Involvement of 3-Phosphoinositide-dependent Protein Kinase-1 in the MEK/MAPK Signal Transduction Pathway*

Received for publication, February 25, 2004, and in revised form, June 1, 2004
Published, JBC Papers in Press, June 2, 2004, DOI 10.1074/jbc.M402055200

Saori Sato‡, Naoya Fujita‡, and Takashi Tsuruo‡§

From the Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan and Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 170-8455, Japan

The phosphatidylinositide-3-OH kinase/3-phosphoinositide-dependent protein kinase-1 (PDK1)/Akt and the Raf/mitogen-activated protein kinase (MAPK/ERK) pathway have central roles in the regulation of cell survival and proliferation. Although their importance, however, the cross-talk between these two pathways has not been fully understood. Here we report that PDK1 promotes MAPK activation in a MEK-dependent manner. In vitro kinase assay revealed that the direct targets of PDK1 in the MAPK pathway were the upstream MAPK kinases MEK1 and MEK2. The identified PDK1 phosphorylation sites in MEK1 and MEK2 are Ser222 and Ser226, respectively, and are known to be essential for full activation. To date, these sites are thought to be phosphorylated by Raf kinases. However, in vivo gene silencing using small interference RNA demonstrates that PDK1 is associated with maintaining the steady-state phosphorylated MEK level and cell growth. The small interference RNA-mediated down-regulation of PDK1 attenuated maximum MEK and MAPK activities but could not prolong MAPK signaling duration. Stable and transient expression of constitutively active MEK1 overcame these effects. Our results suggest a novel cross-talk between the phosphatidylinositide-3-OH kinase/PDK1/Akt pathway and the Raf/MEK/MAPK pathway.

Many growth factors and cytokines have been reported to promote cell survival. Interaction between these factors and their specific receptors trigger the activation of phosphatidylinositol-3-OH kinase (PI3K), and Ras (1). Activated PI3K generates the phospholipid second messenger molecules phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate (2–4). These lipids, then, induce the activation of several members of the protein kinase A, G, and C (AGC) family of protein kinases including Akt/protein kinase B, p70 ribosomal protein S6 kinase (p70S6K), protein kinase C isofoms (PKCs), and serum- and glucocorticoid-inducible kinases (SGKs). Activated Ras stimulates Raf translocation from cytosol to the cell membrane by the direct interaction (5, 6), and the membrane-translocated Raf is phosphorylated and activated by multiple kinases. Activated Raf catalyzes the phosphorylation of its downstream kinases, MEK1 and MEK2 (MEK1/2), through phosphorylation at Ser218 and Ser222 of MEK1 and at Ser222 and Ser226 of MEK2 in their activation loops (7, 8). Then activated phospho-MEK1/2 triggers MAPK signaling pathways (9, 10). Activated kinases including Akt and MAPK1/2 mediate survival signal transduction and cell cycle progression by phosphorylating downstream key regulatory proteins (11–13). Because it has been reported that the activity of Akt or MAPK or both are elevated in many cancer cells, these molecules are thought to be suitable as molecular targets of anticancer drugs (1, 14–16).

PDK1 was originally identified as a kinase that could phosphorylate Akt on its activation loop (residue Thr308) (17–19). Later works, however, have shown that PDK1 is not only an Akt kinase but also a kinase responsible for phosphorylating members of the AGC family of protein kinases: p70S6K, SGKs, PKCs, and p90 ribosomal protein S6 kinases (RSKs) at the equivalent residues of Thr308 of Akt (reviewed in Ref. 13). PDK1 itself is also a member of the AGC subfamily of protein kinases and is phosphorylated at the Ser241 residue (equivalent to Thr308 of Akt) in the activation loop. Since PDK1 expressed in bacteria was active and was phosphorylated at Ser241, it is thought that PDK1 phosphorylates itself at this site (20).

During our analysis of the PDK1-mediated signal transduction pathway, we discovered an increase in the phosphorylated MAPK level of PDK1-transfected cells and saw that MAPK activation depended upon MEK activation. Sequence comparison of the residues around the PDK1-mediated phosphorylation sites in AGC kinases and MEK1/2 revealed that MEK1/2 possessed the PDK1-mediated phosphorylation sites. In vitro and in vivo analysis revealed that PDK1 could directly phosphorylate the sites (Ser222 of MEK1 and Ser226 of MEK2) to date, these sites were thought to be phosphorylated by Raf kinases. Because silencing of the PDK1 gene by siRNA decreased the phospho-MEK and phospho-MAPK levels and suppressed cell proliferation, PDK1 regulated the MEK/MAPK pathway by directly phosphorylating MEK1/2. Our results indicate the importance of PDK1 as a MAPK kinase kinase in MEK/MAPK signaling pathways.

EXPERIMENTAL PROCEDURES

Reagents, Cell Culture Conditions, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay—The recombinant, inactive
MAPK2/EKR2, inactive MEK1, active Raf-1, MIP, and purified P2PA proteins were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The PKC inhibitor rolletrin, myristoylated PKCδ pseudosubstrate inhibitor, and myristoylated PKCδ pseudosubstrate inhibitor were obtained from Calbiochem. Our previously identified PD1K inhibitor UCN-01 (21) was kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant human epidermal growth factor (EGF) and phospholipase C (PLC) were purchased from Roche Applied Science and Sigma, respectively. Human embryonic kidney 293T and human fibrosarcoma HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human lung cancer A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. To assess cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was employed. In brief, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide for 4 h. Formazan products were solubilized with Me2SO, and the optical density was measured at 525 nm using a microplate spectrophotometer (Benchmark Plus; Bio-Rad).

