Insulin Stimulates Increased Catalytic Activity of Phosphoinositide-Dependent Kinase-1 by a Phosphorylation-Dependent Mechanism

Hui Chen,[‡] Fredrik H. Nystrom,[‡] Lily Q. Dong,[§] Yunhua Li,[‡] Sunbin Song,[‡] Feng Liu,[§] and Michael J. Quon*,[‡]

Cardiology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, and Department of Pharmacology, The University of Texas Health Science Center, San Antonio, Texas 78284

Received April 12, 2001; Revised Manuscript Received July 3, 2001

ABSTRACT: Phosphoinositide-dependent kinase-1 (PDK-1) is a serine−threonine kinase downstream from PI 3-kinase that phosphorylates and activates other important kinases such as Akt that are essential for cell survival and metabolism. Previous reports have suggested that PDK-1 has constitutive catalytic activity that is not regulated by stimulation of cells with growth factors. We now show that insulin stimulation of NIH-3T3^{IR} cells or rat adipose cells may significantly increase the intrinsic catalytic activity of PDK-1. Insulin treatment of NIH-3T3^{IR} fibroblasts overexpressing PDK-1 increased both phosphorylation of recombinant PDK-1 in intact cells and PDK-1 kinase activity in an immune-complex kinase assay. Insulin stimulation of rat adipose cells also increased catalytic activity of endogenous PDK-1 immunoprecipitated from the cells. Both insulin-stimulated phosphorylation and activity of PDK-1 were inhibited by wortmannin and reversed by treatment with the phosphatase PP-2A. A mutant PDK-1 with a disrupted PH domain (W538L) did not undergo phosphorylation or demonstrate increased kinase activity in response to insulin stimulation. Similarly, a PDK-1 phosphorylation site point mutant (S244A) had no increase in kinase activity in response to insulin stimulation. Thus, the insulin-stimulated increase in PDK-1 catalytic activity may involve PI 3-kinase- and phosphorylation-dependent mechanisms. We conclude that the basal constitutive catalytic activity of PDK-1 in NIH-3T3^{IR} cells and rat adipose cells can be significantly increased upon insulin stimulation.

Phosphoinositide-dependent kinase-1 (PDK-1)[1] is a serine−threonine kinase immediately downstream from phosphatidylinositol 3-kinase (PI3K) that directly phosphorylates and activates PI3K effectors including Akt, PKC-ζ, p70 S6 kinase, p90-RSK, and SGK (for reviews see refs *1* and *2*). PI3K signaling pathways are required for mediating metabolic and vasodilator actions of insulin (*3*−*8*) and other crucial biological functions regulated by growth factors and cytokines (*9*). However, the mechanisms by which PDK-1 activates downstream effectors such as Akt are complex and not fully understood. Some studies suggest that PDK-1 is a constitutively active kinase localized to the plasma membrane upon binding of its PH domain to the PI3K product PI(3,4,5)P_3 (*10*, *11*). The PH domain of Akt also binds PI(3,4,5)P_3, resulting in colocalization of PDK-1 and Akt and unmasking of the regulatory Thr^{308} phosphorylation site in Akt (*11*−*13*). Similarly, while interaction of PI(3,4,5)P_3 with PDK-1 in vitro does not alter its ability to phosphorylate p70 S6 kinase or SGK, PI3K-dependent modification of these two downstream kinases makes them better substrates for PDK-1 (*14*−*16*). In contrast, increased catalytic activity of PDK-1 in the presence of PI(3,4,5)P_3 has been reported (*11*, *13*), and PI3K-dependent regulation of PDK-1 is important for its ability to phosphorylate PKC-ζ under some conditions (*17*). Moreover, Akt without a PH domain can still be activated in response to insulin (*18*). These latter studies suggest that products of PI3K may help to regulate PDK-1 activity. In addition, Prasad et al. recently reported that oxidative stress or vanadate treatment of 293T cells results in tyrosine phosphorylation and increased catalytic activity of PDK-1 (*19*). Nevertheless, although Ser^{241} in human PDK-1 has been identified as an autophosphorylation site that is required for PDK-1 activity, a number of investigators have failed to detect effects of insulin, IGF-1, or wortmannin treatment to modulate either phosphorylation or catalytic activity of PDK-1 (*12*, *15*, *20*−*22*). In the present study, we now demonstrate that insulin can stimulate increased catalytic activity of PDK-1 in a PI3K-dependent manner that may be linked to phosphorylation of PDK-1. Thus, in addition to recruitment of PDK-1 to signaling complexes and modification of PDK-1 substrates, PI3K-dependent insulin signaling pathways may be regulated by additional mechanisms that enhance the intrinsic catalytic activity of PDK-1.

MATERIALS AND METHODS

Plasmid Constructs. pBEX: An expression vector containing SRα promoter (*23*) was used as the parent vector for various PDK-1 constructs. PDK1-WT: Murine PDK-1 cDNA (except for sequence coding for the last four amino acids) was ligated into pBEX with either a hemagglutinin (HA) epitope tag (*24*) or a myc epitope tag in-frame with

* Address correspondence to this author at the Cardiology Branch, NHLBI, NIH, Building 10, Room 8C-218, Bethesda, MD 20892-1755. Tel: (301) 496-6269. Fax: (301) 402-1679. E-mail: quonm@nih.gov.

[‡] National Heart, Lung, and Blood Institute, National Institutes of Health.

[§] The University of Texas Health Science Center.

[1] Abbreviations: PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase.

