PDK1 Protein Phosphorylation at Thr^{354} by Murine Protein Serine-Threonine Kinase 38 Contributes to Negative Regulation of PDK1 Protein Activity

Hyun-A Seong, Haiyoung Jung, Ravi Manoharan, and Hyunjung Ha

From the Department of Biochemistry, School of Life Sciences, Chungbuk National University, Cheongju 361-763, Republic of Korea

Background: This study was done to elucidate the biochemical mechanisms underlying phosphorylation-dependent regulation of PDK1.

Results: MPK38 inactivates PDK1 activity and function by phosphorylating PDK1 at Thr^{354}.

Conclusion: MPK38 acts as a putative protein kinase to negatively regulate PDK1.

Significance: This study defines a novel mechanism in which MPK38 directly interacts with and phosphorylates Thr^{354} of PDK1, thereby inhibiting PDK1 activity.

Murine protein serine-threonine kinase 38 (MPK38) is a member of the AMP-activated protein kinase-related serine/threonine kinase family, which acts as cellular energy sensors. In this study, MPK38-induced PDK1 phosphorylation was examined to elucidate the biochemical mechanisms underlying phosphorylation-dependent regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) activity. The results showed that MPK38 interacted with and inhibited PDK1 activity via Thr^{354} phosphorylation. MPK38-PDK1 complex formation was mediated by the amino-terminal catalytic kinase domain of MPK38 and the pleckstrin homology domain of PDK1. This activity was dependent on insulin, a PI3K/PDK1 stimulator, as well as various apoptotic stimuli, including TNF-α, H₂O₂, thapsigargin, and ionomycin. MPK38 inhibited PDK1 activity in a kinase-dependent manner and alleviated PDK1-mediated suppression of TGF-β (or ASK1) signaling, probably via the phosphorylation of PDK1 at Thr^{354}. In addition, MPK38-mediated inhibition of PDK1 activity was accompanied by the modulation of PDK1 binding to its positive and negative regulators, serine/threonine kinase receptor-associated protein and 14-3-3, respectively. Together, these findings suggest an important role for MPK38-mediated phosphorylation of PDK1 in the negative regulation of PDK1 activity.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a member of the protein kinase A, G, and C subfamily of protein kinases. PDK1 acts via a PH domain and binds phosphoinositides such as phosphatidylinositol 3,4-P₂ and phosphatidylinositol 3,4,5-P₃ to phosphorylate Thr^{308} of PKB/Akt. Phosphorylation of both Thr^{308} and Ser^{473} is required for maximal activation of PKB/Akt (1, 2). These residues are phosphorylated independently and are dephosphorylated by PDK1/protein phosphatase 2A (PP2A) (for Thr^{308}) and mammalian target of rapamycin complex 2 (mTORC2)/PH domain leucine-rich repeat protein phosphatases (for Ser^{473}), respectively (3–6). In addition, analysis of cellular proteins that interact with PDK1 shows that the activity and function of PDK1 is regulated by PDK1-interacting proteins within cells (7–10). Although an important role for PDK1 in cell survival signaling is well characterized, the mechanism by which PDK1 activity is regulated through phosphorylation remains largely unknown. It was previously thought that PDK1 is constitutively active because it shows a high level of basal activity in unstimulated cells and cannot be further activated by growth factor stimulation (11). However, recent studies strongly suggest that PDK1 activity is regulated in a phosphorylation-dependent manner. Multiple phosphorylation sites on PDK1 (Ser^{25}, Ser^{241}, Ser^{393}, Ser^{396}, and Ser^{410}) have been identified in unstimulated HEK293 cells, but only the phosphorylation of Ser^{241} (Ser^{244} in mouse PDK1) within the activation loop is responsible for PDK1 activity (12). Phosphorylation of Ser^{396} is necessary for nuclear shuttling of PDK1 (13). Phosphorylation of mouse PDK1 at Ser^{163} is also involved in fine-tuning PDK1 activity (14). In addition, PDK1 in HEK293 cells treated with pervanadate, a tyrosine phosphatase inhibitor, undergoes tyrosine phosphorylation at Tyr^{9}, Tyr^{373}, and Tyr^{776}, leading to its activation (15). RET/PTC, a thyroid-specific oncogenic kinase (16), stimulates PDK1 activity by phosphorylating Tyr^{9} (17). Protein kinase Cθ (PKCθ), a kinase implicated in hyperlipidemia-induced insulin resistance, negatively regulates PDK1 activity via phosphorylation at Ser^{204} and Ser^{512} (18). Reduced PDK1 phosphorylation at Ser^{244}, which is stimulated by insulin, occurs in the liver of obese/obese mice (19). We recently demonstrated that PDK1 undergoes apoptosis signal-regulating kinase 1 (ASK1)-dependent phosphorylation at Ser^{394} and Ser^{398}, which suppresses its activity (20).
These findings suggest that the phosphorylation of PDK1 plays an important role in regulating PDK1 activity and function.

Murine protein serine-threonine kinase 38 (MPK38)/maternal embryonic leucine zipper kinase (MELK) is a member of the AMP-activated protein kinase-related serine/threonine kinase family (21, 22) and is activated by apoptotic cellular stresses, such as \( \text{H}_2\text{O}_2 \) and transforming growth factor-\( \beta \) (TGF-\( \beta \)), suggesting functional cross-talk between MPK38 and the ASK1 or TGF-\( \beta \) signaling pathways (23, 24). MPK38 is thought to play a role in various cellular processes, including the cell cycle, embryonic development, spliceosome assembly, gene expression, cell proliferation, hematopoiesis, oncogenesis, and apoptosis; however, its precise function is unclear (23–28).