**Phasgon Clonmg**—The rat wild-type (WT) MEK1 cDNA, rat WT-MEK2 cDNA, and NH-terminally myristoylated (myr) mouse active form of Akt1 cDNA in pUSEamp vectors were purchased from Upstate Biotechnology. The active form of human v-Raf-1 cDNA containing the membrane-targeting CAAX motif (CAAX-Raf-1) in a pCMV vector was purchased from Clontech (Palo Alto, CA). Substitution of Lys for Ala (Raf17, Ser221 for Ala or Ser221 for Asp, Ser227 for Ala or Asp (S221A and S227A)) in both Ser221 and Ser227 in MEK1 cDNA were achieved using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Myc-tagged human full-length PDK1 cDNA (WT-PDK1) in a pCMV3 vector was kindly provided by Drs. P. Hawkins and K. Anderson (The Babraham Institute, Cambridge, UK) (22). The pleckstrin homology (PH) domain-deleted MEK1 cDNA (ΔPH-MEK1) in a pUSEamp vector, and the PDK1 cDNA (ΔN51-PDK1) with Me2SO, and the optical density was measured at 525 nm using a microplate spectrophotometer (Benchmark Plus; Bio-Rad).

**Activation of MEK-MAPK Pathway by PDK1**

In brief, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was employed. In some experiments, MAPK activity was measured using a MAPK immunoprecipitation protocol according to the manufacturer’s instructions (Roche Applied Science). Blots were scanned with an EPSON ES-2200 scanner supported by Adobe Photos hop 5.5 and quantified with NIH Image 1.62 software.

**siRNA Design and Transfection**—Five siRNAs were designed from the human PDK1 sequence (PD1K-1 to PD1K-5). The coding strands of the siRNAs were as follows: GAAGCGGCCUGAGGACUUC (PDK1-1; directed to residues 225–233), GGUGUGAGACCAGUCUA (PDK1-2; directed to residues 83–101), UCCUUGGGGAAGGCUCUUU (PD1K-3; directed to residues 260–278), GAGACCCUUGGAGAAACU (PDK1-4; directed to residues 929–947), and UGGAAGAUGCGAGCCU (PD1K-5; directed to residues 989–1007). In our experiments to suppress human PDK1 expression in 293T, HT1080, and A549 cells, we used the most effective siRNA (PDK1-4) for further analysis. Nonsilencing control siRNA was purchased from Qiagen. The oligonucleotides had 3'dTdT overhangs. The sequences of siRNAs targeted to both Akt1 and Akt2 (Aktt) or Raf-1 genes were reported previously (27, 28). Cells were transfected with siRNAs using the LipofectAMINE 2000 reagent, according to the manufacturer’s instructions.

**Immunostaining**—Cells were transfected with pUSEamp-WT-MEK2 and pcDNA3-EGFP together with a pFLAG-CMV-2 vector encoding nothing (Mock) or ΔN51-PDK1. 24 h after transfection, cells were plated onto collagen-coated culture dishes. After incubation for 4 h, in the presence of 10 μM of Me2SO, the optical density was measured at 525 nm using a microplate spectrophotometer (Benchmark Plus; Bio-Rad). The cells were fixed with 4% paraformaldehyde for 15 min. Permeabilization was carried out in 0.2% Triton X-100 for 5 min. After incubation in 1 h in phosphate-buffered saline supplemented with 1% bovine serum albumin, the labeling was carried out by incubation overnight with a rabbit polyclonal anti-phospho-MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology) followed by a 90-min incubation with AlexaFluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). After washing the cells, we visualized them using a fluorescence microscope (Olympus IX-70; Olympus, Tokyo, Japan) equipped with a CCD camera.

**Purification of Recombinant GST-ΔN51-PDK1 and GST-ΔN51-PDK1 (K111A/D223A) Proteins**—Cultures of BL21 Star E. coli (Novagen) containing a pGEX 6P-3 plasmid encoding ΔN51-PDK1 or kinase-dead form of ΔN51-PDK1 (ΔN51-PDK1) were grown in E. coli (Novagen). After washing the cells, they were resuspended using a fluorescent microscope (Olympus IX-70; Olympus, Tokyo, Japan) equipped with a CCD camera.

**In Vitro Kinase Assay**—To perform the in situ kinase assay, recombinant, active Raf-1 or immunopurified, FLAG-tagged ΔN51-PDK1 was further treated with PreScission protease (Amersham Biosciences, Inc.) incubated with MAPK activity was measured using a MAPK immunoprecipitation protocol according to the manufacturer’s instructions (Amersham Biosciences), as described previously (24). The proteins were further treated with PreScission protease (Amersham Biosciences) to remove the GST tag.

