Involvement of PDK1 in the MEK/MAPK signal-transduction pathway

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The abbreviations used in this paper: AGC, protein kinase A, G and C; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PI3K, phosphatidylinositol-3-OH kinase; PKC, protein kinase C; RSK, p90 ribosomal protein S6 kinase; SGK, serum and glucocorticoid-inducible kinase; siRNA, small interference RNA; WT, wild type.

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ABSTRACT

The phosphatidylinositide-3-OH kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (PDK1)/Akt and the Raf/mitogen-activated protein kinase (MAPK/Erk) kinase (MEK)/mitogen-activated protein kinase (MAPK) pathways have central roles in the regulation of cell survival and proliferation. Despite their importance, however, the crosstalk 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 Ser^{222} and Ser^{226}, respectively, and are known to be essential for full activation. To date, these sites are thought to be phosphorylated by Raf kinases. However, PDK1 gene silencing using small interference RNA (siRNA) demonstrates that PDK1 is associated with maintaining the steady-state phosphorylated MEK level and cell growth. The siRNA-mediated downregulation 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 crosstalk between the PI3K/PDK1/Akt pathway and the Raf/MEK/MAPK pathway. (182 words)
INTRODUCTION

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)\(^1\) and Ras (1). Activated PI3K generates the phospholipid second messenger molecules phosphatidylinositol 3,4,5-trisphosphate \([\text{PtdIns}(3,4,5)P_3]\) and phosphatidylinositol 3,4-bisphosphate \([\text{PtdIns}(3,4)P_2]\) (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 (p70\(^{56K}\)), protein kinase C isoforms (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 Ser\(^{218}\) of MEK1 and at Ser\(^{222}\) and Ser\(^{226}\) 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 Thr\(^{308}\)) (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: p70\(^{56K}\), SGKs, PKCs, and p90 ribosomal protein S6 kinases (RSKs) at the equivalent residues of Thr\(^{308}\) of Akt (reviewed in Ref. 13). PDK1 itself is also a member of the AGC subfamily of protein kinases and is
phosphorylated at the Ser$^{241}$ residue (equivalent to Thr$^{308}$ of Akt) in the activation loop. As PDK1 expressed in bacteria was active and was phosphorylated at Ser$^{241}$, 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 (Ser$^{222}$ of MEK1 and Ser$^{226}$ 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 an MAPK kinase kinase in MEK/MAPK signaling pathways.
EXPERIMENTAL PROCEDURES

Reagents, Cell Culture Conditions, and MTT Assay—The recombinant, inactive MAPK2/Erk2, inactive MEK1, active Raf-1, MBP, and purified PP2A proteins were obtained from Upstate Biotechnology (Lake Placid, NY). The PKC inhibitor rottlerin, myristoylated PKCζ pseudosubstrate inhibitor, and myristoylated PKCθ pseudosubstrate inhibitor were obtained from Calbiochem (La Jolla, CA). Our previously identified PDK1 inhibitor UCN-01 (21) was kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant human epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (TPA) were purchased from Roche (Basel, Switzerland) and Sigma (St. Louis, MO), respectively. Human embryonic kidney 293T and human fibrosarcoma HT1080 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Human lung cancer A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. To assess cell proliferation, the MTT assay was employed. In brief, cells were incubated with 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) for 4 h. Formazon products were solubilized with DMSO, and the optical density was measured at 525 nm using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Hercules, CA).