Therefore, to explore the phosphorylation-dependent regulation of PDK1, we investigated whether MPK38 contributes to the phosphorylation of PDK1 and whether it plays a regulatory role in the PDK1 activity. We showed that MPK38 physically interacts with and phosphorylates PDK1 at Thr\(^{354} \), thereby inhibiting its activity and function. Our work also suggests that MPK38, like PKC\( \theta \) and ASK1 (18, 20), acts as a putative protein kinase to negatively regulate PDK1 in cells.

**MATERIALS AND METHODS**

**Antibodies and Cell Culture**—Anti-PDK1, anti-phospho-AKT(T308), anti-phospho-BAD(S136), anti-MPK38, anti-GST, anti-His, anti-FLAG (M2), and anti-\( \beta \)-actin antibodies have been described previously (24, 29). The anti-Myc antibody was purchased from Santa Cruz Biotechnology. The anti-rabbit phospho-PDK1(T354) antibody was raised against a synthetic phosphopeptide antigen LHQOTTPPKL, where \( T \) represents phosphothreonine (Young In Frontier, Seoul, Korea). HEK293, 293T, NIH 3T3, R1.1, and HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Invitrogen) at 37 °C in 5% \( \text{CO}_2 \).

**Plasmid Constructs and Inducible MPK38 shRNA Cell Line**—Wild-type (WT) and kinase-dead (KD) PDK1, PDK1(1), PDK1(CA), wild-type, and kinase-dead (K40R) Mpk38, Mcat, Mpkc, ASK1, activator protein 1 (AP-1)-Luc reporter, and c-fos were have been described previously (10, 24). An inducible \( \text{Mpk38} \) shRNA HEK293 cell line was generated as described previously (23).

**PDK1 Mutants and RNA Interference**—PDK1 mutants (T354A, S394A/S398A, and S394A/S398A/T354A) were generated by PCR as described previously (20). In brief, wild-type PDK1 was used as the template for amplification with either primers, in conjunction with one of the following mutant primers containing alterations in the nucleotide sequence of wild-type PDK1: for T354A, sense 5’-CGGAATTCTAGGAGTGTCGTCGTCG-3’ (EcoRI site underlined) or reverse 5’-GGCTCCTCCTGACGGGCTGAG-3’ (SacI site underlined) primers, in conjunction with one of the following mutant primers.

**Preparation of Recombinant Proteins and the PDK1 Kinase Assay**—Recombinant glutathione S-transferase (GST) or His-tagged wild-type and deletion constructs of PDK1 and MPK38 were purified by affinity chromatography on glutathione-Sepharose 4B or His columns (Amersham Biosciences). The PDK1 kinase assay was performed as described previously (29) using immunoprecipitated or recombinant PDK1 proteins. Approximately 500 ng of recombinant SGK (Upstate) was used as the substrate.

**Luciferase Reporter Assay**—Assays were carried out in 293T cells as described previously (24). Luciferase activity was assayed using the Dual-Luciferase assay system (Promega) according to the manufacturer’s instructions and normalized to \( \beta \)-galactosidase activity.

**GFP-based Cell Death Assay**—Green fluorescent protein (GFP)-based cell death assays were carried out in HEK293, 293T, and HaCaT cells as described previously (23, 24). The nuclei of GFP-positive cells were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed for apoptotic morphology under a fluorescence microscope. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells.

**Cell Cycle Analysis**—Assays were performed in HaCaT cells as described previously (23). Cells were transfected with the indicated plasmid combinations using WelFect-Ex™ Plus (WelGENE, Daegu, Korea). The cell fraction at each stage of the cell cycle was analyzed after treatment with 10% serum for 24 h in the presence or absence of 2 ng/ml porcine TGF-\( \beta 1 \) (R & D Systems). Flow cytometry analysis was performed using the FACSCalibur-S system (BD Biosciences).

**Statistical Analysis**—Values represent the means ± S.E. A \( p \) value <0.05 calculated using the Student’s \( t \) test was considered statistically significant.

**RESULTS**

**MPK38 Interacts with PDK1 Both in Vivo and in Vivo**—We previously showed that PDK1 inhibits Smad-mediated signaling via direct interaction with Smad proteins (29). In addition, MPK38 physically interacts with and phosphorylates Smad proteins, resulting in the stimulation of TGF-\( \beta \) signaling (23). Therefore, we speculated that there may be a direct or indirect functional link between MPK38 and PDK1 signaling pathways in cells.
To test this hypothesis, we examined whether MPK38 physically interacts with PDK1 in cells using cotransfection experiments incorporating HEK293 cells expressing GST-MPK38 and FLAG-PDK1. The interaction between MPK38 and PDK1 was analyzed by immunoblotting with an anti-FLAG antibody (left panel). Equal amounts of cell lysate from HEK293, NIH 3T3, and R1.1 cells were immunoprecipitated with either rabbit preimmune serum (Preimm.) or anti-MPK38 antibody (a-MPK38) followed by immunoblotting with an anti-PDK1 antibody to determine endogenous binding (right top panels). A, in vivo association of MPK38 with PDK1. GST alone or GST-MPK38 was cotransfected into HEK293 cells along with FLAG-PDK1. GST fusion proteins were purified on glutathione-Sepharose beads (GST Purification), and complex formation was analyzed by immunoblotting with an anti-FLAG antibody (left panel) and PDK1 (right panel) are shown. HEK293 cells transfected with the indicated expression vectors were lysed, precipitated using glutathione-Sepharose beads, and then immunoblotted with an anti-FLAG antibody to determine the level of MPK38-PDK1 binding. B, mapping of the MPK38 and PDK1 binding domains. The schematic structures of wild-type and deletion constructs of Mpk38 (left panel) and PDK1 (right panel) are shown. HEK293 cells transfected with the indicated expression vectors were lysed, precipitated using glutathione-Sepharose beads, and then immunoblotted with an anti-FLAG antibody to determine the level of MPK38-PDK1 binding. C, in vitro binding of MPK38 and PDK1. For native PAGE of the MPK38-PDK1 complex, autophosphorylated His-tagged PDK1 or MPK38 (each 2–3 μg), prepared in the presence of the respective kinase buffers (24, 29), were incubated with unlabeled recombinant GST-tagged kinase-dead MPK38 or PDK1 and its deletion constructs (for MPK38, MCAT, and MPKC; for PDK1, CA, and PH) (each 5 μg), together with the nonspecific control GST at room temperature for 1 h. The same blot was stripped and re-probed with anti-PDK1 and anti-MPK38 antibodies to confirm the presence of PDK1 and MPK38 on the radioactive band shifts (middle and bottom panels). The purity of recombinant PDK1 and MPK38 proteins used for this experiment was analyzed by Coomassie Blue staining (see supplemental Fig. 4A). IP, immunoprecipitation; re., recombinant; WB, Western blot; Mpk38(K40R), kinase-dead MPK38; Preimm., preimmune serum.
Negative Regulation of PDK1 by MPK38

