transcriptional activity, we investigated the cellular localization of NF-κB. Endogenous p65 was mainly localized in the cytoplasm when IKKβ was expressed alone (Fig. 1C). In contrast, p65 was mainly localized in the nucleus of the cells that had been transfected with IKKβ and PDK1. Quantifying more than 100 transfected cells revealed that about four-fifths exhibited p65 nuclear localization when IKKβ was co-expressed with PDK1 (Fig. 1D). The nuclear cytoplasmic fractionation experiment confirmed that p65 nuclear localization was promoted by PDK1 (Fig. 1E). Interestingly, we found increased Ser(P)180/181-IKKβ levels in the cells that had been transfected with IKKβ and PDK1 (Fig. 1E). Phosphorylation of this serine residue has been well known to be associated with IKKβ activation (8). We also observed that IkBα was phosphorylated and degraded in cells transfected with IKKβ and PDK1 (Fig. 2A). In addition, DN-IκBα that is resistant to stimulus-induced degradation (9) suppressed PDK1-mediated NF-κB activation (Fig. 2B). These results indicate that PDK1 enhances NF-κB transcriptional activity by IKK-dependent IkB phosphorylation and degradation.

**PDK1 Activates NF-κB through Phosphorylation of IKKβ—**Sequence comparison of the residues around Ser180/181 in IKKα and Ser181 in IKKβ in their activation loops showed homology between these residues and the previously reported PDK1-phosphorylation sites in IKKβ, MEK1, Akt1, SKG1, and RSK2 (Fig. 3A and Refs. 14 and 19). Immunoblot analysis with an anti–Ser(P)180/181-IKKa/β antibody revealed that expression of PDK1 increased the level of phospho-IKKβ but not of phospho-IKKα (Fig. 3B). In contrast, expression of NIK, which is known to be an IKK kinase (4), enhanced phosphorylation of IKKα (data not shown). This result indicates that PDK1 preferentially phosphorylates IKKβ in cells. To exclude the possibility that IKKβ phosphorylation was accomplished by autophosphorylation, we generated KD-IKKβ by mutating Lys44 to Ala. Expression of PDK1 also increased the Ser(P)180/181-IKKβ levels in K44M-IKKβ-transfected cells (Fig. 3C), indicating that phosphorylation at the Ser181 residue is not mediated by autophosphorylation. To confirm the IKKβ activation by PDK1, cells were transfected with IKKα or IKKβ together with the NF-κB reporter plasmid. PDK1 greatly induced NF-κB transcriptional activity in a dose-dependent manner in IKKα transfecants compared with that in IKKα transfectants (Fig. 3D). Transfection of PDK1 alone did not affect the NF-κB activity in this experiment. Because PDK1 is thought to be constitutively active (15), the expression level of endogenous IKKα may be sufficient to fully phosphorylate and activate endogenous IKKβ.

IKKβ was previously shown to have far higher IkBα kinase activity than IKKα and to be important for the canonical NF-κB pathway induced by TNF-α and IL-1β (25–27). On the other hand, IKKα was reported to be involved in NF-κB/p100 processing induced by B cell-activating factor and lymphotixin β but not by TNF-α or IL-1β (Refs. 16 and 28; reviewed in Ref. 4). To estimate the requirement of IKKα for PDK1-mediated NF-κB activation, we checked NF-κB reporter activity in IKKα−/−MEF cells. We found that PDK1 elevated NF-κB activity both in the presence and absence of IKKα (Fig. 3E), suggesting that IKKα is not essential for NF-κB activation caused by PDK1.

Akt, a downstream kinase of PDK1, was previously reported to activate NF-κB through IKKα phosphorylation at Thr23 (29, 30). To exclude the possibility that PDK1-induced NF-κB transactivation was mediated by Akt, we examined Akt activity.

**FIGURE 4. Association between PDK1 and IKKβ.** A. 293T cells were transfected with a pcFLAG vector encoding none (−) or WT-IKKβ (+) together with a pCMV3 vector encoding none (−) or WT-PDK1 (+). After transfection for 24 h, the Myc-tagged WT-PDK1 proteins were immunoprecipitated with an anti-Myc antibody. The immunoprecipitated proteins and cell lysates were incubated with or without recombinant NEMO antibody-conjugated agarose (lanes 1 and 2, respectively). The immunoprecipitated proteins were immunblotted with antibodies to IKKα, IKKβ, and NEMO. Expression of PDK1 increased the level of phospho-IKKβ but not of phospho-IKKα (Fig. 3B). In contrast, expression of NIK, which is known to be an IKK kinase (4), enhanced phosphorylation of IKKα (data not shown). This result indicates that PDK1 preferentially phosphorylates IKKβ in cells. To exclude the possibility that IKKβ phosphorylation was accomplished by autophosphorylation, we generated KD-IKKβ by mutating Lys44 to Ala. Expression of PDK1 also increased the Ser(P)180/181-IKKβ levels in K44M-IKKβ-transfected cells (Fig. 3C), indicating that phosphorylation at the Ser181 residue is not mediated by autophosphorylation. To confirm the IKKβ activation by PDK1, cells were transfected with IKKα or IKKβ together with the NF-κB reporter plasmid. PDK1 greatly induced NF-κB transcriptional activity in a dose-dependent manner in IKKα transfecants compared with that in IKKα transfectants (Fig. 3D). Transfection of PDK1 alone did not affect the NF-κB activity in this experiment. Because PDK1 is thought to be constitutively active (15), the expression level of endogenous IKKα may be sufficient to fully phosphorylate and activate endogenous IKKβ.