**Transient and Stable Transfection, Immunoprecipitation, and Western Blot Analysis**—Cells were transfected with the appropriate plasmids using Superfect transfection reagent (Qiagen, Chatsworth, CA) or LipofectAMINE 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Stable mock, WT-MEK1, or ΔMEK1 transfected cells were obtained by transfection with the pUSEamp expression vector, WT-MEK1, or ΔMEK1 into HT1080 cells. The stable transfec tants were selected by cultivating them in medium containing 400 μg/ml Genetin (Invitrogen).

**Activation of MEK-MAPK Pathway by PDK1**

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 μM sodium vanadate, 12 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM aprotinin) (26). Tagged proteins were immunoprecipitated with an anti-FLAG-agarose (Sigma), an anti-HA-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or an anti-Myc-agarose (Santa Cruz Biotechnology, Inc.) (25, 26). In some experiments, cell lysates were immunoprecipitated with normal mouse IgG-conjugated agarose (Santa Cruz Biotechnology), protein A-agarose that had been conjugated with normal rabbit IgG, and an anti-FLAG-conjugated IgG-agarose (clone F-8; Santa Cruz Biotechnology), protein A-agarose that had been conjugated with normal rabbit IgG, or an anti-FLAG-conjugated agarose (clone F-8; Santa Cruz Biotechnology), or an anti-MYC antibody-conjugated agarose (clone 9E10; Santa Cruz Biotechnology), or an anti-MYC antibody-conjugated agarose (clone 9E10; Santa Cruz Biotechnology). Then the immunoprecipitated proteins or the cell lysates were electrophoresed and transferred onto a nitrocellulose membrane. The membrane was incubated with antibodies to Akt, phospho-Akt (Thr308), phospho-MEK1/2 (Ser221/222), phospho-MAPK (Thr202/Tyr204), phospho-c-Myc (Thr58/Ser62), phospho-p90RSK (Thr787) (Cell Signaling Technology, Beverly, MA), or Raf-1 or PD1K (BD Transduction Laboratories), MEK1 or phospho-Raf-1 (Upstate Biotechnology), FLAG tag (clone M2) or HA tag (clone 12CA5; Roche Applied Science), Myc (clone 9E10), ERK2/MAPK2, phospho-MEK1/2 (Ser218/222), (Santa Cruz Biotechnology), RSK2 (Stressgen, Victoria, Canada), or phospho-MEK1/2 (Ser229) (BIOCOURCE, Camarillo, CA). Subsequently, membranes were washed and incubated with horseradish peroxidase-conju- gated secondary antibody. After washing, the membranes were developed with an enhanced chemiluminescence system, according to the manufacturer’s instructions (Roche Applied Science). Blots were scanned with an EPSON ES-2200 scanner supported by Adobe Photos hop 5.5 and quantified with NIH Image 1.62 software.
Fig. 1. Phosphorylation of MEK1/2 by PDK1 in vitro and in cells. A, 293T cells were transfected with a pFLAG-CMV-2 vector encoding nothing (Mock; upper panels) or ΔN51-PDK1 (ΔN51-PDK1; lower panels) together with pUSEamp-WT-MEK2 plus pcDNA3-EGFP plasmids. The active MAPK proteins were detected by staining with an anti-phospho-MAPK antibody, followed by an AlexaFluor 568-conjugated antibody incubation. The transfected cells were observed in green (EGFP, middle panels), and the activated MAPK proteins were observed in red (left panels). B, the recombinant active Raf-1 or immunoprecipitated ΔN51-PDK1 protein was incubated with recombinant inactive MEK1 (0.3 μg/assay), inactive MAPK2 (1 μg/assay), or both for 1 h at 30 °C, following incubation with 20 μg of MBP as a MAPK substrate in the presence of [γ-32P]ATP for 10 min at 30 °C. MAPK kinase activity was measured, as described under “Experimental Procedures.” C, 293T cells were transfected with pFLAG-CMV2 vector encoding nothing (Mock), ΔN51-PDK1 (WT), or kinase-dead form of ΔN51-PDK1 (S241A). The FLAG-tagged PDK1 proteins were immunoprecipitated with an anti-FLAG-agarose, following incubation with recombinant inactive MEK1 (0.8 μg/assay) for 1 h at 30 °C. Reactions were electrophoresed and immunoblotted with antibodies to phospho-MEK and MEK1. Immunoprecipitated PDK1 proteins were also detected with an anti-FLAG antibody. D, the indicated amount of purified recombinant WT-ΔN51-PDK1 or kinase-dead (K111A/D223A) form of ΔN51-PDK1 (KD-ΔN51-PDK1) proteins were incubated with recombinant inactive MEK1 (1 μg/assay) for 2 h at 37 °C. Reactions were electrophoresed and immunoblotted with antibodies to phospho-MEK, MEK1, phospho-PDK1 (Ser241), or PDK1. E, alignment of the amino acid sequence around activation loops of human PDK1, MEK1, MEK2, Akt1, SGK1, and PAK1. Phosphorylated Ser or Thr residues are denoted by red letters. Identical and similar amino acids are denoted by blue and green letters, respectively. F and G, 293T cells were transfected with a pUSEamp vector encoding nothing (Mock), WT-MEK1, WT-MEK2, K97A-MEK1, or S218D/S222D-MEK1 (DD) together with a pCMV3 vector encoding nothing (-) or WT-PDK1 (+). The cell lysates were electrophoresed and immunoblotted using antibodies to phospho-MEK (Cell Signaling), HA tag, or Myc tag. Similar results were obtained when immunoblot analysis was performed with antibodies to phospho-MEK1/2 (Santa Cruz Biotechnology) or phospho-MEK1/2 (Ser222) (BIOSOURCE) (data not shown).
Activation of MEK-MAPK Pathway by PDK1

