Substitution of the Autophosphorylation Site Thr\(^{516}\) with a Negatively Charged Residue Confers Constitutive Activity to Mouse 3-Phosphoinositol-dependent Protein Kinase-1 in Cells*

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3-Phosphoinositide-dependent protein kinase-1 (PDK-1) is a serine/threonine kinase that has been found to phosphorylate and activate several members of the AGC protein kinase family including protein kinase B (Akt), p70 S6 kinase, and protein kinase C.\(\ddagger,\) However, the mechanism(s) by which PDK-1 is regulated remains unclear. Here we show that mouse PDK-1 (mPDK-1) undergoes autophosphorylation in vitro on both serine and threonine residues. In addition, we have identified Ser\(^{399}\) and Thr\(^{516}\) as the major mPDK-1 autophosphorylation sites in vitro. Furthermore, we have found that these two residues, as well as Ser\(^{244}\) in the activation loop, are phosphorylated in cells and demonstrated that Ser\(^{244}\) is a major in vivo phosphorylation site. Abolishment of phosphorylation at Ser\(^{244}\), but not at Ser\(^{399}\) or Thr\(^{516}\), led to a significant decrease of mPDK-1 autophosphorylation and kinase activity in vitro, indicating that autophosphorylation at Ser\(^{399}\) or Thr\(^{516}\) is not essential for mPDK-1 autokinase activity. However, overexpression of mPDK-1\(^{T516E}\), but not of mPDK-1\(^{S244E}\) or mPDK-1\(^{S399D}\), in Chinese hamster ovary and HEK293 cells was sufficient to induce Akt phosphorylation at Thr\(^{308}\) to a level similar to that of insulin stimulation. Furthermore, this increase in phosphorylation was independent of the Pleckstrin homology domain of Akt. Taken together, our results suggest that mPDK-1 undergoes autophosphorylation at multiple sites and that this phosphorylation may be essential for PDK-1 to interact with and phosphorylate its downstream substrates in vivo.

3-Phosphoinositide-dependent protein kinase-1 (PDK-1) is a recently identified protein kinase that functions downstream from PI 3-kinase and upstream of protein kinase B (Akt) in receptor tyrosine kinase signal transduction pathways (1). PDK-1 phosphorylates Thr\(^{308}\) in the activation loop of Akt and contributes to the activation of the enzyme (2, 3). In addition to Akt, PDK-1 also phosphorylates and activates several other downstream effectors of PI 3-kinase including p70 S6 kinase (4, 5), protein kinase C isoforms (6–9), serum and glucocorticoid-inducible kinase (10–12), and protein kinase C-related kinases 1 and 2 (13, 14). These kinases play versatile roles in numerous cellular events; thus, the activation state of PDK-1 toward these substrates may play an important physiological role in the regulation of a variety of biological activities including cell proliferation, differentiation, and apoptosis.

Although the activation of several PDK-1 substrates has been characterized, the mechanism by which PDK-1 activity is regulated in cells is largely unknown. Phosphorylation of protein kinase C\(\ddagger\) and protein kinase C\(\ddagger\) by PDK-1 in vivo has been shown to be inhibited by the PI 3-kinase inhibitor LY294002 (6), suggesting that PDK-1 activity is mediated by a PI3-kinase-dependent mechanism. Consistent with this finding, it has recently been found that insulin stimulates the activity of PDK-1 toward Akt and that this activation is blocked by wortmannin, another inhibitor of PI 3-kinase (15). However, others have found PDK-1 to be constitutively activated (4, 8), and PDK-1-mediated phosphorylation of p70 S6 kinase in cells has been shown to be unaffected by wortmannin treatment (5).

Although activation of PI 3-kinase may be important for the activity of PDK-1 toward some of its downstream substrates, differential phosphorylation of PDK-1 itself may also play an important role in regulating the kinase activity of the enzyme. A recent study identified Ser\(^{244}\), Ser\(^{399}\), Ser\(^{244}\), and Ser\(^{516}\) on human PDK-1 (hPDK-1) as phosphorylated sites in cells (17). Of these sites, phosphorylation at Ser\(^{399}\) was found to be essential for hPDK-1 activity in vitro (17). However, the functional role(s) of PDK-1 phosphorylation at the other sites is unknown.