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by Akt, the cells were transfected with IKKβ together with PDK1, CA-akt, or Myr-akt. Immunoblot analysis revealed that PDK1, but not Akt, could phosphorylate IKKβ at Ser181 (Fig. 3F). Consistently, we did not observe NF-κB activation in cells transfected with IKKβ and akt (Fig. 1A). Therefore, PDK1 activates NF-κB via IKKβ phosphorylation, and the phosphorylation is independent of Akt.

We then examined the association between PDK1 and IKKβ in cells. When Myc-tagged PDK1 was immunoprecipitated with an anti-Myc antibody, IKKβ was co-immunoprecipitated (Fig. 4A). PDK1 was present, in addition to IKKa and IKKβ, in anti-NEMO precipitates (Fig. 4B), indicating that PDK1 is incorporated into endogenous IKK complex including IKKa, IKKβ, and NEMO. Moreover, the association between endogenous IKKα and IKKβ was confirmed (Fig. 4C). The binding seemed to be constitutive because treatment of the cells with TNF-α, TRAIL, or insulin-like growth factor-1 had no effect on the complex formation (data not shown). Pull-down analysis with in vitro-translated PDK1 and recombinant IKKβ clearly indicates that PDK1 directly binds to IKKβ (Fig. 4D).

Phosphorylation at the Ser181 Residue in IKKβ Is Important for PDK1-mediated NF-κB Activation—Phosphorylation at both Ser177 and Ser181 residues in the activation loop was reported to be required for IKKβ activation (8). We examined whether phosphorylation at both sites was required for PDK1-mediated NF-κB activation. NF-κB reporter activity was observed when cells were co-transfected with PDK1 and S177A-IKKβ but not S181A-IKKβ (Fig. 5A). Therefore, we determined that NF-κB activation induced by PDK1 depends on phosphorylation at Ser181 in IKKβ, whereas phosphorylation at Ser177 is dispensable in this case. In contrast to WT-IKKβ, S181A-IKKβ showed no retarded migration when co-expressed with PDK1, strongly indicating that retarded migration is caused by Ser181 phosphorylation (Fig. 5A). In addition, PDK1 promoted phosphorylation at Ser177 in WT-IKKβ but not in S181A-IKKβ (Fig. 5B). The result suggests that PDK1-mediated phosphorylation activates IKKβ and that activated IKKβ may be successively phosphorylated at Ser177 by itself or by other IKKβ kinases. To confirm the requirement of kinase activity of PDK1 for IKKβ activation, we transfected the WT- or KD (K111A/D223A)-PDK1 with IKKβ into cells. Transfection of WT-PDK1 promoted IKKβ phosphorylation at the Ser181 residue in a dose-dependent fashion (Fig. 6A) and induced NF-κB activation (Fig. 6B). In contrast, no IKKβ phosphorylation and no NF-κB activation were seen in KD-PDK1 transfecants (Fig. 6, A and B). In vitro incubation of PDK1 with K44M-IKKβ confirmed that kinase activity of PDK1 was indispensable for IKKβ phosphorylation at Ser181 (Fig. 6, C and D).