Plasmid Construction—The rat wild-type (WT) MEK1 cDNA, rat WT-MEK2 cDNA, or NH2-terminal 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). Substitutions of Lys97 for Ala (K97A), Ser218 for Ala or Asp (S218A or S218D), Ser222 for Ala or Asp (S222A or S222D), or both Ser218 and Ser222 for Asp (S218D/S222D, DD) in MEK1 cDNA were accomplished 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 \( PDK1 \) cDNA (\( \Delta PH-PDK1 \)), NH\(_2\)-terminal–deleted \( PDK1 \) cDNA (\( \Delta N51-PDK1 \)), and the kinase-dead form of \( PDK1 \) cDNAs (D223A, S241A, and V243P) in a pCMV3 vector or a pFLAG-CMV-2 vector were generated as described previously (21, 23, 24). The PDK1-4 siRNA-resistant \( PDK1 \) cDNA (PDK1-4\( \text{Res} \)) in pCMV3 vector was generated by mutating CTCGTG of the PDK1-4 siRNA-targeting sequence (see below) to TTGGTC without mutating the amino acid sequence. The WT- and dominant-negative (DN) \( PKC\zeta \) cDNA in a pcDNA3 vector were kindly provided by Dr. J. Moscat (Universidad Autónoma, Madrid, Spain) (25). The human WT-\( Akt1 \) cDNA in a pHM6 vector, \( EGFP \) cDNA in a pcDNA3 vector, and the active form of NH\(_2\)-terminal–deleted \( SGK \) cDNA that encompassed residues 61–431 (\( \Delta N60-SGK \)) with an S422D mutation in a pFLAG-CMV-2 vector were established in our laboratory (24).

**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 (Gibco Laboratories, Grand Island, NY), according to the manufacturers’ instructions. Stable mock-, WT-MEK1, or DD-MEK1 transfectants were established by transfecting pUSEamp encoding none, WT-MEK1, or DD-MEK1 into HT1080 cells. The stable transfectants were selected by cultivating them in medium containing 400 \( \mu \)g/ml of geneticin (Gibco Laboratories).

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 vanadate, 12 mM \( \beta \)-glycerophosphate, 1 mM PMSF, and 1 mM aprotinin) (26). Tagged proteins were immunoprecipitated with an anti-FLAG agarose (Sigma), an anti-HA agarose (Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-Myc agarose (Santa Cruz
Biotechnology) (23, 26). In some experiments, cell lysates were incubated with normal mouse IgG-conjugated agarose (Santa Cruz Biotechnology), protein A agarose that had been conjugated with normal rabbit IgG, an anti-Raf-1 antibody-conjugated agarose (C-12, Santa Cruz Biotechnology), or an anti-MEK-1 antibody-conjugated agarose (clone H-8; 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, phospho-Akt (Thr<sup>308</sup>), phospho-MEK1/2 (Ser<sup>217/221</sup>), phospho-MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>), phospho-c-Myc (Thr<sup>58</sup>/Ser<sup>62</sup>), phospho-p90RSK (Thr<sup>573</sup>) (Cell Signaling Technology, Beverly, MA), c-Raf-1 or PDK1 (BD Transduction Laboratories), MEK1 or phospho-Raf-1 (Ser<sup>338</sup>) (Upstate Biotechnology), FLAG tag (clone M2) or β-actin (Sigma), HA tag (clone 3F10; Roche), Myc tag (clone 9E10), Erk2/MAPK2, phospho-MEK1/2 (Ser<sup>218/222</sup>), (Santa Cruz Biotechnology), RSK2 (Stressgen, Victoria, Canada), or phospho-MEK1/2 (Ser<sup>222</sup>) (Biosource, Camarillo, CA). Subsequently, membranes were washed and incubated with horseradish peroxidase (HRP)–conjugated secondary antibody. After washing, the membranes were developed with an enhanced chemiluminescence (ECL) system, according to the manufacturer’s instructions (Roche). Blots were scanned with EPSON ES-2200 scanner supported by AdobePhotoshop 5.5 and quantified with NIH Image 1.62 software.

**siRNA Design and Transfection**—Five siRNAs were designed from the human PDK1 sequence (PDK1-1~5). The coding strands of the siRNAs were: GAAGCGGCGUGAGACGACUUC (PDK1-1; directed to residues 228–246), UGGUGAGGACCCAGACUGA (PDK1-2; directed to residues 83–101), UCCUUGGGGAAGGCUCUUU (PDK1-3; directed to residues 260–278), GAGACCUCUGUGGAGAAACU (PDK1-4; directed to residues 929–947), and UGGAAGGAUACCGGACCUCU (PDK1-5; 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. Non-silencing control siRNA was purchased from Qiagen. The oligonucleotides had 3' dTdT overhangs. The sequences of siRNAs targeted to both Akt1 and Akt2 (Aktc) or Raf-1 genes were reported previously (27, 28). Cells were transfected with siRNAs using the LipofectAMINE 2000 reagent, according to the manufacturer’s instruction.