Recently, we found that purified mPDK-1 underwent significant autophosphorylation in vitro (8, 18). However, the identity of these sites and the extent to which phosphorylation of these residues played a role in PDK-1 function remained unclear. In the present study, we report the identification of two major in vitro autophosphorylation sites on mPDK-1: Thr\(^{516}\) and Ser\(^{399}\). Interestingly, we found that Ser\(^{244}\) in the activation loop of mPDK-1 is only marginally phosphorylated in vitro. On the other hand, we demonstrate that Ser\(^{244}\), Ser\(^{399}\), and Thr\(^{516}\) of mPDK-1 are phosphorylated in cells and that Ser\(^{244}\) is a major in vivo phosphorylation site. Overexpression of mPDK-1\(^{T516E}\), but not mPDK-1\(^{S244E}\), mPDK-1\(^{S399D}\), or mPDK-1\(^{T516A}\), resulted in Akt Thr\(^{308}\) phosphorylation to a level similar to that of insulin stimulation. Furthermore, this increase in phosphorylation is independent of the PH domain of Akt. Taken together, our results suggest that phosphorylation of PDK-1 at multiple sites plays distinct and important roles in the regulation...
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MATERIALS AND METHODS

Buffers—Buffer A consisted of 50 mM Hepes, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100. Buffer B consisted of 50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM NaF, 1 mM sodium pyrophosphate, 1 mM Na2HPO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Buffer C consisted of 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM Na3VO4, 1 mM sodium pyrophosphate, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride.

Reagents—cDNAs encoding Myc-tagged full-length mPDK-1, mPDK-1Δ360, Myc-tagged Akt-1, and FLAG-tagged full-length and Akt-1Δ360 were described previously (18). Peptide phosphorylation by PDK-1 was determined by autoradiography of Western blot analysis. The proteins in cell lysates were immunoprecipitated by incubation with the primary antibody conjugated to protein G-Sepharose beads for 6–18 h at 4 °C. The immunoprecipitates were washed three times with ice-cold Buffer A. For immunoblot analysis, the cell lysates or immunoprecipitates were separated by SDS-PAGE using 10 or 15% polyacrylamide gels. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes. The bound proteins were detected by incubating with primary antibody, followed by horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies (Promega, Madison, WI).

In Vitro Phosphorylation Assays—CHO/IR cells transiently expressing Myc-tagged wild type or mutant PDK-1 were washed once with phosphate-buffered saline followed by serum starvation for 1 h at 37 °C. The cells were lysed in Buffer B, and the proteins were immunoprecipitated using PDK-1 antibody to the tag as described above. The bound proteins were washed twice with ice-cold Buffer A and once with Buffer C. Phosphorylation was initiated with the addition of 30 μl of Buffer C plus 2 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) and incubated for 20 min at 30 °C. The bound proteins were then washed by heating at 95 °C for 10 min in SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE, blotted to nitrocellulose or polyvinylidene difluoride membrane, and utilized as described above. Phosphopeptide and Phosphoamino Acid Analysis—In vitro or metabolically [32P]-labeled proteins were separated by SDS-PAGE, transferred to nitrocellulose (phosphopeptide mapping) or polyvinylidene difluoride (phosphoamino acid analysis) membranes, and excised as described above. The bound protein was blocked with 0.5% polyvinylpyrrolidone-360 (Sigma) in 100 mM acetic acid for 30 min at 37 °C. The protein was washed twice with double distilled H2O, washed once with 50 mM ammonium bicarbonate, and subjected to trypsin digest for 18 h at 37 °C. The digestes were collected and lyophilized, and the samples were desalted using C18 ZipTips (Millipore, Bedford, MA) according to a modified protocol by the manufacturer. The samples were loaded on TLC cellulose plates and subjected to electrophoresis and liquid chromatography as described previously (20). Phosphopeptides were visualized by autoradiography using x-ray film. Phosphoamino acid analysis was carried out using a protocol described previously (21).