10.1021/bi010743c CCC: $20.00
Published on Web 08/28/2001

the 3′ end. PDK1-K114A: Kinase-inactive PDK-1 (alanine substituted for lysine in the ATP binding site) was derived from PDK1-WT using MORPH kit (5′ to 3′, Inc., Boulder, CO) and mutagenic oligonucleotide 5′-CCA GAG AAT ATG CTA T**CG CG**A TTC TGG AGA AAC GTC-3′. PDK1-W538L: PH domain mutant of PDK-1 (leucine substituted for tryptophan at position 538) similar to a mutant human PDK-1 with disrupted binding to $PI(3,4,5)P_3$ (*11*) was derived from PDK1-WT using MORPH kit and mutagenic oligonucleotide 5′-GCT CAC AAG **C**T**T** TGC AGA AAG-3′. PDK1-S244A: A myc epitope-tagged point mutant of PDK1-WT with alanine substituted for serine at position 244 was constructed. Akt-WT: cDNA for mouse Akt-1 was ligated into pCIS2 expression vector (*4*). Akt-AA: A mutant Akt (substitution of alanine for threonine at position 308 and alanine for serine at position 473 to disrupt phosphorylation sites in the regulatory region) was created from Akt-WT as described (*25*). All mutant sequences were confirmed by direct sequencing. GLUT4-HA: pCIS2 expression vector containing the cDNA coding for human GLUT4 with the HA epitope tag inserted in the first exofacial loop was constructed as described (*26*).

In Vitro PDK-1 and Akt Kinase Assays. NIH-3T3 fibroblasts stably transfected with human insulin receptors (NIH-$3T3^{IR}$ cells) (*27*) were transiently transfected with pBEX or PDK-1 constructs (4 μg of DNA/dish) using Lipofectamaine Plus Reagent (Life Technology, Gaithersburg, MD). One day after transfection, cells were serum-starved overnight and then treated without or with insulin (100 nM, 3 min, 37 °C). In some cases, cells were treated with wortmannin (100 nM, 90 min) before insulin treatment. After insulin stimulation, cells were lysed with 800 μL of buffer A [50 mM Tris, pH 7.5, 10% glycerol, 1% NP-40, 10 mM EDTA, 300 mM NaCl, 20 mM NaF, 5 mM sodium pyrophosphate, 1 mM vanadate, 1 mM benzamidine, 1 mM okadaic acid, and complete protease inhibitor cocktail tablet (Boehringer Mannheim, Germany)] for 30 min at 4 °C, and lysates were centrifuged (6000*g*, 3 min, 4 °C) to pellet cellular debris. An aliquot of each lysate was immunoblotted to verify overexpression of PDK-1 constructs. Another aliquot (400 μg total protein) was immunoprecipitated by incubation with 4 μg of anti-HA monoclonal antibody (HA-11; Berkeley Antibody Co., Richmond, CA) for ~2 h at 4 °C followed by incubation with 25 μL of washed protein G-conjugated agarose beads (Pierce Chemical Co., Rockford, IL) for 2 h at 4 °C on a rotating wheel. Immune complexes were washed twice with 1 mL of buffer B (20 mM Tris, pH 7.4, 0.1% NP-40, 150 mM NaCl) and once with 1 mL of buffer C (20 mM Tris, pH 7.4, 150 mM NaCl) at 4 °C and then incubated with 0.5 μg of purified unactivated Akt1 protein (Upstate Biotechnology, Lake Placid, NY, catalog no. 14-279), 10 μCi of [γ-^{32}P]ATP, and 30 μL of kinase buffer (100 μM ATP, 50 mM Tris, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 2.5 μM PKI, 1 μM microcystin-LR, 10 mM $MgCl_2$, 100 μM phosphatidylserine, 100 μM phosphatidylcholine) for 45 min at 30 °C. The kinase reaction was stopped by briefly centrifuging to separate the immune complex from the substrate solution, immediately adding 6 μL of 6× Laemmli buffer to the supernatant, and boiling for 5 min. Samples were subjected to 10% SDS−PAGE, and phosphorylated Akt1 was detected by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In addition, immunoprecipitated PDK-1 was eluted from pelleted beads by boiling for 5 min in Laemmli buffer and immunoblotted with HA-11 or anti-Akt antibody. To determine endogenous PDK-1 activity in rat adipose cells, similar experiments were performed using a sheep polyclonal antibody against PDK-1 for immunoprecipitation (Upstate Biotechnology, catalog no. 06-637).

Aliquots of samples from experiments described above were subjected to the kinase assay without [γ-^{32}P]ATP. The supernatant containing Akt was then incubated with the peptide RPRAATF [60 μM, derived from the phosphorylation site on GSK-3 (Upstate Biotechnology)] in a total volume of 50 μL containing 50 μM ATP, 8 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP for 10 min at 30 °C with frequent mixing. The reaction was stopped by transferring to phosphocellulose columns (Pierce Chemical Co.) and spinning (10000*g*, 30 s). Columns were washed twice with 1% phosphoric acid and placed in 10 mL of scintillation fluid, and incorporated radioactivity was measured in a γ counter.

PDK-1 Phosphorylation. NIH-$3T3^{IR}$ cells transiently transfected with PDK-1 constructs were serum-starved overnight and incubated with KRB buffer (107 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Hepes, pH 7.4, 10 mM glucose, 0.1% BSA, 7 mM $NaHCO_3$) for 30 min without or with wortmannin (100 nM). Cells were then incubated for 3 h at 37 °C with [^{32}P]H_3PO_4 (final specific activity of 80 μCi/mL) and treated without or with insulin (100 nM, 5 min, 37 °C). Cell lysates were made as described except that some lysates were treated with 30 milliunits/mL protein phosphatase type 2A (PP-2A, Upstate Biotechnology) (20 min, 30 °C). PP-2A was inactivated by treating samples with 1 μM microcystin-LR for 10 min on ice. An aliquot from each group was immunoblotted with HA-11 while another aliquot was immunoprecipitated with HA-11 and subjected to SDS−PAGE followed by PhosphorImager scanning to assess phosphorylation of PDK-1. This gel was also immunoblotted with HA-11 to assess immunoprecipitation efficiency. In addition, similar experiments were performed with unlabeled KH_2PO_4 instead of [^{32}P]H_3PO_4, and HA-11 immunoprecipitates were used for in vitro PDK-1 kinase assays.