RESULTS

Activation of MEK/MAPK Signal Transduction Pathway by PDK1—To analyze the MEK/MAPK signaling pathway, 293T cells were transfected with pUSEamp-MEK2 plasmid together with EGFP-expressing plasmid to detect the transfected cells. Overexpression of MEK2 alone could not induce MAPK phosphorylation (Fig. 1A, yellow arrowheads). Interestingly, co-expression of ΔN51-PDK1, which possesses almost the same activity as full-length WT-PDK1 (data not shown), resulted in the increased phospho-MAPK level in the transfected cells (Fig. 1A, blue arrowheads). We then examined whether or not PDK1 directly activates MAPK. In vitro incubation of PDK1 with recombinant, inactive MAPK2 did not induce MAPK2 activation (Fig. 1B). We observed PDK1-induced MAPK activation in vitro only in the presence of both inactive MEK1 and inactive MAPK2, as we found with Raf-1 (Fig. 1B). Therefore, PDK1 cannot directly activate MAPK; it needs MEK for in vitro activation. The fact that the immunoprecipitated, kinase-dead form of PDK1 (S241A) failed to phosphorylate and activate MEK1 indicates that PDK1-dependent MEK1 activation is not mediated by other co-precipitated kinases (Fig. 1C) (data not shown). Moreover, in vitro incubation of recombinant inactive MEK1 with purified recombinant ΔN51-PDK1, but not the kinase-dead form of ΔN51-PDK1, increased the phospho-MEK1 levels in a dose-dependent manner (Fig. 1D). The results suggest that the target of PDK1 in the MAPK signaling pathway is not MAPK itself but rather its upstream kinase MEK.

PDK1 was originally isolated as a kinase responsible for the phosphorylation of Akt on its activation loop (Thr308 residue) (17–19). Later studies have shown that PDK1 is not only an Akt kinase, but it also regulates multiple kinases, which belong to the AGC family of protein kinases through phosphorylating Ser or Thr residues equivalent to Thr308 of Akt (13, 29). Sequence comparison of the residues around Ser\textsuperscript{222} in human MEK1 and Ser\textsuperscript{226} in human MEK2 in their activation loops showed that these residues had homology to the previously reported PDK1 phosphorylation sites in human Akt1, SGK1, and PAK1 (Fig. 1E) (13, 29). To prove that MEK phosphorylation was PDK1-dependent, we examined the change in phospho-MEK1/2 levels with an anti-phospho-MEK antibody. The Cell Signaling antibody used recognized MEK1/2 only when MEK1/2 were phosphorylated at Ser\textsuperscript{218} and/or Ser\textsuperscript{222} in MEK1 and at Ser\textsuperscript{222} and/or Ser\textsuperscript{226} in MEK2. Immunoblot analysis showed that co-transfection of PDK1 increased the phospho-MEK1/2 levels in cells (Fig. 1F). Similar results were obtained using a Santa Cruz Biotechnology antibody (sc-7995-R) that specifically recognized phosphorylated MEK1 (Ser\textsuperscript{222}) or MEK2 (Ser\textsuperscript{226}) (data not shown). We also found increased phospho-MEK levels when we used a BIOSOURCE antibody that specifically recognized phosphorylated MEK1 (Ser\textsuperscript{222}) or MEK2 (Ser\textsuperscript{226}) (data not shown). To exclude the possibility that MEK phosphorylation was accomplished by autophosphorylation, we generated a kinase-dead form of MEK1 by mutating Lys\textsuperscript{97} to Ala. As shown in Fig. 1G, PDK1 also increased the phospho-MEK1 (Ser\textsuperscript{218}/Ser\textsuperscript{222}) levels in KD-MEK1. Of note, the anti-phospho-MEK1/2 antibody used did not recognize DD-MEK in which both Ser\textsuperscript{218} and Ser\textsuperscript{222} residues had been substituted for Gln. The phospho-MEK1/2 antibodies used did not recognize MEK1 or MEK2 in their kinase-dead forms (data not shown). Therefore, PDK1 also increased the phospho-MEK1/2 levels in KD-MEK1. We investigated whether PDK1 promotes the phosphorylation of endogenous MEK. When 293T and HT1080 cells were transfected with PDK1 alone, endoge-
ous MEK1/2 were phosphorylated in a PDK1 dose-dependent manner, like Raf-1 did (Fig. 2A). Membrane targeting of PDK1 is important for phosphorylation and activation of Akt (22). However, membrane targeting was not necessary for PDK1-mediated endogenous MEK phosphorylation, because PH domain-deleted PDK1 (PH-PDK1) also increased the phospho-MEK level (Fig. 2B). Transfection of kinase-dead forms of PDK1 (S241A-, V243P-, or D223A-PDK1) (17, 20, 24) did not increase the endogenous phospho-MEK levels (Fig. 2B). Consistent with the results, phosphorylation of MEK was dependent on the amounts of co-transfected WT-PDK1 but not the kinase-dead (S241A) form of ΔN51-PDK1 in cells (Fig. 2C). As reported previously (20), S241A-PDK1 had a weak kinase activity. That might be the reason why a slight increase in phospho-MEK level was observed when MEK1 was co-transfected with S241A-PDK1 (Fig. 2C). Because c-Raf-1 phosphorylation was not affected by WT- or ΔPH-PDK1 transfection (Fig. 2B), PDK1 would phosphorylate MEK without affecting c-Raf-1 activity. Consistent with the results, we previously identified PDK1 inhibitor UCN-01 (21) dose-dependently suppressed the PDK1-mediated MEK phosphorylation, in addition to Akt, in cells (Fig. 2D). Thus, kinase activity of PDK1 was essential for MEK phosphorylation.