![FIGURE 2. MPK38/ASK1 and PDK1 signaling decreases MPK38-PDK1 binding.](image)

HEK293 cells were incubated with or without wortmannin (100 nM, 30 min) and then treated with insulin (100 nM, 20 min). The cell lysates were then immunoprecipitated (IP) with either rabbit preimmune serum (Preimm.) or an anti-MPK38 antibody (α-MPK38) followed by immunoblotting with an anti-PDK1 antibody to determine endogenous MPK38-PDK1 binding (1st panel). Equal amounts of cell lysate were treated with or without the following stimuli (20): H2O2 (2 mM, 30 min), TNF-α (500 ng/ml, 30 min), thapsigargin (Tg) (20 μM, 30 min), or ionomycin (IONO) (1 μM, 24 h). The cell lysates were then immunoprecipitated with the indicated rabbit preimmune serum or anti-MPK38 antibody (α-MPK38) and immunoblotted with an anti-PDK1 antibody to assess endogenous MPK38-PDK1 binding (2nd to 4th panels). WB, Western blot.

MPK38 inhibits PDK1 activity through Thr 254 phosphorylation—
To determine whether MPK38 affected PDK1 kinase activity, HEK293 cells were transfected with either PDK1 alone or with both PDK1 and MPK38. PDK1 kinase activity decreased markedly when PDK1 was coexpressed with wild-type MPK38, whereas coexpression of kinase-dead (K40R) MPK38 had no effect on the PDK1 kinase activity compared with the control expressing wild-type PDK1 alone (Fig. 3A, left panel). However, this effect was not observed under the same conditions in the presence of kinase-dead PDK1, suggesting that PDK1-mediated phosphorylation of the SGK substrate occurred without relying on additional proteins communoprecipitated with PDK1. This result indicates that MPK38 inhibited PDK1 kinase activity in a kinase-dependent manner. Another approach using recombinant PDK1 and MPK38 proteins showed that recombinant wild-type MPK38 directly inhibited PDK1 kinase activity in a dose-dependent manner (Fig. 3A, right panel).

To assess whether the MPK38-mediated inhibition of PDK1 kinase activity influenced PDK1-mediated signaling, we developed a stable system for the tetracycline-inducible expression of MPK38 shRNA in HEK293 cells (inducible MPK38 shRNA). Parental HEK293 cells, HEK293 cells expressing a scrambled shRNA (inducible Sc shRNA), or inducible MPK38 shRNA cells were either untreated or treated with doxycycline to induce the knockdown of endogenous MPK38. Anti-phospho-specific antibodies for PDK1 Ser241, AKT Thr308, AKT Ser473, and BAD Ser136 were then used for immunoblot analysis to assess PDK1 downstream signaling. As shown in Fig. 3B, knockdown of endogenous MPK38 markedly stimulated PDK1 downstream signaling. These findings point to the inhibition of PDK1-mediated signaling upon MPK38 binding and suggest that MPK38 is a potential negative regulator of PDK1 activity.

We next determined whether PDK1 acts as a substrate for MPK38 using the recombinant nonphosphorylated form of the kinase-dead (KD) PDK1 protein as a substrate in the MPK38 kinase assay. Wild-type and kinase-dead MPK38 proteins, purified from GST-MPK38-expressing HEK293 cell lysates using glutathione-Sepharose beads, were incubated with [γ-32P]ATP to allow phosphorylation of recombinant PDK1(KD). Phosphorylation of recombinant PDK1(KD) was clearly detected in the presence of wild-type MPK38 but not in the presence of GST alone or kinase-dead MPK38 (Fig. 3C, left panel), indicating that PDK1 may be a substrate for MPK38. Next, to identify the...
MPK38 phosphorylation sites on PDK1, we performed an alignment analysis using the AMP-activated protein kinase/MPK38 consensus sequence (23, 30); three potential MPK38 phosphorylation sites (Ser92, Thr354, and Ser393) on PDK1 were selected.

In vitro kinase assays using recombinant MPK38 and PDK1(KD) substitution mutants (S92A, T354A, and S393A) showed that MPK38 phosphorylated two PDK1(KD) mutants, S92A and S393A, but not the T354A mutant (Fig. 3C, right panel). This was also confirmed by immunoblot analysis using an anti-phospho-PDK1(T354) antibody. These results suggest that MPK38 physically interacts with and phosphorylates PDK1 at Thr354.

MPK38 Alleviates PDK1-mediated Suppression of Apoptosis in a Kinase-dependent Manner—To investigate whether MPK38 regulates PDK1-mediated suppression of apoptosis, we analyzed the effect of MPK38 on PDK1-mediated suppression of TNF-α-induced apoptosis using the GFP system (10, 29).