Transfection of Rat Adipose Cells and Assay for Cell Surface Epitope-Tagged GLUT4. Isolated adipose cells were prepared from rat epididymal fat pads (CD strain, Charles River Breeding Laboratories, Wilmington, MA) by collagenase digestion (*3*) and transfected by electroporation as described (*3*, *28*). For experiments assessing the effects of insulin on translocation of GLUT4, groups of cells were transfected with the empty expression vector pBEX alone (5 μg of DNA/cuvette) or cotransfected with GLUT4-HA (1 μg of DNA/cuvette) and either pBEX, PDK-1 constructs, or Akt constructs (4 μg of DNA/cuvette). For experiments where PDK-1 and Akt constructs were coexpressed with GLUT4-HA, 4 μg of DNA/cuvette for each construct plus 1 μg of DNA/cuvette GLUT4-HA was used. DNA for pBEX was added to various groups as needed to keep the total DNA concentration constant at 9 μg of DNA/cuvette. Twenty hours after electroporation, adipose cells were processed as described (*3*, *26*, *28*) and treated with insulin at concentrations ranging from 0 to 60 nM at 37 °C for 30 min. Cell surface epitope-tagged GLUT4 was determined by using the HA-11 antibody in conjunction with ^{125}I-labeled sheep anti-mouse IgG as described (*4*, *28*).

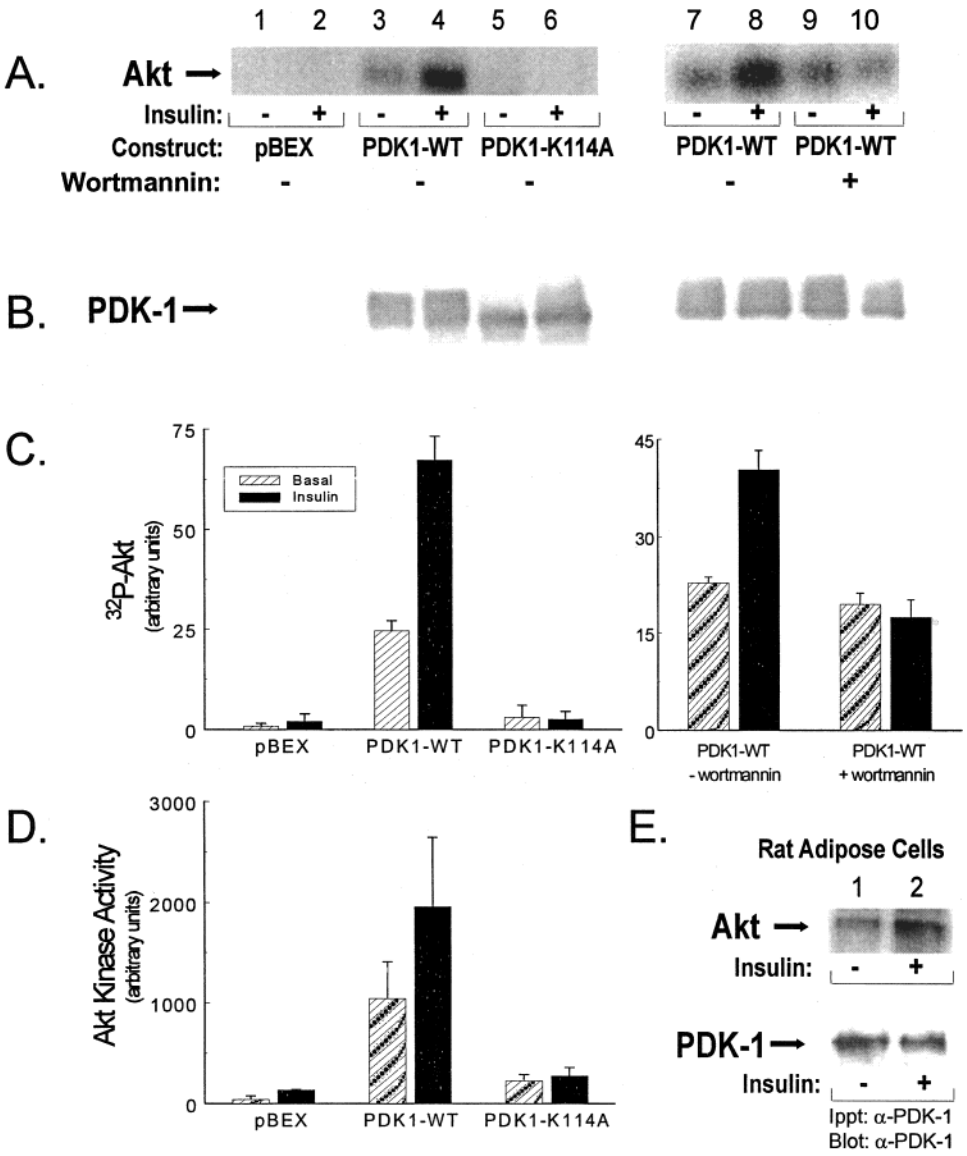

FIGURE 1: Insulin stimulates increased catalytic activity of PDK-1. (A) NIH-3T3^{IR} cells transfected with pBEX, PDK1-WT, or PDK1-K114A were treated without or with wortmannin and insulin as indicated (described in Materials and Methods), and recombinant PDK-1 was immunoprecipitated from cell lysates using an anti-HA antibody. A representative autoradiogram from the in vitro kinase assay using dephosphorylated Akt as the substrate in the presence of [γ-^{32}P]ATP is shown. (B) Anti-HA immunoblot of anti-HA immunoprecipitates demonstrating comparable recovery of recombinant PDK-1 constructs. (C) Mean ± SEM of PhosphorImager quantification of four independent experiments normalized for PDK-1 recovery (determined by scanning densitometry). (D) Akt was separated from the immune complex as described in Materials and Methods and incubated with the peptide RPRAATF in the presence of [γ-^{32}P]ATP to assess Akt activity. Radioactivity incorporated into the peptide substrate was quantified and normalized for the amount of Akt protein determined by immunoblotting (average results from two independent experiments are shown). (E) Insulin stimulates activation of endogenous PDK-1 in rat adipose cells. Lysates of cells treated without or with insulin (1 mg total protein) underwent immunoprecipitation with a sheep polyclonal antibody against PDK-1. The in vitro kinase assay was then performed, and immunoprecipitated samples were also immunoblotted with a rabbit polyclonal antibody against PDK-1. A representative experiment from three independent experiments is shown.