RESULTS

mPDK-1 Undergoes Autophosphorylation on Both Serine and Threonine Residues in Vitro—We (8, 18) and others (22) have previously demonstrated that PDK-1 undergoes significant autophosphorylation in vitro. In an attempt to identify PDK-1 autophosphorylation sites, we carried out phosphoamino acid analysis and two-dimensional phosphopeptide mapping studies on mPDK-1 autophosphorylated wild type mPDK-1. Our results showed that mPDK-1 autophosphorylated in vitro on both serine and threonine residues at approximately a 1:1 ratio (Fig. 1A, left panel). No in vitro phosphorylation was observed for a kinase-inactive mutant of mPDK-1 (mPDK-1K1346E) (data not shown). Phosphopeptide analysis of wild type mPDK-1 revealed three major (Fig. 1A, right panel, 1–3) and nine minor (Fig. 1A, right panel, a–i) phosphopeptides, suggesting that PDK-1 autophosphorylates multiple sites in vitro. Phosphoamino acid analysis of phosphopeptides 1–3 revealed that phosphopeptides 1 and 2 contained exclusively phosphothreonine, whereas phosphopeptide 3 contained only phosphoserine (data not shown).

We have recently shown that Ser244 of mPDK-1 is important for PDK-1 function in cells (15). To determine whether Ser244 is an in vitro autophosphorylation site, we examined the phosphorylation of wild type and Ser244 mutants of mPDK-1 (Fig. 1B). We found that wild type mPDK-1 underwent significant autophosphorylation in vitro. Replacing Ser244 of mPDK-1 with alanine resulted in a dramatic decrease in the in vitro phosphorylation of the protein. This decrease in phosphorylation was partially recovered by replacing Ser244 with a negatively charged glutamate. Interestingly, substitution of Ser244 with a phosphorylatable threonine residue resulted in an overall increase in autophosphorylation compared with wild type. Replacing Ser244 of mPDK-1 with alanine also led to a significant decrease of the in vitro kinase activity of the enzyme, which was partially recovered by substitution of this residue with either glutamate or threonine (Fig. 1C). These findings further support Ser244 as an autophosphorylation site and demonstrate that phosphorylation at this site is important for mPDK-1 autokinase activity.

To further characterize the role of Ser244 in mPDK-1 in vitro autophosphorylation, we performed phosphoamino acid analysis and two-dimensional phosphopeptide mapping studies on mPDK-1 autophosphorylated mPDK-1S244A. Our results showed that although overall autophosphorylation of the mutant PDK-1 was significantly decreased compared with wild type (Fig. 1B), there was no significant change in the overall serine/threonine ratio (Fig. 1, D, left panel versus A, left panel), suggesting that Ser244 is not a major autophosphorylation site in vitro. Consistent with this, two-dimensional phosphopeptide mapping studies revealed that despite the significant decrease in overall phosphorylation of this mutant (Fig. 1B), mutating...
Ser244 to alanine did not abolish the in vitro autophosphorylation of the three major peptides (peptides 1–3) (Fig. 1, D, right panel versus A, right panel). On the other hand, replacing Ser244 of mPDK-1 with threonine led to a notable increase in threonine:serine autophosphorylation compared with the wild type enzyme (Fig. 1, E, left panel versus A, left panel). Two-dimensional phosphopeptide mapping studies of the mPDK-1S244T mutant showed a significant increase in autophosphorylation of phosphopeptide e compared with wild type protein (Fig. 1, E, right panel versus A, right panel). Phosphoamino acid analysis of phosphopeptide e revealed that this peptide contained only phosphoserine for the wild type and exclusively phosphothreonine for the S244T mutant of mPDK-1 (data not shown). Taken together, these results identified phosphopeptide e of wild type mPDK-1 as a Ser 244-containing peptide.