**Immunostaining**—Cells were transfected with pUSEamp-WT-MEK2 and pcDNA3-EGFP together with a pFLAG-CMV-2 vector encoding none (Mock) or ∆N51-PDK1. Twenty-four hours after transfection, cells were plated onto collage-coated culture dishes. After incubation for a further 24 h, cells were washed with PBS and fixed in 3.7% formaldehyde for 15 min. Permealization was carried out in 0.2% Triton X-100 for 5 min. After incubation for 1 h in PBS supplemented with 1% BSA, the labeling was carried out by incubation overnight with a rabbit polyclonal anti-phospho-MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody (Cell Signaling Technology) followed by a 90-min incubation with Alexa Flour 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-∆N51-PDK1 and GST-∆N51-PDK1 (K111A/D223A) Proteins**— Cultures of BL21 Star Escherichia coli (Invitrogen, Carlsbad, CA) containing a pGEX 6P-3 plasmid encoding ∆N51-PDK1 or kinase-dead form of ∆N51-PDK1 (K111A/D223A) were induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C with shaking. Cells were harvested, and recombinant proteins were purified using GST Purification Modules according to the manufacturer’s instructions (Amersham), as described previously (24). The proteins were further treated with PreScission protease (Amersham) to remove GST tag.
In Vitro Kinase Assay—To perform in vitro kinase assay, recombinant, active Raf-1 or immunopurified, FLAG-tagged ΔN51-PDK1 were incubated with recombinant inactive MEK1 (0.3 µg/assay), recombinant inactive MAPK2 (1 µg/assay), or both in 50 µl (final volume) of kinase reaction buffer for PDK1 (40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 1 µM microcystin-LR, 8 mM magnesium acetate, 15 mM magnesium chloride, and 180 µM ATP) or for Raf-1 (16 mM MOPS (pH 7.2), 20 mM β-glycerol phosphate, 4 mM EGTA, 0.8 mM sodium orthovanadate, 0.8 mM dithiothreitol, 15 mM magnesium chloride, and 100 µM ATP) for 1 h at 30°C. Five microliters of the mixture was then reacted with 20 µg of MBP in the presence of 100 µM ATP (final concentration) containing 15 µCi [γ-32P]ATP for 10 min at 30°C. Reactions were spotted onto P81 phosphocellulose paper, washed three times with 0.75% phosphoric acid, air-dried, and subjected to Cerenkov counting. PDK1-mediated MEK phosphorylation was also estimated by incubating immunoprecipitated, FLAG-tagged ΔN51-PDK1 or kinase-dead form of ΔN51-PDK1 (S241A) with recombinant inactive MEK1 (0.8 µg/assay) in kinase reaction buffer for PDK1 for 1 h at 30°C. Appropriate amounts of recombinant ΔN51-PDK1 and kinase-dead form of ΔN51-PDK1 (K111A/D223A) were also incubated with recombinant inactive MEK1 (1 µg/assay) in kinase reaction buffer for PDK1 for 2 h at 37°C. Reactions were electrophoresed and immunoblotted with antibodies to phospho-MEK, MEK1, phospho-PDK1 (Ser241) (Cell Signaling Technology), and PDK1. In some experiments, WT or point-mutated MEK1 proteins were immunoprecipitated and incubated with recombinant, inactive MAPK2 (0.5 µg/assay) in kinase reaction buffer for Raf-1 for 30 min at 30°C, following immunoblot analysis with antibodies to phospho-MAPK and MAPK. In other experiments, MAPK activity was measured using an MAP kinase immunoprecipitation kinase assay kit (Upstate Biotechnology), according to the manufacturers’ instructions.
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 amount in the transfected cells (Fig. 1A, blue arrow heads).