Because PDK1 is known to phosphorylate and activate multiple downstream kinases (13, 29), we investigated the role of PDK1 downstream kinases on MEK phosphorylation. Akt and SGK were known to act as main mediators of PI3K/PDK1-regulated biological responses (12, 13). The increase in phospho-MEK levels was not observed in 293T cells that had been transfected with the active form of Akt1 or SGK1 cDNA although transfection of PDK1 or the active form of Raf-1 cDNA induced MEK phosphorylation (Fig. 3A). Thus, Akt and SGK might not be involved in MEK phosphorylation and MAPK activation. PDK1 is also known to phosphorylate and activate PKCs (13, 18, 29), especially PKCζ, which was reported to activate Raf-1 following MAPK activation in neuronal cells (30, 31). In 293T cells, however, transfection of WT-PKCζ cDNA did not increase phospho-MEK and phospho-MAPK levels (Fig. 3B). Moreover, overexpression of the dominant negative form of PKCζ did not interfere with PDK1-dependent MEK phosphorylation (Fig. 3B). We further examined the effects of PKC inhibitors on Raf/MEK signaling. The PKCδ inhibitor rottlerin and synthetic myristoylated PKCζ and PKCζ pseudosubstrate inhibitor peptides had minimal effects on PDK1-dependent MEK phosphorylation in 293T and HT1080 cells (Fig. 3C). Therefore, PDK1 may only be involved in PDK1-dependent MEK phosphorylation in neuronal cells.

**PDK1 Preferentially Phosphorylates MEK1 at the Ser222 Residue**—Although MEK has been reported to be phosphorylated by several kinases, such as Tpl-2, Mos, and MEKK-1 (32–34), the predominant MEK activator in most cell types is reported to be Raf-1 (35). To identify the PDK1-mediated phosphorylation sites in MEK, we generated several MEK1 point mutants and transfected them into 293T cells together with plasmids containing WT-PDK1 or the active form of Raf-1 cDNA. Consistent with previous reports (7, 8), Raf-1 phosphorylated MEK1 at both Ser218 and Ser222 residues, because MEK phosphorylation was observed whenever either site was converted to Ala or Asp. In contrast, PDK1 phosphorylated MEK1 only at the Ser222 residue, because the anti-phospho-MEK1 antibody did not detect the increases in levels of the phosphorylated form of S222A- or S222D-MEK1, even when cells were co-transfected with WT-PDK1 (Fig. 4A). An in vitro kinase assay revealed that PDK1 transfection increased the kinase activity of S218D-MEK1 but not of S222D-MEK1 (Fig. 4A). Raf-1 overexpression increased MEK kinase activity whenever either Ser218 or Ser222 were mutated to Asp. To confirm the result, we incubated the immunoprecipitated and PP2A-treated WT-, S218A-, and S222A-MEK1 with immunoprecipitated PDK1 in vitro. Immunoblot analysis revealed that PDK1 could phospho-

---

**Fig. 3.** Involvement of PDK1, but not its downstream kinases, in MEK phosphorylation in cells. **A**, 293T cells were transfected with the pUSEamp-WT-MEK1 plasmid together with plasmids encoding WT-PDK1, active Raf-1 (CAAX-Raf1), active Akt (myr-Akt), or active SGK (S422D-SGK). **B**, 293T cells were transfected with the pUSEamp-WT-MEKL plasmid together with a pcDNA3 vector encoding nothing (Mock), WT-PKCζ (WT), or a dominant negative form of PKCζ (DN). In some experiments, 293T cells were further transfected with a pFLAG-CMV-2 vector encoding ΔN51-PDK1 (+). C, 293T cells were transfected with a pcDNA3 vector alone (−) or pCMV3-WT-PDK1 (+). After transfection for 24 h, cells were treated with Me2SO (Mock), 10 μM rottlerin, 30 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ Inh.), or 30 μM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ Inh.) for 1 h at 37 °C. The cell lysates were electrophoresed and immuno-blotted using antibodies to phospho-MEK, phospho-FAK, MAPK, phospho-Raf-1 (Ser338), Raf-1, MEK, HA tag, Myc tag, or FLAG tag.
noprecipitated MEK1 proteins were further incubated with 0.5 μg
proteins were immunoprecipitated with an anti-HA-agarose. The immu-
48 h, cells were harvested. The HA-tagged WT- and point mutated-MEK1
PDK1
mutants. Some cells were further transfected with WT-
Raf-1
(CAA
Ser222 residue.