Expression of PDK1 in the presence of TNF-α resulted in a considerable decrease in apoptotic cell death compared with that in control cells expressing an empty vector. However, wild-type MPK38, but not kinase-dead (K40R) MPK38, alleviated this suppression in a dose-dependent manner (Fig. 4A, compare 6th lane with 7th to 10th lanes). This result implies that MPK38 contributes to the negative regulation of the PDK1-mediated survival signaling pathway through direct interaction and phosphorylation.

To confirm this, we examined the effect of MPK38 on the PDK1(T354A)-mediated signaling because the T354A mutant showed no phosphorylation at Thr354. As expected, knockdown of endogenous MPK38 decreased TNF-α-induced apoptosis in a dose-dependent manner compared with control cells expressing PDK1 alone (Fig. 4A, compare 6th lane with 11th to 14th lanes). To determine the role of MPK38-mediated phosphorylation of PDK1 at Thr354 in the regulation of PDK1 signaling, we also assessed the effect of MPK38 on PDK1(T354A)-mediated signaling because the T354A mutant...
Negative Regulation of PDK1 by MPK38

was found to be defective in MPK38-mediated phosphorylation (see Fig. 3C). Neither wild-type nor kinase-dead MPK38 had an effect on PDK1(T354A)-mediated suppression of apoptosis compared with that in a control expressing PDK1 alone (Fig. 4A, compare 15th lane with 16th to 19th lanes). These results indicate that MPK38 negatively regulates PDK1 activity by phosphorylating it on Thr354. We then extended our analysis to examine whether MPK38 regulated serum-induced cell growth induced by PDK1 activation. Flow cytometry analysis using HaCaT cells showed that coexpression of wild-type MPK38 considerably decreased the percentage of cells in S phase in comparison with that in control cells expressing PDK1 alone (Fig. 4B, upper panel, 3rd lane versus 5th lane, ~51 versus ~41%; see supplemental Fig. 1A), although kinase-dead MPK38 had no effect (Fig. 4B, upper panel, 3rd lane versus 7th lane, ~51 versus ~51%; see supplemental Fig. 1A). These results suggest that the decrease in the number of S phase cells observed in the presence of MPK38 was due to MPK38-mediated phosphorylation of PDK1, because transfected Mpk38 (wild-type or kinase-dead) itself did not influence the percentage of cells in S phase (Fig. 4B, upper panel, 2nd lane versus 4th and 6th lanes, ~37 versus ~37%; see supplemental Fig. 1A). To further confirm the negative role of MPK38 in PDK1-mediated cell growth, we performed flow cytometry analysis using HaCaT cells transfected with Mpk38-specific siRNA. Knockdown of endogenous MPK38 resulted in a marked increase in the percentage of cells in S phase cells compared with that in control cells expressing PDK1 alone (Fig. 5A, upper panel, 3rd lane versus 5th lane, ~58 versus ~72%; see supplemental Fig. 1A). However, we did not observe any change in the percentage of cells in S phase in the presence of control scrambled siRNA (Fig. 4B, lower panel, 3rd lane versus 4th lane, ~58 versus ~59%; see supplemental Fig. 1A). With regard to PDK1 phosphorylation at Thr354, MPK38 had no effect on the accumulation of S phase cells in the presence of the T354A mutant (Fig. 4C, 4th lane versus 7th lane, ~54 versus ~54%; see supplemental Fig. 1B). These results suggest that MPK38-mediated phosphorylation of PDK1 at Thr354 plays an important role in the negative regulation of PDK1-mediated cell growth.

MPK38 Alleviates PDK1-mediated Suppression of TGF-β Signaling in a Kinase-dependent Manner—PDK1 inhibits TGF-β signaling (29); therefore, to investigate whether MPK38 is involved in the regulation of PDK1-mediated TGF-β signaling, we examined the effect of MPK38 on PDK1-mediated suppression of TGF-β-induced apoptosis. Wild-type MPK38, but not kinase-dead MPK38, increased apoptotic cell death in a dose-dependent manner compared with that in control cells expressing PDK1 alone (Fig. 5A, upper panel, 6th lane versus...
Negative Regulation of PDK1 by MPK38

MPK38 inhibits PDK1 activity toward TGF-β-induced apoptosis and cell cycle arrest. A, effect of MPK38 on PDK1-mediated suppression of TGF-β-induced apoptosis. HaCaT cells were transiently transfected with increasing amounts of wild-type and kinase-dead Mpk38 (0.5 and 1.5 μg) and Mpk38 siRNAs (50 and 200 nM) as indicated, together with an expression vector encoding GFP (1 μg) in the presence or absence of the wild-type and mutant form (T354A) of PDK1 (2 μg each). After treatment of the transfected cells with TGF-β1 (2 ng/ml, 20 h), apoptotic cell death was determined using a GFP-based cell death assay. B, effect of MPK38 on PDK1-mediated suppression of TGF-β-induced cell cycle arrest. HaCaT cells (∼2 × 10^5/dish) transfected with wild-type and mutant form (T354A) of PDK1 (0.5 μg each) in the presence or absence of wild-type and kinase-dead Mpk38 (0.4 μg each) were synchronized in G0/G1 by hydroxyurea treatment (2 mM, 20 h). Cells were collected after treatment with 10% serum for 24 h in the presence of TGF-β1, and the percentage of cells in the G0/G1, S, or G2/M phases was analyzed by flow cytometry. Data are representative of at least three independent experiments performed in duplicate.