Identification of Ser399 as a Major Site of mPDK-1 Autophosphorylation in Vitro—Because Ser244 of mPDK-1 was found to be only marginally phosphorylated in vitro, we attempted to identify the major mPDK-1 in vitro autophosphorylation sites. At least five serine residues in hPDK-1, including Ser25, Ser241, Ser393, Ser396, and Ser410 were found phosphorylated in cells (17). To test whether the corresponding sites in mPDK-1 are phosphorylated in vitro, we generated mutants of mPDK-1 in which the corresponding sites, Ser25, Ser244, Ser396, Ser399, and Ser413, were replaced with either alanine or glycine. Mutant mPDK-1 proteins were expressed in CHO/IR cells, purified by immunoprecipitation, and autophosphorylated in vitro in the presence of [γ-32P]ATP. In vitro autophosphorylated mPDK-1 was analyzed by phosphoamino acid analysis (PAA, left panel) or by two-dimensional mapping studies (2D, right panel). Phosphoamino acids and phosphopeptides were visualized by autoradiography. B and C, effect of Ser399 phosphorylation on mPDK-1 autophosphorylation (B) and kinase activity (C) in vitro. Myc-tagged wild type (WT) or Ser399 mutants of mPDK-1 were immunoprecipitated from serum-starved CHO/IR cells transiently expressing these proteins. Bound mPDK-1 proteins were autophosphorylated in vitro in the presence of [γ-32P]ATP (B) or assayed for kinase activity (C) as described under “Materials and Methods.” mPDK-1 autophosphorylation was examined by autoradiography (Autorad, B, upper panel), and relative protein amounts were determined by Western blot (WB) analysis using antibody to the Myc tag (B, lower panel). D and E, phosphoamino acid analysis and two-dimensional mapping of autophosphorylated mPDK-1S244A (D) or mPDK-1S244T (E). Experiments were carried out as described in A. Phosphorylation of mPDK-1 Ser244 mutants was visualized by autoradiography. All of the results are representative of at least four experiments with similar results.
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the presence of \( [\gamma-^{32}P]ATP \). Phosphoamino acid analysis of the in vitro phosphorylated mPDK-1 mutants revealed that mutations at Ser\(^{252}\), Ser\(^{244}\), Ser\(^{399}\), and Ser\(^{471}\) had no significant effect on mPDK-1 autophosphorylation (data not shown). On the other hand, replacing Ser\(^{399}\) with glycine (mPDK-1.S399G) significantly decreased phosphoserine content compared with that of wild type (Fig. 2, left panel versus Fig. 1A, left panel). Phosphopeptide mapping studies revealed that mutation at this site resulted in the loss of phosphopeptide 3 (Fig. 2, right panel), implicating Ser\(^{399}\) of mPDK-1 as a major in vitro autophosphorylation site.

Identification of Thr\(^{516}\) as a Major mPDK-1 in Vitro Autophosphorylation Site—In an attempt to localize potential threonine autophosphorylation site(s), we examined autophosphorylation of a mPDK-1 mutant lacking the carboxyl-terminal PH domain (mPDK-1.\(^{-PH}\), residues 1–459). Phosphoamino acid analysis revealed that deletion of the PH domain resulted in a complete abolishment of threonine autophosphorylation (Fig. 3A, left panel). Phosphopeptide mapping studies of mPDK-1.\(^{-PH}\) revealed a loss of both phosphopeptides 1 and 2 (Fig. 3A, right panel), suggesting that the major mPDK-1 threonine autophosphorylation site(s) might be localized in the PH domain.

Sequence analysis of mPDK-1 revealed the presence of four threonine residues in the PH domain: Thr\(^{482}\), Thr\(^{516}\), Thr\(^{521}\), and Thr\(^{525}\) (Fig. 3B). To identify potential mPDK-1 threonine autophosphorylation sites, we mutated these threonine residues individually to alanine. The mPDK-1 threonine mutants were then expressed in CHO/IR cells, purified by immunoprecipitation, and autophosphorylated in vitro in the presence of \([\gamma-^{32}P]ATP\). We found that mutations at Thr\(^{482}\), Thr\(^{521}\), and Thr\(^{525}\) had no significant effect on mPDK-1 autophosphorylation in vitro (data not shown). On the other hand, replacing Thr\(^{516}\) with alanine significantly decreased phosphothreonine content compared with that of the wild type enzyme (Fig. 3C, left panel versus Fig. 1A, left panel). Interestingly, mutating Thr\(^{516}\) to alanine resulted in the loss of both phosphothreonine-containing peptides 1 and 2 in the two-dimensional phosphopeptide map of this mutant (Fig. 3C, right panel). One possible explanation for this observation may be that two phosphopeptides were generated because of incomplete digestion of the Thr\(^{516}\)-containing tryptic peptide. Consistent with this idea, Thr\(^{516}\) is immediately flanked on the amino terminus by a lysine residue (Lys\(^{515}\)). Phosphorylation at Thr\(^{516}\) could potentially impair the ability of trypsin to cleave the peptide bond between Lys\(^{515}\) and Thr\(^{516}\), resulting in two Thr\(^{516}\)-containing phosphopeptides. To confirm this, we mutated Thr\(^{516}\) of mPDK-1 to serine. mPDK-1.T516S was expressed in CHO/IR cells, immunoprecipitated, autophosphorylated in vitro, and subjected to two-dimensional phosphopeptide mapping analysis. Phosphopeptide mapping showed a significant decrease in phosphorylation at phosphopeptides 1 and 2. Subsequent phosphoamino acid analysis revealed that both phosphopeptides 1 and 2 contained exclusively phosphoserine (data not shown). These results provide further evidence that phosphopeptides 1 and 2 are due to incomplete tryptic digestion of the Thr\(^{516}\)-containing peptide.