When point-mutated MEK1 was phosphorylated by overex-
Fig. 4
PDK1 binds to MEK1 and phosphorylates MEK1 at the
Ser222 residue. A, 293T cells were transfected with a pUSEamp vector
encoding nothing (Mock), WT-MEK1 (WT), or the indicated MEK1 point
mutants. Some cells were further transfected with WT-PDK1 or active
Raf-1
(CAAX-Raf-1) cDNA-containing plasmid. After transfection
for 48 h, cells were harvested. The HA-tagged WT- and point mutated-MEK1
proteins were immunoprecipitated with an anti-HA-agarse. The immu-
noprecipitated MEK1 proteins were further incubated with 0.5 μg of
inactive MAPK2 for 30 min at 30°C. The immunoprecipitated proteins and
reactions were electrophoresed and immunoblotted with antibodies to
phospho-MEK (Cell Signaling), HA tag, phospho-MAPK, or MAPK.
B, 293T cells were transfected with a pUSEamp vector encoding nothing, WT-MEK1, or the indicated MEK1 point
mutants. The Myc-tagged WT-PDK1 proteins were immunoprecipitated
with an anti-Myc-agarse. The immunoprecipitated proteins and
the cell lysates were electrophoresed and immunoblotted with antibodies
to HA tag or Myc tag. C, 293T cells were transfected with pcMV3-WT-
PDK1 together with a pUSEamp vector encoding nothing, WT-MEK1, or the indicated MEK1 point
mutants. D. 293T cells were transfected with a pUSEamp vector
encoding WT-MEK1 or the indicated MEK1 point mutants. The
Myc-
tagged WT-PDK1 proteins were immunoprecipitated with an anti-Myc
agarse. The immunoprecipitated proteins and the cell lysates were
electrophoresed and immunoblotted with antibodies to HA tag, Myc
tag, or c-Raf-1.

PDK1 Gene Silencing Attenuates the MEK/MAPK Signal
Transduction Pathway—To confirm the role of PDK1 in MEK/
MAPK signaling pathway in vivo, we tried to knock down
PDK1 expression using siRNA. We designed five siRNAs from the
human PDK1 gene sequence. Although there was a differ-
ce in silencing ability, all of the siRNAs were able to suppress
endoogenous PDK1 protein expression in 293T and A549 cells
(Fig. 5A). As reported previously (17–19), PDK1 is essential for Akt
phosphorylation at the Thr308 site. Transfection of PDK1-4 siRNA
into 293T cells reduced the phospho-Akt (Thr308) level without affecting the Akt protein amount (Fig. 5B). Therefore,
PDK1-4 siRNA was useful to investigate the phosphorylation
status of PDK1 substrates in cells.

When 293T and HT1080 cells were transfected with PDK1-4 siRNA, endogenous PDK1 expression was significantly suppressed (Fig. 5C). In this condition, we observed the decrease in steady-state levels of phospho-MEK and phospho-MAPK in
293T and HT1080 cells without affecting the MEK and MAPK
protein amount. The inhibitory effects on MEK/MAPK signaling
were also found in cells transfected with siRNA to c-Raf-1,
whereas siRNA to Akt1 and Akt2 (Aktc siRNA) had no effect.
These results clearly showed the connection between PDK1
and MEK/MAPK signaling in cells. For validation of siRNA
data, we generated the pcMV3-PDK1-dfos plasmid in which three bases of PDK1-4 siRNA-targeting sequence were mu-
tated without affecting amino acid sequence of PDK1 protein. As shown in Fig. 5D, PDK1 protein expression was observed in
PDK1-4 siRNA-treated cells when the cells were transfected
with pcMV3-PDK1-dfos. In contrast, no PDK1 protein was
expressed in PDK1-4 siRNA-treated cells even when cells were
co-transfected with pcMV3-WT-PDK1. The PDK1-4 siRNA-
mediated decrease in phospho-MEK levels was rescued by co-
transfection with pcMV3-PDK1-dfos but not with pcMV3-WT-
PDK1. These results indicate that the effect of PDK1-4 siRNA
on lowering the phospho-MEK level is not an artifact.

To exclude the possibility that PDK1 gene silencing affected the
association between Raf-1 and MEK, MEK was immuno-
precipitated from control siRNA- or PDK1-4 siRNA-transfected
cells. As shown in Fig. 5E, the amount of co-immunoprecipitated
c-Raf-1 from PDK1-4 siRNA-transfected 293T cells was almost the same as that from nonsilencing siRNA-transfected
cells. When cells were treated with EGF or phorbol 12-myris-
tate 13-acetate, the activation of MEK signaling was observed
whether or not PDK1 expression was suppressed (Fig. 5, F and G).
However, PDK1 gene silencing attenuated the maximum
MEK phosphorylation level and the functional read-out (phos-
pho-RSK and phospho-MAPK). Because suppression of PDK1
expression could not prolong the duration time of MEK/MAPK/
Akt phosphorylation at the Thr308 site. Therefore, PDK1-4 siRNA was useful to investigate the phosphorylation
status of PDK1 substrates in cells.
maximum MAPK activity that might lead to cell proliferation or differentiation.