Immunodetection of Recombinant HA-Tagged PDK-1 and GLUT4. Whole cell homogenates and membrane (particulate) fractions were prepared from each group of transfected adipose cells after overnight culture as described (*28*). Aliquots from each group containing 50 μg of protein were subjected to SDS−PAGE on a 10% gel. For detection of PDK-1, samples were boiled prior to running on the gel; for detection of GLUT4-HA, samples were not boiled prior to running on the gel. The contents of the gel were transferred to nitrocellulose, and expression of recombinant proteins was determined by immunoblotting with HA-11 as described (*28*).

Statistical Analysis. Paired *t*-tests were used to compare individual points where appropriate. Multiple analysis of variance (MANOVA) was used to compare insulin dose−response experiments. *p* values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Insulin-Stimulated Activation of PDK-1. We performed "immune-complex" PDK-1 kinase assays in vitro using purified unactivated Akt as a substrate and epitope-tagged PDK-1 immunoprecipitated from lysates of transiently transfected NIH-3T3^{IR} cells (Figure 1A). As judged by incorporation of [γ-^{32}P]ATP into Akt, wild-type PDK-1 had basal kinase activity that was increased ~2.7-fold upon insulin stimulation of the cells (Figure 1A,C, lanes 3, 4, 7, and 8).

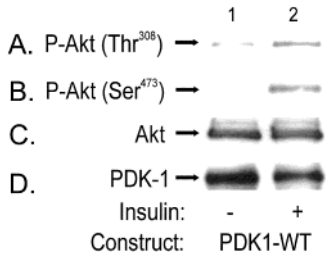

FIGURE 2: Phosphorylation of Akt at both Thr^{308} and Ser^{473} is observed in immune-complex PDK-1 kinase assays derived from insulin-stimulated cells. NIH-3T3^{IR} cells were transfected with PDK1-WT and treated without or with insulin, and in vitro kinase assays as described in Figure 1 were performed in the absence of [γ-^{32}P]ATP. Samples were then immunoblotted with phospho-specific antibodies against Akt phosphorylated at Thr^{308} (A), Ser^{473} (B) (New England Biolabs, Beverly, MA), or antibodies that recognize Akt independent of phosphorylation state (C) (Upstate Biotechnology Inc.). (D) Anti-HA immunoblot of anti-HA immunoprecipitates. Representative blots are shown from experiments that were repeated independently twice.

Although there was some variability in the magnitude of the response from experiment to experiment, insulin treatment consistently resulted in a significant increase in the kinase activity of wild-type PDK-1 (Figure 1C). By contrast, no phosphorylation of Akt by PDK1-K114A (kinase-inactive mutant) was observed (Figure 1A,C, lanes 5 and 6). Interestingly, the immunoblot of PDK1-K114A had a sharper band that ran slightly lower on the gel than wild-type PDK-1, consistent with the idea that wild-type PDK-1 may undergo autophosphorylation. In vitro kinase assays were also done using samples from cells pretreated with wortmannin (an inhibitor of PI3K). While wortmannin did not significantly affect basal PDK-1 activity, it completely blocked the ability of insulin to stimulate increased PDK-1 activity (Figure 1A,C, lanes 9 and 10). We next assessed kinase activity of the Akt substrate used in the PDK-1 kinase assay by incubating it with the peptide RPRAATF. As expected, the kinase activity of Akt toward this substrate correlated with the phosphorylation of Akt by PDK-1 (Figure 1D). We achieved transient transfection efficiencies of ~30% (assessed by expression of green fluorescent protein; data not shown). Thus, when endogenous PDK-1 levels were compared with recombinant PDK-1 expression by immunoblotting, we estimated ~12-fold overexpression of recombinant PDK-1 (data not shown). Since overexpressed PDK-1 may not behave like PDK-1 under physiological conditions, we also assessed activation of endogenous PDK-1 in freshly isolated untransfected rat adipose cells by immunoprecipitating with an anti-PDK-1 antibody rather than an antibody against an epitope tag. Reassuringly, insulin also stimulated ~2-fold activation of endogenous PDK-1 in a bona fide insulin target cell (Figure 1E). To rule out the possibility that endogenous Akt was co-immunoprecipitating with PDK-1 in our immune-complex kinase assays, we also subjected the PDK-1 immunoprecipitates to immunoblotting with anti-Akt antibody. Under our experimental conditions, we did not detect any co-immunoprecipitation of endogenous Akt with PDK-1 (data not shown). Thus, in intact cells, insulin stimulates increased PDK-1 kinase activity which then