Ser\(^{244}\), Ser\(^{399}\), and Thr\(^{516}\) of mPDK-1 Are Phosphorylated in Cells—To determine whether Ser\(^{244}\), Ser\(^{399}\), and Thr\(^{516}\) are phosphorylated in cells, phosphopeptide mapping studies were performed on wild type and point-mutated mPDK-1 overexpressed and metabolically labeled in CHO/IR cells. Two-dimensional phosphopeptide mapping of the metabolically labeled wild type mPDK-1 revealed the presence of phosphopeptides 1–3 (Fig. 4, panel a), indicating that both Ser\(^{399}\) and Thr\(^{516}\) are phosphorylated in intact cells. Consistent with the results from in vitro autophosphorylation and two-dimensional phosphopeptide mapping studies, mutation of Thr\(^{516}\) to alanine resulted in a loss of both phosphopeptides 1 and 2 for the metabolically labeled mPDK-1 (Fig. 4, panel b). Similarly, mutating Ser\(^{399}\) to glycine resulted in the loss of phosphopeptide 3 (Fig. 4, panel c). Our results also showed phosphopeptide e to be
heavily phosphorylated in vivo, indicating that Ser244 is one of the major phosphorylation sites in cells (Fig. 4). Phosphoamino acid analysis of phosphopeptide recovered from the maps of metabolically labeled wild type or the S244T mutant of mPDK-1 indicated that this peptide contained only phosphoserine or phosphothreonine, respectively (data not shown). Interestingly, phosphoamino acid analysis of phosphopeptides and i recovered from the maps of metabolically labeled wild type or mPDK-1-S244T indicated that these two peptides also contained only phosphoserine or phosphothreonine, respectively (data not shown). These findings implicate Ser244 as a major phosphorylation site of mPDK-1 in cells and suggest that phosphopeptides e, f, and i are most likely due to incomplete digestion of Ser244-containing peptide. Interestingly, we found that in vivo phosphorylation pattern of kinase-inactive PDK-1-K144E is similar to that of the wild type enzyme, suggesting that PDK-1 may undergo transphosphorylation in vivo.2

Mutation at Ser244 and Thr316 of mPDK-1 Affects Phosphorylation of mPDK-1 in CHO/IR Cells—We have recently found that insulin stimulates mPDK-1 phosphorylation in NIH3T3 cells and rat adipocytes (15). To test whether a mutation at Ser399 or Thr316 affects the overall phosphorylation of mPDK-1 in vivo, we examined basal and insulin-stimulated phosphorylation of wild type and mPDK-1 phosphorylation site mutants in CHO/IR cells. Consistent with our recent findings (15), insulin treatment resulted in a significant increase in mPDK-1 phosphorylation (Fig. 5A, lane 2 versus lane 1). Interestingly, we found that despite an overall decrease in phosphorylation, the mPDK-1-S244A mutant was still phosphorylated in cells, and the phosphorylation was enhanced in response to insulin stimulation (Fig. 5A, lanes 3 and 4 versus lanes 1 and 2). These findings suggest that insulin stimulates the phosphorylation at a site(s) in addition to or other than Ser244 in CHO/IR cells. Although mutating Ser399 to glycine or aspartate had no significant effect on mPDK-1 phosphorylation in vivo (data not shown), replacing Thr316 of mPDK-1 with glutamate (Fig. 5A, lane 7 versus lane 2), but not alanine (Fig. 5A, lanes 5 versus lane 2), resulted in an increase in the basal phosphorylation of mPDK-1 to the same level as that of the insulin-stimulated wild type protein. Furthermore, this phosphorylation was not further increased following insulin treatment (Fig. 5A, lane 8 versus lane 7). Interestingly, the recently identified constitutively active mutant of mPDK-1, mPDK-1-A280V (18), also showed an enhanced and insulin-independent increase in basal phosphorylation (Fig. 5A, lanes 9 and 10 versus lanes 1 and 2). These findings suggest that phosphorylation of Thr316 may result in a similar conformational change induced by the alanine to valine mutation at position 280, which subsequently leads to enhanced phosphorylation of mPDK-1.