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 and data not shown). Moreover, in vitro incubation of recombinant inactive MEK1 with purified recombinant ΔN51-PDK1, but not 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 (Thr^{308} 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 Thr^{308} of Akt (13, 29). Sequence comparison of the residues around Ser^{222} in human MEK1 and Ser^{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, Ref. 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 used Cell Signaling antibody 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 SantaCruz antibody (sc-7995-R) that could recognize MEK1/2 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 (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 used anti-phospho-MEK1/2 antibody did not recognize DD-MEK in which both Ser\textsuperscript{218} and Ser\textsuperscript{222} residues had been substituted for the phospho-mimic Asp residues. These results indicate that PDK1 activates MEK/MAPK signaling pathway by directly phosphorylating MEK1/2.

**Kinase Activity of PDK1 Is Essential for MEK Phosphorylation**—Since PDK1 increased the phospho-MEK levels in MEK-transfected 293T cells (Fig. 1), we investigated whether PDK1 promoted the phosphorylation of endogenous MEK. When 293T and HT1080 cells were transfected with PDK1 alone, endogenous 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 (ΔPH-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 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 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, our 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 MEK1 phosphorylation (Fig. 3A). Thus, Akt and SGK might not be involved in MEK phosphorylation and MAPK activation. PDK1 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 PKCθ pseudosubstrate inhibitor peptides had minimal effects on PDK1-dependent MEK phosphorylation in 293T and
HT1080 cells (Fig. 3C). Therefore, PKCs may only be involved in PDK1-dependent MEK phosphorylation in neuronal cells.

**PDK1 Preferentially Phosphorylates MEK1 at the Ser^{222} 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 Ser^{218} and Ser^{222} residues because MEK phosphorylation was observed whenever either site was converted to Ala or Asp. In contrast, PDK1 phosphorylated MEK1 only at the Ser^{222} 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). *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 Ser^{218} or Ser^{222} 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 phosphorylate S218A-MEK1 but not S222A-MEK1 (data not shown). These results indicate that PDK1 preferentially phosphorylates MEK1 at the Ser^{222} residue, while Raf-1 can phosphorylate both Ser^{218} and Ser^{222} residues, as reported previously (7, 8).

Interestingly, S218D-MEK1 was constitutively phosphorylated at the Ser^{222} residue in cells (Fig. 4A). To analyze the mechanisms of increased Ser^{222} phosphorylation, we examined the change of PDK1 binding to point-mutated MEK1. The S218D mutation increased its association with PDK1 (Fig. 4B). When point-mutated MEK1 were phosphorylated by overexpressing active form of Raf-1,
PDK1 bound to S218A-MEK1 and S222A-MEK1 almost equally (Fig. 4C). Moreover, PDK1 bound to phosphorylated S218D-MEK1 and S222D-MEK1 (Fig. 4C), and the amounts of co-immunoprecipitated S218D- or S222D-MEK1 were larger than that of co-immunoprecipitated S218A- or S222A-MEK1. Raf-1 binding to MEK1 seemed to be unaffected by point mutation at either the Ser\textsuperscript{218} or Ser\textsuperscript{222} residue (Fig. 4D). These results suggest that phosphorylation of either Ser\textsuperscript{218} or Ser\textsuperscript{222} residue in MEK1 increases its association with PDK1 and that the phosphorylation of both sites in MEK1 further increased its binding ability to PDK1. Because Ser\textsuperscript{222} residue in S218D-MEK1 was constitutively phosphorylated in cells (Fig. 4A), S218D-MEK1 exhibited high affinity to PDK1 in cells (Fig. 4B).

**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 knockdown PDK1 expression using siRNA. We designed five siRNAs from the human *PDK1* gene sequence. Although there was a difference in silencing ability, all of the siRNAs were able to suppress endogenous PDK1 protein expression in 293T and A549 cells (Fig. 5A). As reported previously (17–19), PDK1 is essential for Akt phosphorylation at the Thr\textsuperscript{308} site. Transfection of PDK1-4 siRNA into 293T cells reduced the phospho-Akt (Thr\textsuperscript{308}) 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*, while 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-\textit{PDK1-4}\textsuperscript{Res} plasmid in which three bases of PDK1-4 siRNA-targeting sequence were mutated 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-\textit{PDK1-4}\textsuperscript{Res}. In contrast, no PDK1 protein was expressed in PDK1-4 siRNA-treated cells even when cells were co-transfected with pCMV3-WT-\textit{PDK1}. The PDK1-4 siRNA-mediated decrease in phospho-MEK levels was rescued by co-transfection with pCMV3-\textit{PDK1-4}\textsuperscript{Res} but not with pCMV3-WT-\textit{PDK1}. These results indicate that the effect of PDK1-4 siRNA on lowering phospho-MEK level is not an artifact.