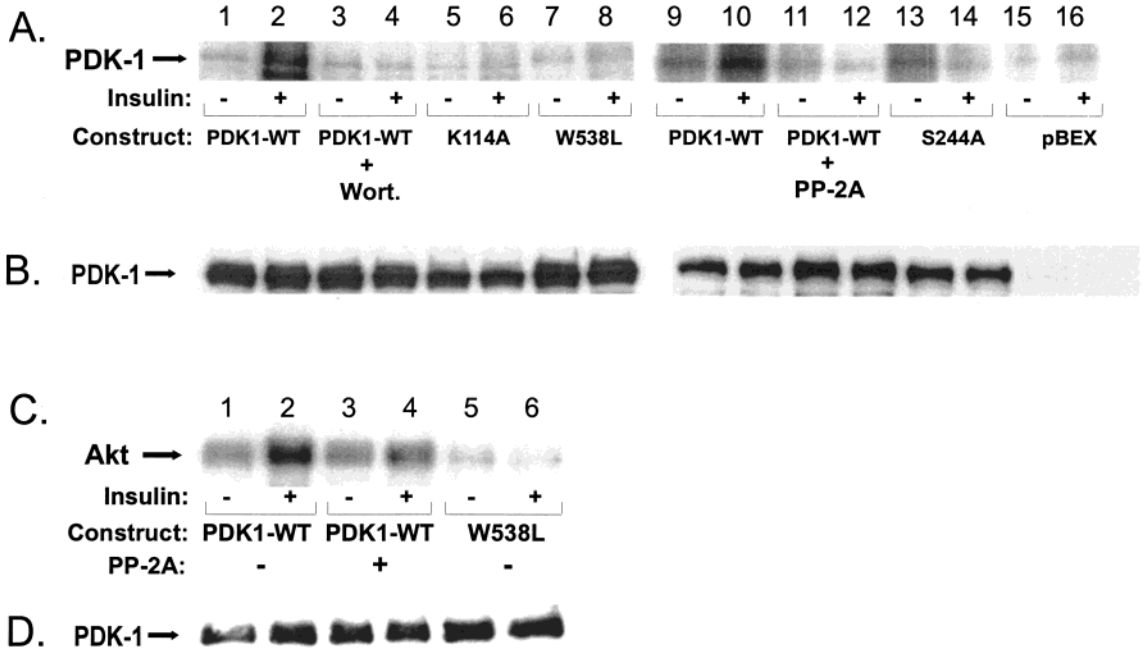

FIGURE 3: (A) Insulin-stimulated phosphorylation of PDK-1 in intact cells. NIH-3T3^{IR} cells were transfected with PDK-1 constructs, labeled with [^{32}P]H_3PO_4, and treated with insulin and wortmannin as indicated. HA-tagged constructs were used in experiments shown in lanes 1−8 while myc-tagged constructs were used in experiments shown in lanes 9−14. Cell lysates were immunoprecipitated with anti-HA or anti-myc antibodies to recover recombinant PDK-1 and subjected to SDS−PAGE, and incorporated radioactivity was quantified with a PhosphorImager. In some cases, cell lysates were treated with PP-2A (lanes 11 and 12). Representative results are shown for experiments that were repeated independently at least three times. (B) Anti-HA immunoblot of cell lysates (lanes 1−8) or anti-myc immunoblot of anti-myc immunoprecipitates (lanes 9−16) from experiments shown in panel A demonstrate comparable expression of PDK-1 constructs. (C) Experiments similar to those in panel A were repeated without [^{32}P]H_3PO_4, and in vitro kinase assays as described in Figure 1 were performed. In some cases, samples were treated with PP-2A prior to the kinase assay. Representative results are shown for experiments that were repeated independently at least five times. Autoradiogram from a representative in vitro kinase assay performed using unactivated Akt as the substrate in the presence of [γ-^{32}P]ATP. (D) Anti-HA immunoblot of anti-HA immunoprecipitates obtained from experiments shown in panel C demonstrate comparable recovery of PDK-1 constructs.

presumably leads to increased phosphorylation and activation of downstream kinases such as Akt.

To determine which of the known regulatory sites on Akt were phosphorylated by insulin-stimulated PDK-1, we repeated the immune-complex kinase assays described above in the absence of [γ-^{32}P]ATP and immunoblotted the purified Akt substrate with phospho-specific antibodies against Akt phosphorylated at Thr^{308} or Ser^{473} (Figure 2). Under basal conditions, when PDK1-WT was immunoprecipitated from untreated cells, we detected phosphorylation only at Thr^{308}. Interestingly, PDK1-WT recovered from insulin-treated cells mediated increased phosphorylation of Akt at Thr^{308} as well as phosphorylation of Ser^{473}.

Relationship between PDK-1 Phosphorylation and Kinase Activity. We next assessed the ability of insulin to stimulate phosphorylation of PDK-1 in vivo (by labeling cells with [^{32}P]H_3PO_4) and correlated this with PDK-1 activity. Insulin stimulation of transiently transfected NIH-3T3^{IR} cells resulted in increased in vivo phosphorylation of PDK1-WT, but not PDK1-K114A or PDK1-W538L [PH domain mutant that does not bind PI(3,4,5)P_3] (Figure 3A, lanes 1, 2, 5−8, 9, and 10). Pretreatment of cells with wortmannin blocked the ability of insulin to stimulate phosphorylation of PDK1-WT (Figure 3A, lanes 3 and 4). Taken together, our data suggest that insulin stimulation results in autophosphorylation of PDK-1 (or phosphorylation of PDK-1 by other downstream kinases) in a manner that depends on PI(3,4,5)P_3 binding to the PH domain of PDK-1. Treatment of cell lysates with the serine-specific phosphatase PP-2A reversed insulin-stimulated phosphorylation of PDK1-WT (Figure 3A, lanes 11 and 12) and also inhibited the insulin-stimulated increase in kinase activity of PDK1-WT (Figure 3C, lanes 3 and 4). Furthermore, the basal activity of PDK1-W538L was unaffected by treating cells with insulin (Figure 3C, lanes 5 and 6). These data are consistent with the hypothesis that binding of lipid products of PI3K such as PI(3,4,5)P_3 to the PH domain of PDK-1 is important for its increased activation in response to insulin.