PDK1 Gene Silencing Attenuates the NF-κB Signal Transduction Pathway—To estimate the role of endogenous PDK1 in IKK/NF-κB signaling in cells, we tried to knock down PDK1 expression using previously designed PDK1 siRNAs (19). The reporter assay showed that PDK1 gene silencing by PDK1-2 siRNA attenuated NF-κB transcriptional activity by half in both A549 and MCF-7 cells (Fig. 7A). On the other hand, targeted disruption of Akt expression by Akt siRNA (23) showed no effect on NF-κB activity. Although Akt was reported to activate NF-κB through IKKα phosphorylation (29, 30), these results indicate that Akt hardly participates in PDK1-mediated NF-κB activation. Moreover, other PDK1 siRNA PDK1–4, in addition to PDK1–2 siRNA, suppressed nuclear localization of endogenous p65 in cells (Fig. 7B). This result strongly indicates that NF-κB activation is dependent on PDK1. We further investigated the effect of PDK1 gene silencing on the expression of NF-κB-dependent genes (2) in various cells. We found that PDK1–2 siRNA decreased the protein expression of XIAP in MCF-7 and A549 cells and FLIP in HT1080, MCF-7, and A549 cells (Fig. 7C). In HT1080, MCF-7, and A549 cells, PDK1–2 siRNA decreased the expression of IκBα (data not shown), which is also known to be induced by NF-κB as an autoregulatory loop (31). In addition, the increase in cleaved fragments of PARP was observed in HT1080 and MCF-7 cells, suggesting that gene silencing of PDK1 promoted cellular apoptosis (Fig. 7C). These results indicate that knockdown of PDK1 expression down-regulates the expression levels of anti-apoptotic proteins, resulting in apoptosis progression. Therefore, PDK1 regulates apoptosis by
modulating IKK/NF-κB signaling in addition to Akt signaling. However, TNF-α stimulation could promote phosphorylation of IKKβ even in PDK1–2 siRNA-transfected cells (Fig. 7D), suggesting that PDK1 is not involved in TNF-α-induced IKKβ activation. Consistently, TNF-α did not enhance kinase activity of PDK1 (Fig. 7E). Moreover, we confirmed that KD-PDK1 had no effect on TNF-α-induced NF-κB activation (data not shown).

To analyze the effect of PDK1 on cell survival via NF-κB activation, PDK1–2 siRNA-transfected cells were treated with TRAIL. TRAIL was reported to induce apoptosis specifically in tumor cells (reviewed in Ref. 32). TRAIL also activates NF-κB signaling, and blockade of NF-κB sensitizes tumor cells to TRAIL-induced apoptosis (33). When cells were treated with PDK1–2 siRNA, the sensitivity to TRAIL was significantly enhanced (Fig. 8A). The TRAIL-mediated p65 nuclear localization was suppressed by PDK1 gene silencing (Fig. 8B). To evaluate the role of IKKβ in the PDK1 siRNA-mediated susceptibility to TRAIL, we examined whether EGFP-fused CA-IKKβ could overcome this susceptibility. We counted more than 100 EGFP-positive cells and considered the cells showing condensed and fragmented nuclei as apoptotic. As a result, expression of CA-IKKβ, but not of CA-Akt, attenuated TRAIL-induced apoptosis in PDK1–2 siRNA-transfected cells comparably with that in control siRNA transfectants (Fig. 8C). These results indicate that PDK1-mediated NF-κB activation plays an important role in cell survival and the sensitivity to TRAIL.

**DISCUSSION**

NF-κB is a critical transcription factor that regulates a number of cell functions including growth, survival, inflammation, and immunity (1–3). It is also clear that PDK1 plays a central role in activating the AGC family of protein kinases, which mediates intracellular signaling such as cell growth, cell survival, protein synthesis, and gene expression (14). In the present study, we revealed a novel cross-talk between the IKK/NF-κB and PI3K/PDK1/Akt pathways. We showed here that PDK1 activates the NF-κB pathway through direct IKKβ phosphorylation, and the phosphorylation is independent of Akt. Three lines of evidence support the assumption. First, overexpression of PDK1 activates NF-κB in cells (Figs. 1 and 2); second, PDK1 directly phosphorylates IKKβ in the activation loop (Figs. 3–6); and third, siRNA directed to PDK1 decreases NF-κB activity in cells (Fig. 7).

Ozes et al. (29) and Romashkova and Makarov (30) have previously reported the cross-talk between the IKK/NF-κB and PI3K/PDK1/Akt pathways. They showed that Akt was involved in IKKα phosphorylation at Thr183, and this phosphorylation was required for TNF-α-induced, NF-κB-dependent gene expression. On the other hand, we revealed that...
IKKα was not required for PDK1-mediated NF-κB activation because PDK1 also increased NF-κB activity in IKKα−/− MEF cells (Fig. 3E). In addition, transfection of active Akt was reported to be able to stimulate the p65 transactivation domain but not IkBα degradation or NF-κB DNA binding (34). The PI3K inhibitor LY294002, which strongly inhibited Akt activation, had no effects on IL-1-stimulated IKKα degradation and NF-κB DNA binding (35). Therefore, Akt activates NF-κB-dependent transcription by stimulating the transactivation domain of p65 rather than inducing NF-κB nuclear translocation via IkB degradation.