Mutating Thr316 of mPDK-1 to Glutamate Constitutively Activates mPDK-1 in Cells—To determine whether autophosphorylation plays a role in the function of mPDK-1, we examined the kinase activity of mPDK-1, both in vitro and in cells. In vitro, we found that mutation of Ser399 of mPDK-1 to glycine or aspartate, or Thr316 to alanine or glutamate, had no significant effect of mPDK-1 kinase activity (data not shown). In addition to or other than Ser244 in CHO/IR cells. Although co-expression of mPDK-1-S244E or mPDK-1-S399D mutants had no effect on Akt phosphorylation at Thr308 under basal conditions (Fig. 5B, lanes 2 and 3 versus lane 1), co-expression of mPDK-1-T516E resulted in a significant increase in Akt phosphorylation to a level similar to that induced by insulin stimulation (Fig. 5B, lane 5 versus lane 7). Under this condition, only a small increase in Akt phosphorylation at Thr308 was observed in cells co-expressing mPDK-1-T516E (Fig. 5B, lane 4 versus lane 1), suggesting that a negatively charged residue at this site plays a critical role in promoting PDK-1 to phosphorylate Akt in cells. Furthermore, insulin stimulation did not increase the elevated Akt Thr308 phosphorylation in cells co-expressing mPDK-1-T516E (Fig. 5B, lane 11 versus lane 5). Similar results were obtained for mPDK-1-T516E co-expressed with Akt in CHO and HEK-293 cells (data not shown).
In agreement with our recent study (18), co-expression of mPDK-1\(^{A280V}\) with Akt also resulted in a similar increase in basal Akt phosphorylation at Thr\(^{308}\) (Fig. 5B, lane 6).

Previous studies have suggested that translocation of Akt to the plasma membrane is required for full phosphorylation and activation of the enzyme (23, 24). Studies have also shown that this translocation requires an intact PH domain (25). To better understand the mechanism by which autophosphorylation of PDK-1 regulates its activation of downstream substrates, we co-expressed FLAG-tagged wild type Akt-1 or the PH domain deletion mutant (Akt-1\(^{APH}\)) with either wild type or mutant mPDK-1 in CHO/IR cells. Insulin stimulation (Fig. 5C, lane 2 versus lane 1) or co-expression of either mPDK-1\(^{T516E}\) (Fig. 5C, lane 3 versus lane 1) or mPDK-1\(^{A280V}\) (Fig. 5C, lane 4 versus lane 1) resulted in significant Akt phosphorylation at Thr\(^{308}\). The PH domain-deleted mutant of Akt was not phosphorylated on Thr\(^{308}\) in response to insulin stimulation (Fig. 5C, lane 6). However, co-expression of PDK-1\(^{T516E}\) was sufficient to restore Akt-1\(^{APH}\) phosphorylation at Thr\(^{308}\) to the same level as that of insulin-stimulated, full-length Akt (Fig. 5C, lane 7 versus lane 2). It is of interest to note that the insulin-independent and Akt PH domain-independent phosphorylation of Akt at Thr\(^{308}\) can be mediated by both PDK-1\(^{T516E}\) and PDK-1\(^{A280V}\) mutants (Fig. 5, B, lanes 5 and 6 and C, lanes 7 and 8). These data suggest that substituting Val\(^{280}\) with alanine or Thr\(^{281}\) with glutamate may lead to a similar conformational change of mPDK-1, which is essential for interaction and phosphorylation of downstream substrates such as Akt in cells.

