Regulation of Kinase Activity of 3-Phosphoinositide-dependent Protein Kinase-1 by Binding to 14-3-3*

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) plays a central role in activating the protein kinase A, G, and C subfamily. In particular, PDK1 plays an important role in regulating the Akt survival pathway by phosphorylating Akt on Thr-308. PDK1 kinase activity was thought to be constitutively active; however, recent reports suggested that its activity is regulated by binding to other proteins, such as protein kinase C-related kinase (PRK2), p90 ribosomal protein S6 kinase (RSK2), and heat-shock protein 90 (Hsp90). Here we report that PDK1 binds to 14-3-3 proteins in vitro and in vivo through the sequence surrounding Ser-241, a residue that is phosphorylated by itself and is critical for its kinase activity. Mutation of PDK1 to increase its binding to 14-3-3 decreased its kinase activity in vivo. By contrast, mutation of PDK1 to decrease its interaction with 14-3-3 resulted in increased PDK1 kinase activity. Moreover, incubation of wild-type PDK1 with recombinant 14-3-3 in vitro decreased its kinase activity. These data indicate that PDK1 kinase activity is negatively regulated by binding to 14-3-3 through the PDK1 auto-phosphorylation site Ser-241.

Many growth factors and cytokines have been reported to promote cell survival. Stimulation of cells with these factors activates phosphatidylinositol 3-oh kinase (PI3K), and activated PI3K generates the phospholipid second messenger molecule phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (1–3). These lipids then induce protein kinase B (PKB/Akt) activation. Activated kinases, then, mediate survival-signal transduction by phosphorylating downstream key regulatory proteins.

3-Phosphoinositide-dependent protein kinase-1 (PDK1) was originally identified as a kinase that could phosphorylate Akt on its activation loop (residue Thr-308) (4–6). Later studies, however, have showed that PDK1 is not just an Akt kinase but also a kinase phosphorylating p70SGK. SGKs, PKC isoforms, and p90 ribosomal protein S6 kinases (RSKs) at the equivalent residues of Thr-308 of Akt (reviewed in Ref. 7). Therefore, PDK1 plays a central role in activating the AGC family of protein kinases. In the case of Akt, the interaction of phosphatidylinositol 3,4,5-trisphosphate with the pleckstrin homology domain recruits Akt to the plasma membrane and promotes conformational change, which results in phosphorylation of Akt at Thr-308 by PDK1 and at Ser-473 by an as yet unidentified kinase (so called PDK2) (7, 8). In the case of p70SGK, SGKs, and PKC isoforms, however, the precise mechanisms for PDK1-dependent phosphorylation and activation are not well understood.

PDK1 itself is also a member of the AGC subfamily of protein kinases and is phosphorylated on the Ser-241 activation loop (equivalent to Thr-308 of Akt) (7). As PDK1 expressed in bacteria is active and is phosphorylated at Ser-241 (9), it is thought to phosphorylate itself at this same site. Further, mutation of Ser-241 to Ala was reported to abolish PDK1 kinase activity, and IGF-1 stimulation did not cause further activation of PDK1 (9). According to these results, PDK1 was thought to be constitutively active. Several recent reports, however, suggested that PDK1 kinase activity is controlled by PDK1-associated proteins, such as PKC-related kinase-1 (PRK1/PRK2 (10), PDK1-interacting fragment of PRK2 (11), RSK2 (12), and Hsp90 (13). The interaction of PDK1 with the PDK1-interacting fragment of PRK2 converts PDK1 from an enzyme that phosphorylates Akt only at Thr-308 into a kinase that phosphorylates both Thr-308 and Ser-473 (11). Further, the association of PDK1 with RSK2 stimulates PDK1 activation and autophosphorylation (12). We recently reported that Hsp90 participates in stability and signaling of PDK1 (13). Therefore, PDK1 is not a constitutively active kinase but a kinase regulated by other interacting proteins.