In our study, PDK1 promoted degradation of IkBα (Fig. 2), DNA binding of NF-κB, and nuclear localization of p65 through IKKβ (Fig. 1). Moreover, co-expression with active Akt did not activate NF-κB through IKKβ (Figs. 1A and 3F), and gene silencing of PDK1, but not of Akt, decreased NF-κB activation (Fig. 7A). These results strongly indicate that PDK1 also activates NF-κB pathway independent of Akt. PDK1 constitutively and moderately activates NF-κB through phosphorylation of IKKβ under nonstimulating conditions, whereas Akt is involved in strong NF-κB activation through IKKα only when cells are stimulated with TNF-α. Recently, Hu et al. (36) reported a novel cross-talk between the IKK/NF-κB and PI3K/PDK1/Akt pathways. They showed that IKKβ phosphorylated and caused proteolysis of the Forkhead family transcription factor FOXO3α (FKHRL1), which was known to be phosphorylated and excluded from nuclei by Akt (37). On the other hand, we found that an upstream Akt kinase PDK1 activated IKKβ. These results suggest that PDK1 may strongly inhibit the Forkhead family of transcription factor with two pathways: nuclear exclusion through Akt and degradation through IKK.

Although it was previously shown that IKKβ activation required phosphorylation at both Ser177 and Ser181 residues in the activation loop (8), we observed that substitution of Ser177, but not of Ser181, for Ala failed to activate NF-κB when IKKβ was co-expressed with PDK1 (Fig. 5A). The result indicates that only Ser181 is essential and that Ser177 is dispensable for IKKβ activation at least induced by PDK1.

We showed that siRNA directed to PDK1 suppressed the expression of several anti-apoptotic genes regulated by NF-κB and induced apoptosis in various cancer cells (Fig. 7C). Although recent reports suggest its regulatory mechanism, PDK1 is fundamentally thought to be constitutively active in cells (15). Thus, it is suggested that PDK1 constitutively maintains an IKK/NF-κB pathway in cancer cells to protect them from apoptosis. Because sensitization to TRAIL was observed in PDK1 siRNA-transfected cells and overcome by expression of CA-IKKβ, PDK1 may also protect cells from TRAIL-induced apoptosis through IKK/NF-κB pathway (Fig. 8). Recently, the cyclooxygenase-2 inhibitor celecoxib was reported to act as PDK1 inhibitor (38). Celecoxib has been shown to induce apoptosis in various cancer cells in vitro and reduce the formation of polyps in familial adenomatous polyposis patients (38, 39).

In the present study, we showed that siRNA directed to PDK1 inhibited NF-κB activation (Fig. 7). Therefore, it is possible that celecoxib suppresses NF-κB through PDK1 inhibition, leading to down-regulation of genes needed for inflammation, proliferation, and carcinogenesis. We found that PDK1 gene silencing sensitized cells to TRAIL-induced apoptosis (Fig. 8). Combining the PDK1 inhibitor with anticancer drugs is expected to enhance their anticancer effects (40).

PDK1 was previously shown to mediate mammary epithelial cell transformation and tumorigenesis (41). Activity of β-catenin/T cell factor and levels of its target genes c-myc and cyclin D1 were shown to be markedly elevated in PDK1-expressing cells (42). Intriguingly, c-myc and cyclin D1 were also reported to be regulated by NF-κB (2, 4). According to our observations, PDK1 overexpression would induce c-Myc and cyclin D expression via activating NF-κB. In the case of Ras-mediated transformation in rat liver epithelial cells, IKK-mediated NF-κB activation was involved in the process of transformation (43).
Thus, PDK1 might contribute to mammary epithelial cell transformation by activating NF-κB.

Hinton et al. (44) provided evidence that PDK1, as a rate-limiting upstream activator of AGC kinases, regulated T cell development. Complete PDK1 loss blocked T cell differentiation in the thymus, whereas reduced PDK1 expression allowed T cell differentiation but blocked proliferative expansion. NF-κB has been reported to play broad roles in immune functions (1, 6). For example, mice lacking the c-rel, which is expressed predominantly in hematopoietic cells and encodes a subunit of the NF-κB family, showed proliferation defects as well as decreased production of cytokines and immunoglobulins in mature T and B cells (45). Transgenic mice expressing a dominant-negative form of IkBα, able to repress multiple NF-κB proteins, showed perturbation of T cell lineage (46). Recently, PDK1 was reported to play a critical role in the T cell receptor-induced NF-κB signaling (47). Although the report focused on PDK1-associated complex formation, IKK kinase activity of PDK1 was not studied at all. Taking our findings into consideration, PDK1 may contribute to NF-κB activation both to promote complex formation and to phosphorylate and activate IKKβ. Because PDK1 was reported to activate PKCs (48), it is possible that activation of PKCs leads to IKKβ activation. Previous reports have also suggested that PKCζ and PKCδ activated NF-κB through phosphorylation of IKKβ (22). In our hands, transfection of dominant-negative forms of IKKα and IKKß, able to repress multiple NF-κB, showed higher levels of NF-κB transcription by activating NF-κB. Thus, PDK1 might contribute to mammary epithelial cell transformation.