Recent evidence suggests that Ser^{244} (equivalent to Ser^{241} in human PDK-1) may be a critical autophosphorylation site (*29*). Therefore, we tested the ability of the mutant PDK1-S244A to undergo insulin-stimulated phosphorylation and activation in transfected NIH-3T3^{IR} cells using our immune-complex kinase assay. By contrast with PDK1-WT, insulin did not stimulate either increased phosphorylation of PDK1-S244A (Figure 3A, lanes 13 and 14) or increased kinase activity of PDK1-S244A (Figure 4). These results suggest that phosphorylation of PDK-1 at Ser^{244} may be involved with the mechanism underlying the activation of PDK-1 by insulin.

Role of PDK-1 in Insulin-Stimulated Translocation of GLUT4. To evaluate the role of PDK-1 in insulin-stimulated translocation of GLUT4, we cotransfected rat adipose cells with GLUT4-HA and pBEX, PDK1-WT, or PDK1-K114A. We confirmed comparable overexpression of the HA-tagged PDK-1 constructs by immunoblotting whole cell lysates derived from transfected cells with anti-HA antibody (data not shown). In addition, membrane fractions were immunoblotted with HA-11 to demonstrate that total cellular levels of GLUT4-HA were comparable in all groups (data not shown). When control cells (cotransfected with pBEX and GLUT4-HA) were stimulated with insulin, we observed a

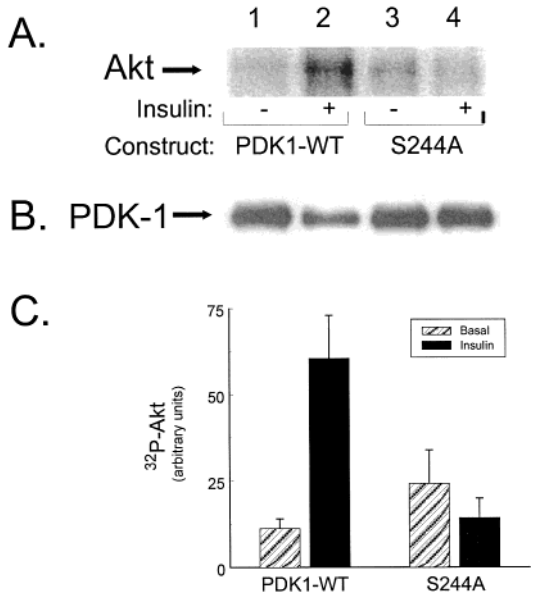

FIGURE 4: Ser^{244} in the activation loop of PDK-1 is required for insulin-stimulated activation of PDK-1. NIH-3T3^{IR} cells were transfected with myc-tagged PDK1-WT or the S244A point mutant and treated without or with insulin, and in vitro kinase assays as described in Figure 1 were performed. (A) Autoradiogram from a representative kinase assay using unactivated Akt as the substrate in the presence of [γ-^{32}P]ATP. (B) Anti-myc immunoblot of anti-myc immunoprecipitates demonstrating comparable recovery of PDK-1 constructs. (C) Mean ± SEM of PhosphorImager quantification of three independent experiments normalized for PDK-1 recovery (determined by scanning densitometry).

dose-dependent increase in cell surface GLUT4-HA with an ED_{50} of ~0.1 nM and a ~2-fold increase upon maximal insulin stimulation (Figure 5A,B). Interestingly, in the absence of insulin, overexpression of PDK1-WT was sufficient to cause a significant increase in cell surface GLUT4 (to levels ~80% of those seen in control cells treated with a maximally stimulating dose of insulin) (Figure 5A). In these cells, insulin stimulation caused a further small increase in cell surface GLUT4 to levels similar to those observed in the insulin-stimulated control cells. Wortmannin pretreatment of these cells inhibited the effects of overexpression of PDK1-WT on translocation of GLUT4 (data not shown). By contrast with PDK1-WT, overexpression of the kinase-inactive PDK1-K114A did not significantly change the insulin dose−response curve (Figure 5B). Similar results were observed with overexpression of PDK1-S244A (data not shown). Furthermore, coexpression of PDK1-K114A with wild-type Akt did not impair the ability of overexpressed Akt to recruit GLUT4-HA (data not shown). Taken together, our results suggest that effects of overexpression of PDK-1 to recruit GLUT4 to the cell surface are dependent upon intact PDK-1 kinase activity.

Since Akt can be phosphorylated and activated by PDK-1 (cf. Figure 1), we also investigated the effects of coexpressing wild-type PDK-1 and a dominant inhibitory mutant of Akt. Adipose cells overexpressing PDK1-WT were compared with cells coexpressing PDK1-WT and Akt-AA (point mutations T308A and S473A in the regulatory domain of Akt) (Figure 5C). Interestingly, coexpression of Akt-AA partially impaired the ability of PDK-1 to recruit GLUT4 to the cell surface in the absence of insulin. Moreover, a partial inhibitory effect of Akt-AA on translocation of GLUT4 was also observed