Members of the 14-3-3 protein family are highly conserved and widely expressed 28–31 kDa proteins that naturally assemble as homodimers or heterodimers. They consist of at least seven isoforms in mammals (β, ε, η, θ, τ, and χ). The 14-3-3 proteins have been shown to interact with and to regulate proteins controlling a wide array of signaling pathways, including Raf-1, Bad, FKHR1, and Cdc25c (reviewed in Ref. 14). Binding of 14-3-3 to its partners depends on phosphorylation of the Ser or Thr residue in the recognition domains. Using peptides derived from Raf-1, Muslin et al. (15) identified that the motif optimal for association with 14-3-3 proteins was RXXpSPX, where pR represents phosphorylated Ser and X represents any amino acid. Moreover, using phosphopeptide libraries, Yaffe et al. (16) and Rittinger et al. (17) revealed that there are two preferred 14-3-3 binding motifs, RXXpSPX and RXXpSPX.
**Plasmids—**14-3-3, β, γ, and α cDNAs were generated by PCR with human fetal brain cDNA library (Invitrogen, Carlsbad, CA) as the template. The sense (5'-TGGAGAAGACTGAGCTGATCCAG-3') and antisense (5'-TTAGTGGTCAGCCCCCTGCGG-3') primers for 14-3-3-α were synthesized according to the sequence from GenBank™ accession number L20422). The sense (5'-TGGATAAAAA-TH9258) and antisense (5'-TATTTTTCCCC-TCCTTTCCTG-3') primers for 14-3-3-β were synthesized according to the sequence from GenBank™ accession number M66400). The sense (5'-ACACTGGGGGACGGAGGACTGCTGCA-3') and antisense (5'-CCAGGGGATCGAAGGATCTTGCA-3') primers for 14-3-3-γ were synthesized according to the sequence from GenBank™ accession number L20422). The sense (5'-TGGATGACGAGGATCGGTG- ACC-3') and antisense (5'-TCAGCTGTTCCGCTGCA-3') primers for 14-3-3-ε were synthesized according to the sequence from GenBank™ accession number U28936). The PCR products were cloned into a plasmid vector, pCMV vector was purchased from Clontech (Palo Alto, CA). All the plasmid DNA for transfection were purified using a Qiagen plasmid Maxi kit, according to the manufacturer’s protocol (Qiagen, Chatsworth, CA).

**RESULTS**

14-3-3 Proteins Bind to PKD1 In Vivo and In Vitro—In order to find novel PKD1-binding proteins that regulate PKD1 kinase activity, we searched protein-protein interaction motifs in PKD1. We found that it has four 14-3-3 binding motifs (RXXpS, pS represents phosphorylated Ser, and X represents any amino acid). Among the four (RXXpS, RXXpS, RXXpS, and RXXpS), two Ser residues (Ser-241 and Ser-410) have been reported to be phosphorylated in cells (9). First, we confirmed the presence of 14-3-3 binding motifs in PKD1 by immunoblotted
Fig. 1. PDK1 binding to 14-3-3 isoforms in vivo and in vitro. A, 293T cells were transfected with pFLAG-CMV-2 vector encoding nothing (Mock; lane 1), ΔN51-PDK1 (lane 2), or WT-Akt (Akt; lane 3), or with pCMV vector encoding v-Raf-1 (Raf-1; lane 4). The immunoprecipitated FLAG-tagged proteins (lanes 1–3) and Raf-1 protein (lane 4) were immunoblotted with the indicated antibodies. B, 293T cells were transfected with pCMV3 vector containing nothing (Mock) or WT-PDK1 cDNA (WT-PDK1) together with pHM6 vector containing nothing (Mock; lanes 1 and 7), 14-3-3θ (θ; lanes 2 and 8), 14-3-3β (β; lanes 3 and 9), 14-3-3ζ (ζ; lanes 4 and 10), 14-3-3η (η; lanes 5 and 11), or 14-3-3ε (ε; lanes 6 and 12) cDNAs. Proteins were immunoprecipitated with an anti-Myc agarose and were immunoblotted with the indicated antibodies. Expression level of HA-tagged 14-3-3 θ (lanes 3 and 7) was confirmed by immunoblot analysis (lower panel). C and D, 293T cells were transfected with pFLAG-CMV-2 vector containing nothing (Mock), 14-3-3θ (θ), 14-3-3β (β), or 14-3-3ζ (ζ) cDNAs together with pCMV-raf-1 (C) or pHM6-akt (D) plasmid. Proteins were immunoprecipitated with an anti-FLAG agarose and were immunoblotted with the indicated antibodies. E, endogenous 14-3-3θ proteins were immunoprecipitated from 293T and NIH3T3 cells with an anti-14-3-3θ antibody (α-14-3-3θ). For control experiments, the cell lysates were incubated with protein A-Sepharose conjugated with control rabbit antibody (Cont. IgG) (lanes 1 and 2) and cell lysates (lanes 3 and 6) were immunoblotted with the indicated antibodies. F, GST (lane 1) or GST-14-3-3θ (lanes 2 and 3) bound to glutathione-Sepharose was incubated with lysates of COS-7 cells expressing WT-PDK1 for 2 h at 30 °C. The precipitate proteins (lanes 1 and 2) and cell lysates (lane 3) were immunoblotted with the indicated antibodies. The positions of molecular mass standards (kDa) are shown on the right.