MPK38 Alleviates PDK1-mediated Suppression of ASK1 Signaling in a Kinase-dependent Manner—Because PDK1 interacts with ASK1 and inhibits ASK1-mediated signaling (20), we also examined whether MPK38 had an effect on PDK1-mediated suppression of H2O2-induced apoptosis in a dose-dependent manner (Fig. 6A, upper panel, 7th lane versus 8th to 11th lanes). To further confirm this observation, we performed knockdown experiments using Mpk38 siRNA. Transfection of Mpk38 siRNA into HEK293 cells potentiated the PDK1-mediated suppression of H2O2-induced apoptosis in a dose-dependent manner (Fig. 6A, lower panel, 7th lane versus 8th to 11th lanes). These results suggest that MPK38 contributes to the alleviation of PDK1-mediated suppression of ASK1 signaling by inhibiting PDK1 activity via direct interaction and phosphorylation. To verify this, we compared the effect of MPK38 on PDK1-mediated suppression of H2O2-induced apoptosis in the presence of wild-type PDK1 with its effect in the presence of the T354A mutant, which is defective in MPK38-mediated phosphorylation. In contrast with the negative effect of wild-type MPK38 on PDK1-mediated suppression of H2O2-induced apoptosis (Fig. 6A, lower panel, 7 to 11 lanes), neither wild-type nor
kinase-dead MPK38 affected PDK1(T354A)-mediated suppression of H2O2-induced apoptosis (Fig. 6A, lower panel, 12th to 16th lanes), indicating a crucial role for PDK1 phosphorylation at Thr354 in the MPK38-mediated regulation of H2O2-induced apoptosis suppressed by PDK1.

Because PDK1 suppresses ASK1-induced AP-1 transcriptional activity (20), it is possible that MPK38 also enhances AP-1 transcriptional activity. To test this hypothesis, we performed an AP-1-responsive luciferase reporter assay to determine whether MPK38 affected the transcriptional activity of AP-1. As expected, wild-type MPK38, but not kinase-dead MPK38, increased AP-1-dependent luciferase activity in the presence of ASK1 and PDK1 in a dose-dependent manner (Fig. 6B, upper panel, 8th to 12th lanes). However, this effect was not observed in the presence of ASK1 and T354A mutants (Fig. 6B, lower panel, 13th to 17th lanes), suggesting that MPK38-mediated phosphorylation of PDK1 at Thr354 is also required for the alleviation of PDK1-mediated suppression of AP-1 transcriptional activity.

Phosphorylation of PDK1 at Thr354, Ser394, and Ser398 Functions Cooperatively to Inhibit PDK1 Activity—ASK1-mediated phosphorylation of PDK1 at Ser394 and Ser398 contributes to the inhibition of PDK1 activity (20). Therefore, to establish whether the phosphorylation of PDK1 at Thr354 induced by MPK38 has a similar effect on the regulation of PDK1 activity, we first analyzed the kinase activity of the T354A mutant using an in vitro kinase assay. The results showed that the T354A mutant induced PDK1 kinase activity at a level comparable with that of the S394A/S398A double mutant, which is defective in ASK1-mediated phosphorylation (Fig. 7A, 1st to 3rd lanes). Next, to determine the cooperative effects of PDK1 phosphorylation at Thr354, Ser394, and Ser398 in the negative regulation of PDK1 activity, we measured the kinase activity of the PDK1 S394A/S398A/T354A triple mutant and compared it with that of two other PDK1 mutants, T354A and S394A/S398A. The S394A/S398A/T354A triple mutant showed higher phosphorylation of SGK than the T354A single mutant or the S394A/S398A double mutant (Fig. 7A, 1st lane versus 2nd to 4th lanes). These results provide evidence that the phosphorylation of PDK1 at Thr354, Ser394, and Ser398 is cooperatively involved in the negative regulation of PDK1 activity. If this is the case, the increased kinase activity of the PDK1 mutants defective in MPK38- and/or ASK1-mediated phosphorylation may influence PDK1-mediated cell survival functions. To verify this, we

FIGURE 6. MPK38 inhibits PDK1 activity toward H2O2-induced apoptosis and JNK-mediated transcription. A, effect of MPK38 on PDK1-mediated suppression of H2O2-induced apoptosis. HEK293 cells were transiently transfected with increasing amounts of wild-type and kinase-dead Mpk38 (0.4 and 0.8 μg) or Mpk38 siRNAs (100 and 200 nM) in the presence of the wild-type or mutant form (T354A) of PDK1 (1.5 μg each). Apoptotic cell death was determined in the GFP-based cell death assay. Cells exposed to 1 mM H2O2 for 9 h were used as a positive control. B, effect of MPK38 on PDK1-mediated suppression of JNK-mediated transcription. 293T cells were transfected with PDK1 (wild-type and mutant form (T354A), 1 μg each), Mpk38 (wild-type and kinase-dead (K40R), 0.6 and 1.2 μg), ASK1 (0.8 μg), and Mpk38 siRNAs (50 and 200 nM), together with 0.2 μg of AP-1 luciferase plasmid and β-galactosidase plasmid (0.2 μg) as an internal control in the presence or absence of c-fos (0.7 μg) as indicated. Luciferase activity was measured 48 h after transfection and normalized to β-galactosidase activity. Data represent the mean (± S.E.) of three independent experiments. RLU, relative light unit; WB, Western blot.
analyzed the effect of three PDK1 mutants (T354A, S394A/S398A, and S394A/S398A/T354A) on PDK1-mediated suppression of TNF-α-induced apoptosis. As expected, the suppressive effect of the S394A/S398A/T354A triple mutant was stronger than that of the T354A or S394A/S398A mutants (Fig. 7B, left panel). A similar result was also obtained when we analyzed the effect of the three PDK1 mutants on PDK1-mediated suppression of TGFB-β-induced apoptosis (Fig. 7B, right panel). Together, these results indicate that the phosphorylation of PDK1 at Thr354, Ser394, and Ser398 by MPK38 and ASK1 has a cooperative effect on the negative regulation of PDK1 activity.

**DISCUSSION**

In this study, we show that MPK38 inhibits PDK1, a master kinase for regulating multiple signaling pathways (34, 35), through direct interaction and phosphorylation. MPK38 inactivates PDK1 by phosphorylating PDK1 at Thr354. This suggests that the MPK38-dependent phosphorylation of PDK1 at Thr354, like Ser394/Ser398 phosphorylation by ASK1 and Ser394/Ser398 phosphorylation by PKCθ (18, 20), plays a negative role in the regulation of PDK1 activity and function.