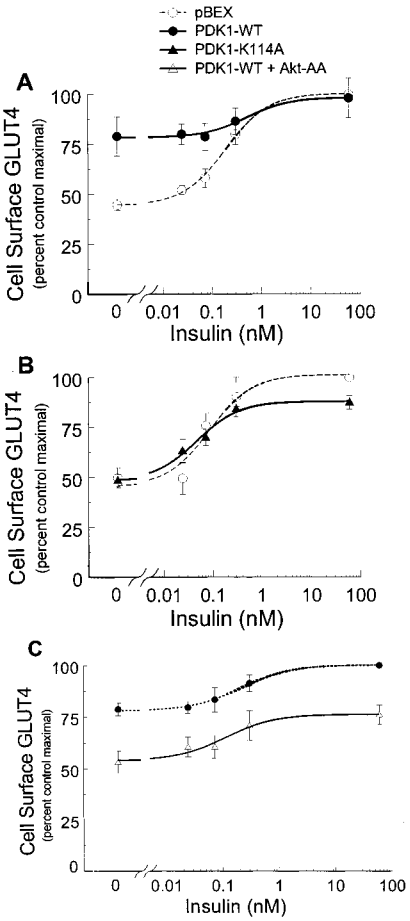

FIGURE 5: Insulin-stimulated recruitment of GLUT4-HA to the cell surface of rat adipose cells overexpressing PDK1-WT or PDK1-K114A. Cells were cotransfected with GLUT4-HA and pBEX (empty vector) (○), PDK1-WT (●), PDK1-K114A (▲), or PDK1-WT plus Akt-AA (△). GLUT4-HA at the cell surface was measured using the HA-11 antibody as described in Materials and Methods. Data are expressed as a percentage of cell surface GLUT4 in the presence of a maximally effective insulin concentration for the control group (pBEX). **(**A) Overexpression of PDK1-WT in the absence of insulin significantly increased recruitment of GLUT4-HA to the cell surface. Results shown are the mean ± SEM of five independent experiments. The difference between the dose−response curves was statistically significant by MANOVA ($p < 1 \times 10^{-5}$). **(**B) Overexpression of PDK1-K114A did not significantly affect insulin-stimulated recruitment of GLUT4-HA to the cell surface. Results shown are the mean ± SEM of six independent experiments. The difference between the dose−response curves was not statistically significant by MANOVA ($p \geq 0.2$). (C) Overexpression of Akt-AA partially inhibits recruitment of GLUT4 caused by coexpression of PDK1-WT in rat adipose cells. The two dose−response curves are significantly different when analyzed by MANOVA ($p < 1 \times 10^{-10}$). Results shown are the means ± SEM of four independent experiments.

in the presence of insulin. These results are consistent with the hypothesis that Akt is downstream from PDK-1 and that both kinases may play a role in insulin-stimulated translocation of GLUT4.

DISCUSSION

Elucidation of insulin signaling mechanisms downstream from PI3K is of great interest because PI3K plays a central role in mediating metabolic and vascular actions of insulin (*3*, *6*−*8*, *30*). PDK-1 directly phosphorylates and activates effectors of PI3K pathways such as Akt and PKC-ζ that are important for regulating physiological actions of insulin (*17*, *20*, *24*, *31*). Many previous studies concluded that PDK-1 is a constitutively active enzyme whose catalytic activity is not increased by growth factors that stimulate PI3K activity such as insulin, IGF-1, or EGF (*12*, *15*, *22*, *29*, *32*). Moreover, in some studies, inhibition of PI3K activity with wortmannin does not decrease catalytic activity of PDK-1 (*15*, *20*). Lipid products of PI3K such as $PI(3,4,5)P_3$ bind to the PH domain of PDK-1 and may help to localize PDK-1 to signaling complexes at the cell membrane (*11*). Indeed, stimulation of HeLa cells with insulin promotes localization of PDK-1 to the plasma membrane (*33*). Direct or indirect interactions of $PI(3,4,5)P_3$ with a variety of downstream targets also makes these molecules better substrates for PDK-1 (*10*, *12*, *14*−*16*, *24*). However, other reports suggest that $PI(3,4,5)P_3$ may play an additional role to directly increase catalytic activity of PDK-1 (*11*, *13*, *17*). In keeping with these studies, there is also evidence that the PH domain of PDK-1 may function as a negative regulator of PDK-1 activity (*33*, *34*). Very recently, it has been demonstrated that sphingosine can stimulate increased catalytic activity of PDK-1 [independent of $PI(3,4,5)P_3$] (*35*) and that tyrosine phosphorylation of PDK-1 may also regulate its catalytic activity (*19*). Thus, a number of separate mechanisms for regulating catalytic activity of PDK-1 may exist. In the present study, we have demonstrated the ability of insulin to increase both phosphorylation and catalytic activity of PDK-1 and explored the role of PDK-1 in translocation of GLUT4 in the physiologically relevant rat adipose cell.

Insulin-Stimulated Activation of PDK-1. Recombinant PDK-1 derived from unstimulated cells overexpressing PDK1-WT phosphorylated purified Akt in vitro. This in vitro phosphorylation of Akt correlated with Akt activity. In addition, treatment of cells with wortmannin did not affect the basal level of PDK1-WT activity. As expected, the kinase-inactive PDK1-K114A did not phosphorylate Akt in our assay (in the absence or presence of insulin). Thus, it is unlikely that kinase activity in our immune-complex assay with PDK1-WT is due to some other co-immunoprecipitated kinase. In addition, the basal activity of PDK1-W538L [a PH domain mutant that does not bind $PI(3,4,5)P_3$] was unaffected by treating cells with insulin. These results demonstrating constitutive kinase activity of PDK-1 in the absence of insulin stimulation that is independent of activation of PI3K or interaction of $PI(3,4,5)P_3$ with the PH domain of PDK-1 are consistent with previous reports (*10*, *12*, *14*−*16*). Nevertheless, when samples derived from cells treated with insulin were examined, we observed a significant increase in PDK1-WT activity that could be blocked by wortmannin pretreatment of the cells. Taken together, our data strongly suggest that, in addition to constitutive basal kinase activity, insulin stimulation causes an increase in PDK-1 activity via a PI3K-dependent mechanism that may involve interaction of $PI(3,4,5)P_3$ with the PH domain of PDK-1. Importantly, insulin treatment of freshly isolated rat adipose cells caused a significant stimulation of endogenous PDK-1 activity. This result confirms that insulin-stimulated activation of PDK-1 occurs not only with overexpressed recombinant PDK-1 but also with the native protein in a physiologically relevant insulin target cell. It is possible that differences in the types of cells studied may explain why other investigators failed to observe changes in PDK-1

activity with insulin or IGF-1 stimulation of 293 cells (*12*, *15*, *22*, *29*).