Analysis using an anti-phospho-14-3-3 binding motif antibody that could preferentially recognize the conserved 14-3-3 recognition motif (RX(XS)T) in which X may be any amino acid) only when Ser or Thr was phosphorylated (14, 18). As shown in Fig. 1A, ΔN51-PDK1, which lacks the NH$_2$-terminal 51 amino acids, was recognized by the anti-phospho-14-3-3 binding motif antibody (lane 2). The full-length PDK1 was also recognized by the antibody (data not shown). Raf-1 is well known to form a complex with 14-3-3 (14, 18), and we observed that it was also recognized by the antibody (Fig. 1A, lane 4). To date, it has not been reported whether Akt forms a complex with 14-3-3. Fig. 1A shows that Akt was barely recognized in the same analysis (lane 3) although it contains one potential 14-3-3 binding motif (120-RSGpS-124).

To confirm the interaction between PDK1 and 14-3-3 in cells, we transfected Myc-tagged PDK1 cDNA together with HA-tagged 14-3-3 isoform cDNAs into 293T cells, following immunoprecipitation of PDK1 with an anti-Myc antibody. Immunoblot analysis revealed that 14-3-3θ and η, but not 14-3-3β, ζ, or ε, were co-immunoprecipitated with PDK1 (Fig. 1B). This result indicates that among the five 14-3-3 isoforms we tested, only 14-3-3θ and η bind to PDK1 in cells, and 14-3-3θ binds to PDK1 more tightly than 14-3-3η does (Fig. 1B). We then examined the binding of 14-3-3θ, β and ζ to Raf-1 and found that it bound to all the three isoforms with almost the same affinity (Fig. 1C). On the other hand, Akt bound to none of the three isoforms under the same conditions (Fig. 1D), which is in agreement with the data that Akt was not recognized by the anti-phospho-14-3-3 binding motif antibody (Fig. 1A, lane 3).

To further examine the association between endogenous PDK1 and endogenous 14-3-3θ in cells, we immunoprecipitated the endogenous 14-3-3θ proteins from 293T and NIH3T3 cells with an anti-14-3-3θ antibody, following immunoblot analysis with an anti-PDK1 antibody (Fig. 1E). We found that endogenous PDK1 was co-immunoprecipitated with 14-3-3θ. In order to reconstitute the interaction between PDK1 and 14-3-3θ in vitro, purified recombinant GST alone or GST-fused 14-3-3θ was incubated for 2 h at 30 °C (Fig. 1F) or at 4 °C (data not shown).
shown) with the lysate of COS-7 cells that express Myc-tagged PDK1. Then, GST or GST-fused 14-3-3 proteins were precipitated by glutathione-Sepharose. PDK1 was co-precipitated with GST/H18528 14-3-3 under both conditions (Fig. 1F, upper panel, lane 2 and data not shown) and, to lesser degree, with GST alone (lane 1). Raf-1 was also co-precipitated with 14-3-3, and its association was more specific than PDK1 (Fig. 1F, middle panel, lane 2). These results indicate that PDK1 binds to 14-3-3 in cells and in vitro.