We investigated whether MPK38 directly phosphorylates PDK1 using an in vitro kinase assay because MPK38 physically interacted with PDK1 both in vivo and in vitro (Fig. 1). We found that MPK38-mediated phosphorylation of PDK1 occurred exclusively at Thr354, leading to the inhibition of PDK1 activity and function (Figs. 4–7). These findings indicate that the Thr354 of PDK1 is a MPK38 phosphorylation site for the negative regulation of PDK1.

Our results also showed that the T354A mutant itself had a comparatively minor effect on PDK1 function when compared with the control wild-type PDK1 (Figs. 4–7), suggesting the possibility of other potential phosphorylation sites on PDK1 that regulate its activity. Indeed, a similar trend was observed in our previous study showing that ASK1-dependent phosphorylation of PDK1 at Ser394 and Ser398 plays a negative regulatory role in the PDK1 function (20). This observation, together with that of PKCθ (18), strongly indicates that the phosphorylation of PDK1 at Thr354 stabilized complex formation between PDK1 and its negative regulator 14-3-3, but destabilized complex formation between PDK1 and its positive regulator STRAP, thereby inhibiting PDK1 activity (Fig. 8, A and B, right panels). This finding suggests that the phosphorylation of PDK1 at Thr354 induced by MPK38 has an important impact on the interaction of PDK1 with its regulators (STRAP and 14-3-3) and plays a key role in the progress of PDK1-mediated signaling.

**FIGURE 7. Cooperative effect of PDK1 phosphorylation at Thr354, Ser394, and Ser398 on the negative regulation of PDK1 activity.** A, effect of mutations of Thr354, Ser394, and Ser398 to Ala on PDK1 kinase activity. After 48 h of transfection with GST-PDK1 or one of its substitution mutants (S394A/S398A, T354A, or S394A/S398A/T354A), HEK293 cell lysates were subjected to precipitation with glutathione-Sepharose beads (GST purification) and analyzed in an in vitro kinase assay with recombinant SGK as the substrate to assess the kinase activity of PDK1. The relative level of kinase activity was quantitated by densitometric analysis, and the fold increase relative to the control expressing wild-type PDK1 was calculated. B, effect of mutating Thr354, Ser394, and Ser398 to Ala on PDK1-mediated suppression of TNF-α (or TGFB-β)–induced apoptosis. 293T (left panel) or HEK293 (right panel) cells were transfected with the indicated plasmid vectors. Cells were treated with TNF-α (20 ng/ml) and cycloheximide (10 μg/ml) for 14 h or TGFB-β1 (2 ng/ml) for 20 h to induce apoptosis. The data shown are representative of at least three independent experiments. WB, Western blot.

**Cooperative effect of PDK1 phosphorylation at Thr354, Ser394, and Ser398 on the negative regulation of PDK1 activity.** A, effect of mutations of Thr354, Ser394, and Ser398 to Ala on PDK1 kinase activity. After 48 h of transfection with GST-PDK1 or one of its substitution mutants (S394A/S398A, T354A, or S394A/S398A/T354A), HEK293 cell lysates were subjected to precipitation with glutathione-Sepharose beads (GST purification) and analyzed in an in vitro kinase assay with recombinant SGK as the substrate to assess the kinase activity of PDK1. The relative level of kinase activity was quantitated by densitometric analysis, and the fold increase relative to the control expressing wild-type PDK1 was calculated. B, effect of mutating Thr354, Ser394, and Ser398 to Ala on PDK1-mediated suppression of TNF-α (or TGFB-β)–induced apoptosis. 293T (left panel) or HEK293 (right panel) cells were transfected with the indicated plasmid vectors. Cells were treated with TNF-α (20 ng/ml) and cycloheximide (10 μg/ml) for 14 h or TGFB-β1 (2 ng/ml) for 20 h to induce apoptosis. The data shown are representative of at least three independent experiments. WB, Western blot.
of PDK1 plays an important role in the regulation of PDK1 activity and function. To examine whether the phosphorylation sites (Thr\(^{354}\), Ser\(^{394}\), and Ser\(^{398}\)) on PDK1, which are known to inhibit the PDK1 activity (20), had a cooperative effect on the negative regulation of PDK1 activity and function, we analyzed the kinase activity and apoptotic suppression of a PDK1 triple mutant (S394A/S398A/T354A), together with two other PDK1 mutants, T354A and S394A/S398A, which are defective in MPK38- and ASK1-mediated phosphorylation, respectively.