To exclude the possibility that \textit{PDK1} gene silencing affected the association between Raf-1 and MEK, MEK was immunoprecipitated 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 non-silencing siRNA-transfected cells. When cells were treated with EGF or TPA, the activation of MEK signaling was observed whether PDK1 expression was suppressed or not (Fig. 5F and G). However, \textit{PDK1} gene silencing attenuated the maximum MEK phosphorylation level and the functional read out (phospho-RSK and phospho-MAPK). Because suppression of PDK1 expression could not prolong the duration time of MEK/MAPK/RSK signaling, Raf-1 might compensate MEK phosphorylation in EGF- or TPA-stimulated cells (Fig. 5F and G). Therefore, PDK1 was involved in MEK/MAPK signaling as a MAPK kinase kinase (MAPKKK) to increase the maximum MAPK activity that might leads to cell proliferation or differentiation.

\textbf{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-\textit{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). In contrast, PDK1-4 siRNA or c-Raf-1 siRNA could not decrease the phopho-MAPK or phospho-RSK level in DD-MEK1 cDNA-transfected cells, but these same siRNAs downregulated phospho-MEK levels (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-RSK, or phospho-c-Myc levels (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 human, and they control such fundamental cellular processes as proliferation, differentiation, survival, and apoptosis. The cascades consist of three kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. There are four subgroups in the MAPK family: MAPK/Erk, p38, Erk5, JNK/SAPK, each MAPK has its own MAPKKK and MAPKK. 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 (CRE)-binding protein (CREB), Elk-1, and c-Jun (36). Growth factors also stimulate the activation of PI3K. The activated PI3K, then, generates phospholipid second messenger molecules PtdIns(3,4,5)P3 and PtdIns(3,4)P2, which raise a diverse set of cellular responses. The major targets of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 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 (Thr308) and the other in the COOH-terminal of the catalytic domain (Ser473), in a region termed the hydrophobic motif (12, 13). Phosphorylation of Akt at Thr308 is catalyzed by the ubiquitously expressed PDK1, and the kinase responsible for phosphorylation of Akt at Ser473 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\textsuperscript{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 crosstalk exists between these pathways. For example, Akt was reported to inactivate Raf-1 through phosphorylation at Ser\textsuperscript{259} (38) although this inhibitory effect appears to depend 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 crosstalk 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 \textit{in vivo} and \textit{in vitro} (Fig. 1); second, PDK1 directly phosphorylates MEK1/2 in the activation loop (Figs. 1 and 2); third, PDK1 binds to MEK \textit{in vivo} (Fig. 4); fourth, siRNA directed to \textit{PDK1} decreased phospho-MEK and phospho-MAPK levels \textit{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\textsubscript{ζ} 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\textsubscript{ζ} transfection or PKC inhibitors had no effects on PDK1-mediated MEK phosphorylation (Fig. 3).

Although \textit{PDK1} gene silencing did not affect the association between Raf-1 and MEK (Fig. 4\textit{D}), 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, it is no doubt that PDK1 is associated with MEK phosphorylation, as PDK1 directly phosphorylates MEK \textit{in vitro} (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 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 coworkers reported that MEK/MAPK activity was consistently about twofold higher in PDK1^-/- ES cells than in PDK1^+/+ ES cells (41). 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 (pT represents PDK1-targeting Thr) (Fig. 1E, Ref. 13 and 29). PDK1 could also phosphorylate p21-activated protein kinase-1 (PAK1) at the Thr^{423} residue of the pTMVGT motif (pT represents PDK1-targeting Thr; Ref. 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^{241} residue (Fig. 1E; Ref. 20) 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 activation loop. Since several proteins, other than the AGC family of protein kinases, were also
phosphorylated by PDK1 in cells (N.F., T.T.; unpublished results), PDK1 might be involved in the regulation of many kinases in addition to the AGC kinases.