Mutation in 14-3-3 Impairs the Association between 14-3-3 and PDK1—The co-crystal structure of 14-3-3 with synthetic phosphopeptide demonstrated that the phosphopeptide binds along a groove on the inner surface of 14-3-3 (16, 17). Mutational analysis revealed that alteration of the residues exposed on the binding surface, such as Arg-56, Arg-60, and Arg-127, impairs the phosphopeptide binding ability (17, 22). To confirm the interaction of PDK1 and 14-3-3/H9258 mutants in which its binding surface residues were changed to Ala (R56A/R60A and R127A), we transfected these mutant cDNAs into 293T cells together with N51-PDK1 cDNA. Immunoprecipitation of HA-tagged 14-3-3/H9258 or 14-3-3/H9258 mutants followed by immunoblot analysis revealed that PDK1 was co-precipitated with the

Fig. 2. Specificity of PDK1 binding to 14-3-3θ mutants. A and B, 293T cells were transfected with pHM6 vector encoding nothing (Mock), 14-3-3θ (WT), R56A/R60A-14-3-3θ (R56,60A), and R127A-14-3-3θ (R127A) together with pFLAG-CMV-2 vector encoding nothing (A, lane 1) or ΔN51-PDK1 (A, lanes 2–5), or pCMV vector encoding v-Raf-1 (B, lanes 1–4). Proteins were immunoprecipitated with an anti-HA agarose and were immunoblotted with the indicated antibodies (upper and middle panels). Expression level of transfected FLAG-tagged ΔN51-PDK1 and Raf-1 proteins were confirmed by immunoblot analysis with an anti-FLAG antibody or an anti-Raf-1 antibody, respectively (lower panels). The positions of molecular mass standards (kDa) are shown on the right.

Fig. 3. Identification of phospho-Ser-241 in PDK1 as a 14-3-3θ recognition site. A, schematic representation of the 14-3-3 binding motifs of the known 14-3-3 binding partners, the potential 14-3-3 binding sequences in PDK1, and the sequences of the generated PDK1 mutants. *S represents phosphorylated Ser, and X represents any amino acids. B, 293T cells were transfected with pFLAG-CMV-2 vector encoding nothing (Mock, lane 1), ΔN51-PDK1 (WT, lane 2), S241A-PDK1 (S241A, lane 3), S410A-PDK1 (S410A, lane 4), and S549A-PDK1 (S549A, lane 5) together with pHM6 vector containing 14-3-3θ cDNA (+; lanes 1–5). Proteins were immunoprecipitated with an anti-FLAG agarose and were immunoblotted with the indicated antibodies. Expression level of transfected HA-tagged 14-3-3θ protein was confirmed by immunoblot analysis with an anti-HA antibody (lower panel). C and D, 293T cells were transfected with pCMV3 vector encoding nothing (Mock), WT-PDK1 (WT), or the indicated PDK1 mutants together with pHM6 vector containing 14-3-3θ cDNA. Double stands for a double PDK1 point mutant (S241A/V243P-PDK1). Proteins were immunoprecipitated with an anti-Myc agarose and were immunoblotted with the indicated antibodies. Expression level of transfected HA-tagged 14-3-3θ protein was confirmed by immunoblot analysis with an anti-HA antibody (lower panels). The positions of molecular mass standards (kDa) are shown on the right.
Cells were treated as described in indicated PDK1 point mutants (with either of the 14-3-3 its mutants. Consistent with the previous report using a phospho-PDK1, and the ability to bind to 14-3-3 Ala (S241A, S410A, or S549A, respectively) was introduced to was not recognized by the anti-phospho-14-3-3 binding motif (Fig. 2A). As a control, we also examined Raf-1 binding to 14-3-3 or its mutants. Consistent with the previous report using a phospho-Ser-containing Raf-1 peptide, Raf-1 did not co-purify with either of the 14-3-3 mutants (Fig. 2B, lanes 3 and 4 and Ref. 23). This result indicates that PDK1 associates with 14-3-3 in the binding pocket of 14-3-3 and that Arg-127 is important for binding.