Overcoming PDK1 Gene Silencing by Expressing Constitutively Active MEK—To confirm that PDK1 regulates MAPK signaling by directly phosphorylating MEK, we transfected PDK1-4 siRNA to 293T cells that had been transfected with WT-MEK1 or constitutively active DD-MEK1 cDNA. Transfection of PDK1-4 siRNA or c-Raf-1 siRNA decreased the phosphorylated forms of MAPK, MEK, and RSK in WT-MEK1 cDNA-transfected cells (Fig. 6A). However, PDK1-4 siRNA or c-Raf-1 siRNA could not decrease the phospho-MAPK or phospho-ERK level in DD-MEK1 cDNA-transfected cells, but these same siRNAs down-regulated phospho-MEK protein (Fig. 6A). We generated HT1080 cell clones that stably transfected with mock (M1 and M2 cells), WT-MEK1 cDNA (WT29 cells), or DD-MEK1 cDNA (DD5 cells), and we examined the PDK1 gene silencing in these stable transfectants. Transfection of PDK1-4 siRNA suppressed MEK/MAPK/RSK or MEK/MAPK/c-Myc signaling in M1 and WT29 cells (Fig. 6B). In DD5 cells, PDK1-4 siRNA attenuated the phospho-MEK level but not the phospho-MAPK, phospho-ERK, or phospho-c-Myc level (Fig. 6B). Measurement of MAPK activity confirmed that it was suppressed by PDK1-4 siRNA in mock transfectants (M2) but not in DD-MEK1 cDNA transfectants (DD5; Fig. 6C). When we examined the growth of stable transfectants, we found that PDK1 gene silencing by PDK1-4 siRNA attenuated M1 (filled circles) and WT29 (filled squares) cell growth but not DD5 (filled triangles) cell growth (Fig. 6D). These results strongly indicate that PDK1 plays an important role in MEK/MAPK signaling that is involved in cell proliferation by regulating MEK activity.

**DISCUSSION**

The MAPK cascades are evolutionarily conserved signaling pathways from yeast to humans, and they control such fundamental cellular processes as proliferation, differentiation, survival, and apoptosis. The cascades consist of three kinases: MAPK kinase kinase, MAPK kinase, and MAPK. There are four subgroups in the MAPK family: MAPK/ERK, p38, ERK5, and the c-Jun N-terminal kinase/stress-activated protein kinase. Each MAPK has its own MAPK kinase and MAPK kinase. Of the four MAPK cascades, the most extensively studied pathway is the Raf/MEK/MAPK cascade (35–37).

Upon growth factor stimulation, Raf is translocated to the membrane and activated in a Ras-dependent manner. Activated Raf stimulates dual specificity protein kinase MEK activation by phosphorylating two serine residues in their activation loops, which results in MAPK activation. Activated MAPK mediates survival and growth signals by phosphorylating RSKs and transcriptional factors such as CAMP response element-binding protein, Elk-1, and c-Jun (36). Growth factors also stimulate the activation of PI3K. The activated PI3K, then, generates phospholipid second messenger molecules.
phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, which raise a diverse set of cellular responses. The major targets of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate are PH domain-containing proteins, such as Akt (also known as protein kinase B) (12, 13). Akt mediates many PI3K-regulated biological responses including glucose uptake, protein synthesis, cell cycle progression, and apoptosis inhibition. For Akt activation, Akt needs to translocate from cytosol to plasma membrane and to be phosphorylated at two specific phosphorylation sites, one in the T-loop of the kinase domain (Thr$^{308}$) and the other in the COOH-terminal of the catalytic domain (Ser$^{473}$) in a region termed the hydrophobic motif (12, 13). Phosphorylation of Akt at Thr$^{308}$ is catalyzed by the ubiquitously expressed PDK1, and the kinase responsible for phosphorylation of Akt at Ser$^{473}$ is called PDK2 (13, 29). Although PDK1 was originally identified as an Akt kinase, later works revealed that PDK1 also participates in the activation of members of the AGC family of protein kinases by phosphorylating their equivalent residues of Thr$^{308}$ of Akt (13, 29).

Although Raf/MEK/MAPK and PI3K/PDK1/Akt pathways have been thought to organize distinct cascades, recent reports suggested that a certain degree of cross-talk exists between these pathways. For example, Akt was reported to inactivate Raf-1 through phosphorylation at Ser$^{209}$ (38) although this inhibitory effect appears to depend on cell type and stage of differentiation (39). Moreover, MAPK and PDK1 coordinately activate RSK2, and the activated RSK2, in turn, activates PDK1 (40). In this report, we described a novel cross-talk between these two cascades. Four lines of evidence support the assumption that PDK1 is involved in MEK/MAPK signaling. First, overexpression of PDK1 activates MAPK in vivo and in vitro (Fig. 1); second, PDK1 directly phosphorylates MEK1/2 in the activation loop (Figs. 1 and 2); third, PDK1 binds to MEK in vivo (Fig. 4); fourth, siRNA directed to PDK1 decreased phospho-MEK and phospho-MAPK levels in vivo (Figs. 5 and 6). Because PDK1 was reported to activate PKCs (13, 18, 29), it is possible that PDK1 activates PKCs, leading to activation of Raf-1. Previous reports have also suggested that PKC$\gamma$ is associated with PDK1-dependent MAPK activation in neuronal cells (30, 31). In our hands, PDK1-dependent MAPK activation was not dependent on PKCs, because DN-PKC$\gamma$ transfection or PKC inhibitors had no effects on PDK1-mediated MEK phosphorylation (Fig. 3).