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REFERENCES

**FIGURE LEGENDS**

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 none (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 Alexa Flour 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 none (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 none (Mock),
WT-MEK1, WT-MEK2, K97A-MEK1, or S218D/S222D-MEK1 (DD) together with a pCMV3 vector encoding none (-) 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 (Ser\textsuperscript{222}) (Biosource) (data not shown).

Fig. 2. Requirement of PDK1 kinase activity in endogenous MEK phosphorylation. A, 293T and HT1080 cells were transfected with the indicated amounts of plasmids. B, 293T cells were transfected with a pCMV3 vector encoding none (-), WT-PDK1, PH domain-deleted PDK1 (\(\Delta\)PH), or a kinase-dead form of PDK1 (S241A, V243P, and D223A). After transfection for 24 h, cell lysates were electrophoresed and immunoblotted using antibodies to phospho-MEK (Cell Signaling), MEK, Myc tag, phospho-Raf-1 (Ser\textsuperscript{338}), or Raf-1. C, 293T cells were transfected with a pFLAG-CMV2 vector encoding \(\Delta\)N51-PDK1 (open circles) or kinase-dead (S241A) form of \(\Delta\)N51-PDK1 (closed triangles) together with a pUSEamp vector encoding WT-MEK1. After transfection for 24 h, cells were harvested. The HA-tagged WT-MEK1 proteins were immunoprecipitated with an anti-HA agarose. The immunoprecipitated proteins were electrophoresed and immunoblotted with antibodies to phospho-MEK (Cell Signaling) or HA tag. The intensities of phospho-MEK bands were quantified with NIH Image 1.62 software, as described under ‘Experimental Procedures.’ Phospho-MEK1 level in PDK1 non-transfected cells was taken as 1. D, 293T cells were transfected with the pCMV3-WT-PDK1 plasmid together with pUSEamp-WT-MEK1 or pHM6-WT-akt. After transfection for 24 h, cells were treated with the indicated amounts of UCN-01 for 2 h. The cell lysates were electrophoresed and immunoblotted using antibodies to phospho-MEK (Cell Signaling), phospho-Akt (Thr\textsuperscript{308}), HA tag, or Myc tag.
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-Raf-1), active Akt (myr-Akt), or active SGK (S422D-SGK). B, 293T cells were transfected with the pUSEamp-WT-MEK2 plasmid together with a pcDNA3 vector encoding none (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 DMSO control (Vehicle), 10 µM rottlerin, 30 µM myristoylated PKCζ pseudosubstrate inhibitor (PKCζ Inh.), or 30 µM myristoylated PKCθ pseudosubstrate inhibitor (PKCθ Inh.) for 1 h at 37°C. The cell lysates were electrophoresed and immunoblotted using antibodies to phospho-MEK (Cell Signaling), phospho-MAPK, MAPK, phospho-Raf-1 (Ser338), Raf-1, MEK, HA tag, Myc tag, or FLAG tag.

Fig. 4. PDK1 binds to MEK1 and phosphorylates MEK1 at the Ser222 residue. A, 293T cells were transfected with a pUSEamp vector encoding none (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 agarose. The immunoprecipitated 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 pCMV3-WT-PDK1 together with a pUSEamp vector encoding none (Mock), WT-MEK1 (WT), or the indicated MEK1 point mutants. The Myc-tagged WT-PDK1 proteins were immunoprecipitated with an anti-Myc agarose. 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 and pCMV-CAAX-Raf-1 together with a pUSEamp vector encoding none (Mock), WT-MEK1 (WT), or the indicated MEK1 point mutants. The Myc-tagged WT-PDK1 proteins were immunoprecipitated with an anti-Myc agarose. The immunoprecipitated proteins and the cell lysates were electrophoresed and immunoblotted with antibodies to HA tag, Myc tag, or c-Raf-1. D, 293T cells were transfected with a pUSEamp vector encoding WT-MEK1 (WT) or the indicated MEK1 point mutants. Cell lysates were incubated with protein A agarose that had been conjugated with a normal rabbit IgG (Cont. IgG) or an anti-Raf-1 antibody-conjugated agarose (α-Raf-1). The immunoprecipitated proteins were electrophoresed and immunoblotted with antibodies to HA tag or c-Raf-1. IP, immunoprecipitation.