**Identification of the Ser-241 Residue in PDK1 as the 14-3-3 Recognition Site**—As shown in Fig. 3A, 14-3-3 recognizes conserved motifs containing phospho-Ser (e.g. RXRXXpSXP, RXSXP, and RXpS). PDK1 has several reported 14-3-3 binding motifs in its amino acid sequence (\(^\text{RTT}^{\text{550}, \text{218pRANP}^{\text{241, 497RSgpS}^{\text{510, 546RYQS}^{\text{529}}}}, \text{Ref. 9}\)), so we tried to identify the sites. Substitution at residue Ser-241, Ser-410, or Ser-549 with Ala (S241A, S410A, or S549A, respectively) was introduced to PDK1, and the ability to bind to 14-3-3 was examined. We did not generate a mutant in which Ser-6 was converted to Ala, since \(\Delta N 51\)-PDK1 that lacks Ser-6 could also interact with 14-3-3 with similar affinity as WT-PDK1 (data not shown). Co-immunoprecipitation analysis indicated that mutation at Ser-241 impairs the PDK1 binding ability for 14-3-3 and 14-3-3\(\gamma\) (Fig. 3B, upper panel, lane 3, and data not shown). On the other hand, mutations at Ser-410 and Ser-549 did not affect the PDK1–14-3-3\(\gamma\) binding (Fig. 3B, upper panel, lanes 4 and 5, respectively). Consistent with the result of this analysis, S241A was not recognized by the anti-phospho-14-3-3 binding motif antibody (Fig. 3B, second panel, lane 3), but S410A and S549A were recognized (lanes 4 and 5). This result indicates the importance of Ser-241 in PDK1 interaction with 14-3-3 and 14-3-3\(\gamma\).

To further confirm the role of residues surrounding Ser-241 of PDK1, we generated other PDK1 point mutants in which these residues were changed (Fig. 3A). Substitution of Arg-238, equivalent to conserved Arg in the 14-3-3 binding motif, with Glu (R238E) decreased 14-3-3\(\gamma\) binding to PDK1 (Fig. 3C, upper panel, lane 4). In contrast, substitution of Val-243 with Pro (V243P), a residue conserved in many 14-3-3 targets, such as Raf-1 and Bad (Fig. 3A), dramatically increased the amount of 14-3-3 bound to PDK1 (Fig. 3C, upper panel, lane 7). Changes in the binding ability of R238E and V243P were similarly observed when these mutants were incubated in vitro with GST-14-3-3 (data not shown). Additional mutation of Ser-241 to Ala in V243P (V243P/S241A; Double) reduced the binding affinity of PDK1 to 14-3-3 (Fig. 3D, upper panel, lane 4), indicating the importance of phospho-Ser-241 for PDK1–14-3-3 binding. The mutation of Ala-239 to Glu (A239E) or to Gln (A239Q) did not affect the PDK1–14-3-3 complex formation (Fig. 3C, upper panel, lane 5 or 6, respectively). Therefore, 14-3-3\(\gamma\) and 14-3-3\(\gamma\) recognized and bound to the 238-RANPS-241 residue in PDK1. Moreover, Arg-238 and phospho-Ser-241 residues are important for 14-3-3 binding.