**DISCUSSION**

The mechanism(s) by which PDK-1 activity is regulated has remained, for a large part, unclear. Subcellular localization and substrate conformation have been hypothesized as the primary means for regulating PDK-1 activity enzyme (22, 26, 27); however, some studies have indicated that phosphorylation of PDK-1 may also play a role (8, 15). PDK-1 is phosphorylated both in vitro and in cells (2, 17), and treatment of PDK-1-expressing cells with insulin or hydrogen peroxide has been shown to increase phosphorylation of PDK-1 on specific residues (15, 28). Several studies have focused on Ser\(^{244}\) (mPDK-1) or Ser\(^{241}\) (hPDK-1) in the activation loop as a critical phosphorylation site, because phosphorylation upon this residue is essential for PDK-1 kinase activity (15, 17). Some studies have indicated that phosphorylation at Ser\(^{241}\) is not regulated by growth factor stimulation (17). On the other hand, we have found that in certain cell types, mPDK-1 phosphorylation and activity increase following insulin stimulation in a Ser\(^{244}\)-dependent manner (15). However, in CHO/IR cells, we found that the mPDK-1\(^{S244A}\) mutant still underwent insulin-stimulated phosphorylation (Fig. 5A). These findings suggest that insulin-stimulated phosphorylation of mPDK-1 occurs at sites other than or in addition to Ser\(^{244}\), and this phosphorylation may function in conjunction with Ser\(^{244}\) to regulate mPDK-1 activity in cells.

In the present study, we identify Ser\(^{249}\) and Thr\(^{256}\) on mPDK-1 as two major in vitro autophosphorylation sites, which can also be phosphorylated in cells. This finding is in agreement with a recent study by Park et al. (26), who showed that the Thr\(^{256}\)-corresponding site in hPDK-1 to be phosphorylated in vitro. However, our results also show that phosphorylation at these sites may play different roles in PDK-1 function in cells. Indeed, substitution of Thr\(^{256}\) with glutamate increases basal PDK-1 phosphorylation in cells to that seen with insulin stimulation, whereas replacing Ser\(^{249}\) with aspartate does not significantly affect either basal or insulin-stimulated phosphorylation of the enzyme. In addition, overexpression of mPDK-1\(^{T516E}\), but not mPDK-1\(^{S249D}\), led to Akt phosphorylation at Thr\(^{308}\) to the same level as that induced by insulin treatment. Taken together, these findings suggest that autophosphorylation at Thr\(^{308}\), but not Ser\(^{249}\), regulates PDK-1 phosphorylation upon additional residues in cells and subsequently functions to regulate the activity of PDK-1 toward its substrates.

Previous studies have shown that hPDK-1 isolated from cells is constitutively active in vitro, presumably as a result of autophosphorylation upon Ser\(^{244}\) (4, 8, 22). In agreement with this, we have found that a mutation of the corresponding Ser\(^{244}\) on mPDK-1 renders the kinase inactive in vitro (Fig. 1C) and in cells (Ref. 15 and data not shown). Interestingly, although we found the kinase activity of mPDK-1 to be significantly reduced by a mutation at Ser\(^{244}\), we found the ratio of serine/threonine autophosphorylation unchanged in its absence. This suggests that Ser\(^{244}\) is not a major site for in vitro autophosphorylation. Low autophosphorylation at Ser\(^{244}\) may result from phosphorylation at this site in cells that remains stable in vitro. In support of this, phosphorylation at Ser\(^{244}\) is less stable if the residue is replaced with threonine, resulting in an increase in autophosphorylation at this site in vitro (Fig. 1B). Although Ser\(^{244}\) is a minor phosphorylation site, phosphopeptide maps of metabolically labeled wild type and S244T mutant of mPDK-1 indicated that Ser\(^{244}\) is highly phosphorylated in CHO/IR cells, which is in agreement with our recent studies showing that Ser\(^{244}\) is a major phosphorylation site in NIH-3T3 cells (15). In contrast, autophosphorylation upon the major in vitro sites, Ser\(^{249}\) and Thr\(^{256}\), is less stable in cells, which indicates that phosphorylation upon these residues is reversible and may be tightly regulated in cells.