Although PDK1 gene silencing did not affect the association between Raf-1 and MEK (Fig. 4D), we could not exclude the possibility that PDK1-4 siRNA transfection decreased phospho-MEK levels by suppressing c-Raf-1 kinase activity. In fact, we found a slight decrease in c-Raf-1 kinase activity and phospho-c-Raf-1 level in PDK1-4 siRNA-transfected 293T cells (data not shown). Because the role of PDK1 in the regulation of c-Raf-1 kinase activity has not been reported yet, we have to clarify the mechanism in future. However, there is no doubt that PDK1 is associated with MEK phosphorylation, since PDK1 directly phosphorylates MEK in vivo (Fig. 1), and PDK1 binds to MEK in cells (Fig. 4). We observed nearly equal decrease in phospho-MEK and phospho-MAPK levels in PDK1-4 siRNA- and c-Raf-1 siRNA-transfected cells (Fig. 5C). It does not mean that PDK1 and c-Raf-1 contribute equally in the activation of MEK, because the gene silencing efficiency of PDK1-4 siRNA seemed to be stronger than that of c-Raf-1 siRNA. Thus, c-Raf-1 might play the main role in activating MEK/MAPK signaling; PDK1 might contribute to maintaining the steady-state phosphorylated MEK level.

Recently, Alessi and co-workers (41) reported that MEK/ MAPK activity was consistently about 2-fold higher in PDK1$^{-/-}$ ES cells than in PDK1$^{-/-}$ ES cells. They discussed in their report (41) that PDK1 might suppress the basal MEK/MAPK activity through Akt-mediated Raf inactivation. In our experiments, however, interference of PDK1 expression by siRNA attenuated the basal levels of phospho-MEK and phospho-MAPK (Fig. 5C). Moreover, transfection of PDK1 cDNA increased the MEK/MAPK activation (Figs. 1 and 2). It was not clear why PDK1$^{-/-}$ ES cells showed this increased MEK/MAPK activity. Because ES cells express ES cell-specific ras and other genes (42), the PDK1-dependent MEK/MAPK activation seemed to be dependent on cell type and stage of differentiation.

All of the reported PDK1 phosphorylation sites in the AGC family of kinases possessed the conserved pTFCGT motif (where pT represents PDK1-targeting Thr) (Fig. 1E) (13, 29). PDK1 could also phosphorylate p21-activated protein kinase-1 (PAK1) at the Thr$^{423}$ residue of the pTMVGT motif (where pT represents PDK1-targeting Thr) (43), suggesting that there is some redundancy in the PDK1 targeting site. Our identified PDK1 phosphorylation sites in MEK1 and MEK2 had homology to the motif, although the PDK1-phosphorylation site was
not the Thr but rather the Ser residue (Fig. 1E). Because PDK1 was known to phosphorylate by itself at the Ser residue (21) and Ser residues in RSK2 and MSK1/2 (40), it was no surprise to us that PDK1 phosphorylated MEK1/2 at the Ser residues in the activation loop. Since several proteins, other than the AGC family of protein kinases, were also phosphorylated by PDK1 in cells, PDK1 might be involved in the regulation of many kinases in addition to the AGC kinases.

Acknowledgments—We thank Drs. Philip Hawkins and Karen Anderson of the Babraham Institute for providing the pCMV-3-PDK1. We also thank Dr. Jorge Moscat of the Universidad Autónoma for kindly providing WT- and DN-PKC \( ^{\alpha} \) H9256/5 and PDK1 might be involved in the regulation of many kinases in addition to the AGC kinases.

REFERENCES

1. Lee, J. T., Jr., and McCubrey, J. A. (2002) Leukemia 16, 486–507
2. Rodriguez-Vieiana, P., Warne, P. H., Kitwana, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cell 89, 457–467
3. Toker, A., and Cantley, L. C. (1997) Nature 387, 673–676
4. Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends. Biochem. Sci. 22, 267–272
5. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
6. Winkler, D. G., Cutler, R. E. Jr., Drugan, J. K., Campbell, S., Morrison, D. K., and Cooper, A. J. (1998) J. Biol. Chem. 273, 21578–21584
7. Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) EMBO J. 13, 1610–1619
8. Zheng, C. F., and Guan, K. L. (1994) EMBO J. 13, 1123–1131
9. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) Science 258, 478–480
10. Crews, C. M., and Erikson, R. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5205–5209
11. Allan, L. A., Mourice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) Nat. Cell Biol. 5, 647–654
12. Brazit, D. P., Park, J., and Hemmings, B. A. (2002) Cell 111, 293–303
13. Vanhaesebroeck, B., and Alessi, D. R. (2000) Biochem. J. 346, 561–576
14. Shenh-Leoold, J. S. (2000) Oncogene 19, 6584–6599
15. Vivanco, I., and Sawyers, C. L. (2002) Nat. Rev. Cancer 2, 489–501
16. West, K. A., Castillo, S. S., and Denu, P. A. (2002) Drug Resist. Updat. 5, 234–248

2. N. Fujita and T. Tsuruo, unpublished results.