**Regulation of PDK1 Kinase Activity by Binding to 14-3-3**—Ser-241 of PDK1 was reported to be phosphorylated by itself (9). Phosphorylation of PDK1 at Ser-241 is suggested to be essential for its kinase activity because the conversion of Ser-241 to Ala dramatically reduces PDK1 kinase activity (9). Therefore, it is possible that 14-3-3 regulates PDK1 kinase activity by associating with PDK1 at Ser-241 residue. We then investigated the role of PDK1–14-3-3 complex formation on PDK1 kinase activity. PDK1 mutant cDNAs were co-transfected with WT-akt cDNA into COS-7 cells, and the kinase activity of PDK1 and its mutants were estimated by the amount of the phosphorylated form of Akt at Thr-308 after serum stimulation. Consistent with a previous report (9), S241A showed weak phosphorylation activity when compared with WT-PDK1 (Fig. 4A, upper panel, compare lanes 4 and 3). It is important to note that mutating Arg-238 to Glu (R238E) to decrease the interaction between PDK1 and 14-3-3 resulted in a slight increase in PDK1 kinase activity (Fig. 4A, upper panel, lane 4).
decreased the phospho-Akt (Thr-308) level of transfected Myr
with 14-3-3 form of Akt was also observed when WT-Akt was co-transfected Akt protein. A decrease in the amount of the phosphorylated in Fig. 4 plasma membrane in a PI3K-independent manner. As shown R238E-PDK1, which associated less with 14-3-3 substrate, SGK (Fig. 5 a) decreased PDK1 autophosphorylation activity by 14-3-3, we incubated recombinant PDK1 with recombinant GST-fused 14-3-3 in vitro—To confirm the negative regulation of PDK1 kinase activity by 14-3-3, we incubated recombinant PDK1 with recombinant GST-14-3-3 in vitro and estimated the change of PDK1 autophosphorylation activity. After the incubation with GST or GST-fused 14-3-3θ at 30 °C for 2 h, a condition sufficient to reconstitute specific interaction of PDK1 with 14-3-3θ in vitro (Fig. 1F), PDK1 was incubated with phosphorylated or non-phosphorylated RSK2 peptide. Then, the autophosphorylation activity was examined by autoradiography. As shown in Fig. 5A, preincubation with GST-14-3-3θ down-regulated PDK1 autophosphorylation activity in a dose-dependent manner. As reported previously (12), PDK1 kinase activity was elevated by adding phosphorylated RSK2 peptide (pS386 peptide). Under this condition, GST-14-3-3θ also decreased PDK1 autophosphorylation activity (Fig. 5A). Similarly, incubation of the immunoprecipitated Myc-tagged WT-PDK1 with GST-14-3-3θ caused a reduction of PDK1 kinase activity to phosphorylate a PDK1 substrate, SGK (Fig. 5B). In contrast, the kinase activity of R238E-PDK1, which associated less with 14-3-3θ (Fig. 3C), was not affected by preincubation with 14-3-3θ (Fig. 5B). These results indicate that 14-3-3 negatively regulates PDK1 kinase activity by association with the residues surrounding the PDK1 Ser-241 residue.

**FIG. 5.** Incubation of PDK1 with 14-3-3θ in vitro decreases PDK1 kinase activity. A, the recombinant PDK1 was incubated with the indicated amount of GST or GST-14-3-3θ for 2 h at 30 °C, following incubation with 15 μM non-phosphorylated RSK2 peptide (non-phos- pho) or phosphorylated RSK2 peptide (phospho) for 20 min at 30 °C, PDK1 autophosphorylation assay was performed, as described under "Experimental Procedures." The relative amounts of incorporated radioactivity were quantified with a BAS1500 Bio-Imaging analyzer. B, COS-7 cells were transfected with pCMV3 vector encoding nothing (-), WT-PDK1 (WT) or R238E-PDK1 (R238E). The immunoprecipitated Myc-tagged proteins were incubated with the indicated amounts of GST or GST-14-3-3θ for 2 h at 4 °C, following incubation with 15 μM phosphorylated RSK2 peptide (phospho-RSK2 peptide). Then, 500 ng of recombinant inactive SGK was added to the reactions. PDK1 kinase assay was performed, as described under “Experimental Procedures.” The relative amounts of 32P-SGK were quantified with a BAS1500 Bio-Imaging analyzer. The amount of the immunoprecipitated Myc-tagged PDK1 proteins was confirmed by immunoblot analysis with an anti-Myc antibody (lower panel). The positions of molecular mass standards (kDa) are shown on the right.

**DISCUSSION**

It is clear that PDK1 plays a central role in activating the AGC subfamily of protein kinases (7, 24). These kinases then mediate intracellular signaling such as cell survival, cell growth, protein synthesis, and gene expression. PDK1 phosphorylates AGC kinase members at the residues equivalent to...
Thr-308 of Akt (known as activation loop or T-loop) (7). PDK1 is, itself, a member of the AGC subfamily of protein kinases. Thus, PDK1 phosphorylates itself at its activation loop (Ser-241), thereby activating itself (9). Although PDK1 kinase activity has been thought to be constitutively active and not further activated by growth factor stimulation, recent findings suggest that its activity and its character are controlled by interaction with other proteins. For example, when PDK1 interacts with the PDK1-interacting fragment of PRK2, it is converted to exhibit PDK2-like activity (11). The association of PDK1 with RSK2 up-regulates PDK1 kinase activity and autophosphorylation (12). We recently reported that PDK1 binds to Hsp90, and its binding prevents PDK1 from proteasome-dependent degradation and keeps it in a soluble and active conformational state (13). Moreover, PDK1 kinase activity is promoted by phosphorylation at tyrosine residues, presumably by a member of the Src kinase family (25, 26).