Fig. 5. Suppression of MEK/MAPK signaling by siRNA-mediated PDK1 gene silencing. A-C, Cells were transfected with non-silencing control siRNA (Non sil.), PDK1 siRNAs (PDK1-1~PDK1-5), c-Raf-1 siRNA (c-Raf-1), or Akt1/2 siRNA (Aktc). After transfection for 72 h, cells were harvested. The cell lysates were electrophoresed and immunoblotted with antibodies to PDK1, β-actin, phospho-Akt (Thr 308), Akt, phospho-MEK, MEK, phospho-MAPK, MAPK, or Raf-1. D, HT1080 cells were transfected with non-silencing control siRNA (Non sil.) or PDK1-4 siRNA together with 1 µg of pUSEamp-WT-MEK1 and the indicted amounts of pCMV3-PDK1-4Res or pCMV3-WT-PDK1. After transfection for 72 h, cells were harvested. The cell lysates were electrophoresed and immunoblotted with antibodies to phospho-MEK, MEK, or Myc tag. E, 293T cells were transfected with non-silencing control siRNA (Non sil.) or PDK1 siRNA (PDK1-4). After transfection for 72 h, cells were harvested. Cell lysates were incubated with a mouse IgG-conjugated agarose (Cont. IgG) or an anti-MEK-1 antibody-conjugated agarose (α-MEK). Then the immunoprecipitated proteins were electrophoresed and immunoblotted with antibodies to Raf-1 or MEK. IP,
immunoprecipitation. F and G, HT1080 cells were transfected with non-silencing control siRNA (Non sil.) or PDK1 siRNA (PDK1-4). After transfection for 72 h, cells were treated with 100 ng/ml of EGF or 500 nM TPA. At the indicated time points, cells were harvested, and the cell lysates were electrophoresed and immunoblotted with antibodies to phospho-MAPK, MAPK, phospho-MEK, MEK, phospho-RSK (Thr^{573}), RSK2, or PDK1.

Fig. 6. Overcome of PDK1 gene silencing by expressing constitutively active MEK. A, 293T cells were transfected with a pUSEamp vector encoding WT-MEK1 (WT) or S218D/S222D-MEK1 (DD). After transfection for 72 h, cells were further transfected with non-silencing control siRNA (Non sil.), PDK1 siRNA (PDK1-4), or c-Raf-1 siRNA (c-Raf-1). After incubation for additional 72 h, cells were harvested. B, HT1080 cell clone that stably expressed none (M1), WT-MEK1 (WT29), or S218D/S222D-MEK1 (DD5) were transfected with non-silencing control siRNA (Non sil.) or PDK1 siRNA (PDK1-4). After transfection for 72 h, cells were harvested. The cell lysates were electrophoresed and immunoblotted with antibodies to phospho-MAPK, MAPK, phospho-MEK, MEK, phospho-RSK (Thr^{573}), RSK2, PDK1, phospho-c-Myc, or c-Myc. Exo MEK, exogenous MEK1; Endo MEK, endogenous MEK. C, M2 and DD5 cells were treated as described in B. The MAPK activities in the cells were measured using MAP kinnase assay kit. D, M1 (circles), WT29 (squares), and DD5 (triangles) cells were transfected with non-silencing control siRNA (Non sil.; open symbols) or PDK1 siRNA (PDK1-4; filled symbols). After transfection for 24 h, cells were replated onto a 12 well plate at 1 X 10^4 cells/well. Forty-eight, 72, and 96 h after transfection, MTT assay was performed as described under “Experimental Procedures”.
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Involvement of PDK1 in the MEK/MAPK signal-transduction pathway
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