The results showed that the PDK1 triple mutant (S394A/S398A/T354A) had a stronger effect on the kinase activity and apoptotic suppressive function of PDK1 than T354A or S394A/S398A (Fig. 7), indicating that PDK1 phosphorylation at Thr\(^{354}\), Ser\(^{394}\), and Ser\(^{398}\) has a cooperative effect on the negative regulation of PDK1 signaling. Recent studies suggest the involvement of docking interactions between protein kinases and their substrates for achieving substrate specificity and regulation of protein kinase activities (36). Based on this, one may raise the argument that the regulation of PDK1 activity by MPK38 is due to the direct docking interaction between PDK1 and MPK38. However, MPK38, unlike other protein family kinases A, G, and C interacting with PDK1, was found to interact with PDK1 via the amino-terminal kinase domain of MPK38 (see Fig. 1B). In addition, as in the case of SGK (Fig. 7A), similar results (data not shown) were obtained when we analyzed the effect of the three PDK1 mutants on PDK1 kinase activity using other PDK1 substrates that do not possess docking sites, including Smads (Smad2, -3, -4, and -7) (29), STRAP (10), and ZPR9 (see Ref. 31 and data not shown). In this context, it seems that the most likely mechanism by which MPK38 may inhibit the PDK1 activity would be through the change of PDK1 intrinsic activity, probably via direct phosphorylation of PDK1 at Thr\(^{354}\) by MPK38, rather than docking interaction-mediated regulation of PDK1 activity.
Modulating the association between ASK1 and its regulators, such as TRX and 14–3–3, has been proposed as a potential mechanism for the MPK38-mediated stimulation of ASK1 activity (24). Therefore, we speculated that MPK38 may inhibit PDK1 signaling, possibly by influencing the association between PDK1 and its regulators, STRAP (10) and 14–3–3 (8). To test this hypothesis, we examined the effect of MPK38 on STRAP and 14–3–3 binding to PDK1 using in vivo binding assays. Coexpression of MPK38 markedly decreased the association between PDK1 and its positive regulator STRAP and increased the association between PDK1 and its negative regulator 14–3–3 (Fig. 8). These results indicate that the MPK38-mediated inhibition of PDK1 signaling is accompanied by the modulation of PDK1 binding to its regulators, STRAP and 14–3–3, similar to the MPK38-mediated stimulation of ASK1 signaling reported previously (24). As PDK1 phosphorylation at Thr^{354} plays an important role in the negative regulation of PDK1 activity and function, it is likely that Thr^{354} phosphorylation of PDK1 may influence binding between PDK1 and its regulators (STRAP and 14–3–3). We found that complex formation between PDK1 and its positive regulator STRAP increased in the presence of the T354A mutant compared with the control expressing wild-type PDK1, whereas complex formation between PDK1 and its negative regulator 14–3–3 decreased (Fig. 8, A and B, right panels). These results suggest that MPK38-mediated phosphorylation of PDK1 at Thr^{354} modulates the interaction between PDK1 and its regulators (STRAP and 14–3–3), which is crucial for determining the manner of PDK1 signaling and eventually inhibits PDK1 activity and function.

The fact that PDK1 physically interacts with both ASK1 and MPK38 led us to hypothesize that a ternary complex consisting of PDK1, MPK38, and ASK1 occurs within cells (supplemental Fig. 3). In fact, the PDK1-MPK38-ASK1 complex was detected in unstressed cells; however, treatment with H_{2}O_{2} disrupted this ternary complex and led to the formation of a binary complex between MPK38 and ASK1, which allowed the stimulation of ASK1 signaling, probably by stabilizing the MPK38-ASK1 complex in the presence of H_{2}O_{2}. In addition to the ternary complex, it is possible that the binary PDK1-ASK1 complex exists in unstressed cells because the interaction domains of ASK1 responsible for PDK1 and MPK38 binding, as well as the interaction domains of PDK1 responsible for ASK1 and MPK38 binding, are equivalent (20, 24). Treatment with H_{2}O_{2} also disrupted the binary complex between PDK1 and ASK1 (20), promoting complex formation between MPK38 and ASK1 (24) and subsequently leading to ASK1 activation.

Collectively, the results of this study define a novel mechanism in which MPK38 directly interacts with and phosphorylates Thr^{354} of PDK1, thereby inhibiting PDK1 activity. The results also provide evidence that Thr^{354} of PDK1, like Ser^{394} and Ser^{398} of ASK1 (20) and Ser^{504} and Ser^{532} of PKCθ (18), represents a potential phosphorylation site for the negative regulation of PDK1 activity. Furthermore, the finding that MPK38 phosphorylates PDK1 on Thr^{354}, thereby negatively regulating PDK1 activity, will contribute to a better understanding of the regulatory mechanism(s) involved in PDK1 activity and function.