Insulin stimulation has been shown to increase PDK-1 phosphorylation in both NIH-3T3 and rat adipose cells (15). Consistent with this, we observed insulin-stimulated phosphorylation of mPDK-1 when overexpressed in CHO/IR cells (Fig. 5A); however, we found that this insulin-stimulated increase could occur in the absence of Ser\(^{244}\) phosphorylation. Mutations at Ser\(^{249}\) and Thr\(^{256}\) did not significantly affect insulin-stimulated mPDK-1 phosphorylation (Fig. 5A and data not shown), suggesting the presence of other insulin-stimulated phosphorylation site(s). On the other hand, replacement of Thr\(^{256}\) with a negatively charged glutamate residue resulted in a significant increase in basal phosphorylation. The increase in basal mPDK-1\(^{T516E}\) phosphorylation also correlates with an increase in mPDK-1\(^{T516E}\)-mediated phosphorylation of Akt at Thr\(^{308}\) (Fig. 5B), which is in agreement with our recent finding that insulin-stimulated phosphorylation of mPDK-1 correlates with an increase in activity (15).

It has been previously shown that growth factor-mediated phosphorylation and activation of Akt requires a translocation event via an intact PH domain (23–25, 29). Recently, we (18) and others (22, 30) have shown that phosphorylation at Thr\(^{308}\) of Akt by PDK-1 is facilitated by an intact PDK-1-PH domain, whereas others have suggested that translocation of PDK-1 to the membrane may play a role in the phosphorylation and activation of the enzyme (31). In the present study, we have found that mutating Thr\(^{256}\) to glutamate allows PDK-1 to phosphorylate Thr\(^{308}\) not only in a growth factor-independent fashion but also without the requirement for the PH domain of Akt (Fig. 5C). These data suggest that the T516E mutation of mPDK-1 bypasses the normal requirement for translocation and allows it to interact with and subsequently phosphorylate Akt at Thr\(^{308}\). One possible explanation for this observation may be that the introduction of a negatively charged residue at Thr\(^{256}\) results in a conformational change in mPDK-1, which normally occurs after growth factor-mediated translocation of the enzyme to the plasma membrane. This resulting conformation may result in an increase in autophosphorylation at Ser\(^{244}\).
and a subsequent increase in mPDK-1 activity. Consistent with this, a mPDK-1 \(^{S244A/T516E}\) mutant was unable to increase phosphorylation of mPDK-1 or Thr\(^{308}\) of Akt in cells (data not shown), suggesting that phosphorylation at Thr\(^{516}\) modifies activity of mPDK-1 through a Ser\(^{244}\) phosphorylation-dependent mechanism.

In Caenorhabditis elegans, a natural point mutation (A277V) allows the mammalian PDK-1 homolog to bypass the need for PI 3-kinase for its activation (16). We recently reported that a corresponding mutation in mPDK1 (A280V) resulted in an increase in the \textit{in vitro} autophosphorylation of mPDK-1 and constitutive activation of the kinase toward Akt in cells (18). Whereas this mutation has not been found to occur naturally in higher species, it is interesting to note the similarity between mPDK-1\(^{A280V}\) and mPDK-1\(^{T516E}\). We found that although neither had any significant effect on mPDK-1 activity \textit{in vitro} (data not shown), both elevated mPDK-1 basal phosphorylation in cells (Fig. 5A). Furthermore, overexpression of either mPDK-1\(^{A280V}\) or mPDK-1\(^{T516E}\) was sufficient to elevate Akt phosphorylation at Thr\(^{308}\) in a growth factor- and Akt PH domain-independent manner (Fig. 5, B and Fig. C). These findings suggest that each mutation may result in a conformational change and/or cellular relocalization of mPDK1, which allows the enzyme to interact with and phosphorylate its downstream substrates such as Akt.

In conclusion, our results suggest that phosphorylation at Thr\(^{516}\) plays an important role in the regulation of mPDK-1 \textit{in vivo} activity toward its substrates. Although it remains to be established whether phosphorylation upon Thr\(^{516}\) is directly regulated by insulin stimulation, autophosphorylation at this site may specifically direct the extent of phosphorylation at other site(s) and subsequently the activity of mPDK-1 in response to insulin or other growth factors. Autophosphorylation at Thr\(^{516}\) may be specifically regulated in cells by various cellular events, such as conformational changes of the kinase following growth factor stimulation. Alternatively, Thr\(^{516}\) may be constitutively autophosphorylated in cells, and this phosphorylation may be tightly controlled by a serine/threonine phosphatase, allowing dynamic regulation of this pivotal kinase in response to growth factor stimulation. Further studies will be needed to test these possibilities.

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