Here we provide evidence that PDK1 binds to 14-3-3 in vitro and in vitro through the residues surrounding the autophosphorylation site Ser-241 and that the association is achieved only when Ser-241 has been phosphorylated (Fig. 3). Although PDK1 contains other 14-3-3-binding motifs in its amino acid sequence (14-3-3 motifs (Raf-1) and the one that had only one (PDK1). The differences we observed above were a result of the differences between the ligand that possessed at least two 14-3-3 binding motifs (Raf-1) and the one that had only one (PDK1). The distinct mechanism of isoform-specific interaction is a problem to be solved in future studies.

The general mechanisms by which 14-3-3 regulates partner protein functions are 4-fold: 1) regulation of subcellular localization of the target protein; 2) direct regulation of catalytic activity of the binding partner; 3) protection of the ligands from proteolysis or dephosphorylation; and 4) regulation of interaction between bound protein and other molecules. The 14-3-3 binding residue of PDK1 (Ser-241) is thought to be phosphorylated by itself and it be essential for its kinase activity (9). Consistent with previous reporting (9), we confirmed that S241A-PDK1 exhibited weak Akt and SGK phosphorylation activity when compared with WT-PDK1 (Fig. 4). We thus generated PDK1 point mutants in which residues other than Ser-241 are mutated (Fig. 3C) and examined their activity to phosphorylate Akt (Fig. 4A) or SGK (Fig. 4C). Mutation of PDK1 to promote 14-3-3 (V243P-PDK1) association exhibited reduced PDK1 kinase activity to phosphorylate Akt and SGK in cells, while mutation of PDK1 to inhibit the interaction (R238E-PDK1) slightly increased its activity (Fig. 4). Moreover, incubation with recombinant 14-3-3 decreased PDK1 autophosphorylation activity (Fig. 5A) and kinase activity to phosphorylate SGK (Fig. 5B) in a dose-dependent manner in vitro. These results indicate that 14-3-3 is not only a PDK1-associating protein but also a protein that negatively regulates PDK1 kinase activity.

Several reports indicate that PDK1 binds to phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate through its pleckstrin homology domain (19, 30). Because mutants of PDK1 deleted in its pleckstrin homology domain have been reported to prevent translocation of Akt, the relocalization of PDK1 from cytosol to the plasma membrane is suggested to play an important role in the recruitment of Akt to the plasma membrane and the subsequent Akt activation in stimulated cells (30). PDK1 catalytic activity is not required for the PDK1 translocation to the plasma membrane (30). Although mutating PDK1 to increase its binding to 14-3-3 (V243P) reduced kinase activity to phosphorylate Akt at Thr-308, the mutation did not affect the binding affinity of PDK1 to Akt (Fig. 4). Therefore, the decrease in kinase activity of V243P-PDK1 to phosphorylate Akt in cells may be caused by down-regulation of its kinase activity but not by suppressing translocation. This notion was supported by the fact that the amount of the phosphorylated form of Myr-Akt was reduced by transfection of PDK1 to increase its binding to 14-3-3 (30). Although mutating PDK1 to increase its binding to 14-3-3 (V243P) reduced kinase activity to phosphorylate Akt at Thr-308, the mutation did not affect the binding affinity of PDK1 to Akt (Fig. 4). Therefore, the decrease in kinase activity of V243P-PDK1 to phosphorylate Akt in cells may be caused by down-regulation of its kinase activity but not by suppressing translocation. These results strongly suggest that inhibition of PDK1 activity but not PDK1 translocation is the main mechanism of 14-3-3-mediated inactivation of PDK1-dependent signal transduction.

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