REFERENCES
1. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 15, 6541–6551
2. Downward, J. (1998) Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. 10, 262–267
3. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase that phosphorylates and activates protein kinase B. Science 279, 710–714
4. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998) Protein kinase B kinases that mediate phosphorylidyinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 279, 710–714
5. Sarbassov, D. D., Gueratin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307, 1098–1101
6. Bayascas, J. R., and Alessi, D. R. (2005) Regulation of Akt/PKB Ser^{473} phosphorylation. Mol. Cell 18, 143–145
7. Fujita, N., Sato, S., Ishida, A., and Tsuruo, T. (2002) Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. J. Biol. Chem. 277, 10346–10353
8. Sato, S., Fujita, N., and Tsuruo, T. (2002) Regulation of kinase activity of 3-phosphoinositide-dependent kinase-1 by binding to 14–3–3. J. Biol. Chem. 277, 39360–39367
9. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. Curr. Biol. 9, 393–404
10. Seong, H. A., Jung, H., Choi, H. S., Kim, K. T., and Ha, H. (2005) Regulation of transforming growth factor-β signaling and PDK1 kinase activity by physical interaction between PDK1 and serine-threonine kinase receptor-associated protein. J. Biol. Chem. 280, 42897–42908
11. Toker, A., and Newton, A. C. (2000) Cellular signaling. Pivoting around PDK-1. Cell 103, 185–188
12. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1. Identification of five sites of phosphorylation in vivo. Biochem. J. 342, 287–292
13. Scheid, M. P., Parsons, M., and Woodgett, J. R. (2005) Phosphoinositide-dependent phosphorylation of PDK1 regulates nuclear translocation. Mol. Cell Biol. 25, 2347–2363
14. Riojas, R. A., Kikani, C. K., Wang, C., Mao, X., Zhou, L., Langlais, P. R., Hu, D., Roberts, J. L., Dong, L. Q., and Liu, F. (2006) Fine-tuning PDK1 activity by phosphorylation at Ser^{393}. J. Biol. Chem. 281, 21588–21593
15. Yang, K. J., Shin, S., Pao, L., Shin, E., Li, Y., Park, K. A., Byun, H. S., Won, M., Hong, J., Kweon, G. R., Hur, G. M., Seok, J. H., Chun, T., Brazzi, D. P., Hemmings, B. A., and Park, J. (2008) Regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) by Src involves tyrosine phosphorylation of PDK1 and Src homology 2 domain binding. J. Biol. Chem. 283, 1490–1491
16. Ito, T., Seyama, T., Iwamoto, K. S., Hayashi, T., Mizuno, T., Tsuyama, N., Dohi, K., Nakamura, N., and Akiyama, M. (1993) In vivo irradiation is able to cause RET oncogene rearrangement. Cancer Res. 53, 2940–2943
17. Kim, D. W., Hwang, J. H., Suh, J. M., Kim, H., Song, J. H., Hwang, E. S., Hwang, I. Y., Park, K. C., Chung, H. K., Kim, J. M., Park, I., Hemmings, B. A., and Shong, M. (2003) RET/PTC (rearranged in transformation/cancer gene) in papillary thyroid carcinomas: tyrosine kinase phosphorylations and activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1). J. Biol. Chem. 278, 26226–26234
18. Wang, C., Liu, M., Riojas, R. A., Xin, X., Gao, Z., Zeng, R., Wu, J., Dong, L. Q., and Liu, F. (2009) Protein kinase Cθ (PKCθ)-dependent phosphorylation of PDK1 at Ser^{504} and Ser^{532} contributes to palmetine-induced insulin resistance. J. Biol. Chem. 284, 2038–2044
19. Kondo, T., and Kahn, C. R. (2004) Altered insulin signaling in retinal tissue
in diabetic states. J. Biol. Chem. 279, 37997–38006
20. Seong, H. A., Jung, H., Ichijo, H., and Ha, H. (2010) Reciprocal negative regulation of PDK1 and ASK1 signaling by direct interaction and phosphorylation. J. Biol. Chem. 285, 2397–2414
21. Gil, M., Yang, Y., Lee, Y., Choi, I., and Ha, H. (1997) Cloning and expression of a cDNA encoding a novel protein serine/threonine kinase predominantly expressed in hematopoietic cells. Gene 195, 295–301
22. Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B., and Ackerman, S. L. (1997) New member of the Snf1/AMPK kinase family, Melk, is expressed in the mouse egg and preimplantation embryo. Mol. Reprod. Dev. 47, 148–156
23. Seong, H. A., Jung, H., and Ha, H. (2010) Murine protein serine-threonine kinase 38 stimulates TGF-β signaling in a kinase-dependent manner via direct phosphorylation of Smad proteins. J. Biol. Chem. 285, 30959–30970
24. Jung, H., Seong H. A., and Ha, H. (2008) Murine protein serine-threonine kinase 38 activates apoptosis signal-regulating kinase 1 via Thr838 phosphorylation. J. Biol. Chem. 283, 34541–34553
25. Saito, R., Tabata, Y., Muto, A., Arai, K., and Watanabe, S. (2005) Melk-like kinase plays a role in hematopoiesis in the zebrafish. Mol. Cell. Biol. 25, 6682–6693
26. Beullens, M., Vancauwenbergh, S., Morrice, N., Derua, R., Ceulemans, H., Waclenkens, E., and Bollen, M. (2005) Melk-like kinase plays a role in hematopoiesis in the zebrafish. Mol. Cell. Biol. 25, 6682–6693
27. Gray, D., Jubb, A. M., Hogue, D., Dowd, P., Klijavin, N., Yi, S., Bai, W., Frantz, G., Zhang, Z., Koeppen, H., de Sauvage, F. J., and Davis, D. P. (2005) Maternal embryonic leucine zipper kinase/murine protein serine-threonine kinase 38 is a promising therapeutic target for multiple cancers. Cancer Res. 65, 9751–9761
28. Vulsteke, V., Beullens, M., Boudrez, A., Keppens, S., Van Eynde, A., Rider, M. H., Stalmans, W., and Bollen, M. (2004) Inhibition of spliceosome assembly by the cell cycle-regulated protein kinase MELK and involvement of splicing factor NIP1. J. Biol. Chem. 279, 8642–8647
29. Seong, H. A., Jung, H., Kim, K. T., and Ha, H. (2007) 3-Phosphoinositide-dependent PDK1 negatively regulates transforming growth factor-β-induced signaling in a kinase-dependent manner through physical interaction with Smad proteins. J. Biol. Chem. 282, 12272–12289
30. Jakobsen, S. N., Hardie, D. G., Morrice, N., and Tornqvist, H. E. (2001) 5′-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. J. Biol. Chem. 276, 46912–46916
31. Seong, H. A., Jung, H., Manoharan, R., and Ha, H. (2011) Positive regulation of apoptosis signal-regulating kinase 1 signaling by ZPR9 protein, a zinc finger protein. J. Biol. Chem. 286, 31123–31135
32. Datta, P. K., Chytil, A., Gorska, A. E., and Moses, H. L. (1998) Identification of STRAP, a novel WD domain protein in transforming growth factor-β signaling. J. Biol. Chem. 273, 34671–34674
33. Jung, H., Seong H. A., and Ha, H. (2007) NM23-H1 tumor suppressor and its interacting partner STRAP activate p53 function. J. Biol. Chem. 282, 35293–35307
34. Kikani, C. K., Dong, L. Q., and Liu, F. (2005) “New”-clear functions of PDK1. Beyond a master kinase in the cytosol? J. Cell Biochem. 96, 1157–1162
35. Fayard, E., Tintignac, L. A., Baudry, A., and Hemmings, B. A. (2005) Protein kinase B/Akt at a glance. J. Cell Sci. 118, 5675–5678
36. Biondi, R. M., and Nebreda, A. R. (2003) Signaling specificity of Ser/Thr protein kinases through docking site-mediated interactions. Biochem. J. 372, 1–13