With respect to a role for PI3K in regulating PDK-1 activity, our results are consistent with a previous report showing that the PI3K inhibitor LY294002 blocked the ability of full-length PDK-1 to phosphorylate a kinase-domain fragment of PKC-ζ while the inhibitor had no effect on the ability of a kinase-domain fragment of PDK-1 to phosphorylate full-length PKC-ζ (*17*). Similarly, PI(3,4,5)-P_3 has been reported to enhance the ability of PDK-1 to phosphorylate an Akt mutant lacking a PH domain (*13*) while PH domain mutants of PDK-1 have a greatly decreased ability to activate Akt in vitro in the presence of PI(3,4,5)P_3 (*11*). Our data are also consistent with studies demonstrating that the PH domain of PDK-1 is a negative regulator of PDK-1 catalytic activity (*33*, *34*).

Intriguingly, a recent report suggests that interaction of PDK-1 with a region of protein kinase C-related kinase-2 (PRK2) converts PDK-1 into an enzyme that is capable of phosphorylating both the previously defined Thr^{308} regulatory site on Akt and the Ser^{473} site that is the target of a putative PDK-2 (*36*). Moreover, the acquired PDK-2 activity of PDK-1 appears to be stimulated by PI(3,4,5)P_3. It has also been suggested that Ser^{473} is an autophosphorylation site for Akt (*37*). While both of these mechanisms for phosphorylating Akt on Ser^{473} may exist, data from mouse embryonic stem cells lacking PDK-1 also suggest that other kinases in addition to PDK-1 are capable of phosphorylating Ser^{473} in a PI3K-dependent fashion (*38*). When we used phospho-specific antibodies to examine the ability of insulin-stimulated PDK-1 to phosphorylate Akt, we observed increased phosphorylation at both Thr^{308} and Ser^{473} of Akt. Thus, our data are consistent with the possibility that the increased PDK-1 activity we observed in response to insulin stimulation may include acquired PDK-2 activity. Alternatively, we cannot completely rule out the possibility that there may be another kinase that is activated upon insulin stimulation that co-immunoprecipitates in our immune complex. However, this seems unlikely because, under similar conditions, PDK-1 mutants PDK1-K114A, PDK1-W538L, and PDK1-S244A did not phosphorylate Akt in response to insulin in our in vitro kinase assay (Figures 1A,C, 3B, and 4A,C).

Relationship between PDK-1 Phosphorylation and PDK-1 Activity. To further investigate the mechanism of insulin-stimulated activation of PDK-1, we examined the relationship between phosphorylation of PDK-1 and PDK-1 activity by labeling intact cells with ^{32}P. Since only PDK1-WT, but not the kinase-inactive PDK1-K114A, showed increased phosphorylation in response to insulin, it is likely that insulin stimulation results in autophosphorylation of PDK-1 (or phosphorylation of PDK-1 by other downstream kinases). Wortmannin pretreatment blocked insulin-stimulated phosphorylation of PDK1-WT. Furthermore, the PH domain mutant PDK1-W538L did not undergo phosphorylation in response to insulin. These data are consistent with the hypothesis that binding of lipid products of PI3K such as PI(3,4,5)P_3 to the PH domain of PDK-1 may be important for its phosphorylation in response to insulin. Increased phosphorylation of PDK-1 in intact cells corresponded to an increase in its kinase activity in vitro. Moreover, dephosphorylation of PDK-1 with the serine-specific phosphatase PP-2A reversed the insulin-stimulated activation of PDK-1 (but was without effect on basal PDK-1 activity). It is conceivable that, similar to other serine−threonine kinases, phosphorylation of regulatory sites in the activation loop of the kinase domain of PDK-1 may be important for increased catalytic activity. Indeed, recent evidence suggests that Ser^{244} in PDK-1 is an autophosphorylation site in the activation loop that is critical for PDK-1 activity (*29*). In the present study, we have demonstrated that the S244A point mutant of PDK-1 is unable to increase its activity in response to insulin stimulation. Thus, in addition to localizing PDK-1 to appropriate signaling complexes and inducing changes in downstream substrates as previously suggested by others (*10*−*13*), insulin-stimulated activation of PI3K may result in conformational changes in PDK-1 that are induced by binding of PI(3,4,5)P_3 to the PH domain of PDK-1, rendering it a better substrate for phosphorylation (either by itself or by another as yet unidentified kinase). Phosphorylation of Ser^{244} (and possibly other sites) may then lead to increased catalytic activity. In agreement with our results, Casamayor et al. found that phosphorylation of Ser^{241} in human PDK-1 was important for catalytic activity (*29*). However, they also found that IGF-1 stimulation of 293 cells activated Akt without altering phosphorylation of Ser^{241} or other residues in PDK-1. Our findings of increased phosphorylation and activation of PDK-1 in response to insulin stimulation were observed not only in NIH-3T3 fibroblasts overexpressing insulin receptors but also in physiologically relevant rat adipose cells in primary culture. It remains possible that phosphorylation of PDK-1 at other sites in addition to Ser^{244} may also contribute to increased catalytic activity in response to insulin stimulation. Thus, phosphorylation of PDK-1 may be required for its increased catalytic activity, and this may represent a general mechanism for regulation of PDK-1 activity by a variety of upstream inputs.

Role of PDK-1 in Translocation of GLUT4. Overexpression of wild-type PDK-1 in rat adipose cells was sufficient to cause an increase in translocation of GLUT4 in the absence of insulin and is consistent with the hypothesis that PDK-1 plays a role in mediating metabolic effects of insulin such as increased glucose transport. These results are in agreement with published studies where PDK-1 was overexpressed in rat adipose cells (*5*, *39*). We have previously demonstrated that the downstream substrates of PDK-1, Akt and PKC-ζ, both play a role in mediating effects of insulin to promote translocation of GLUT4 in rat adipose cells (*4*, *5*, *25*). It is likely that there is a small signal upstream from PDK-1 in the absence of insulin that can be amplified by overexpression of PDK-1. It is also possible that the basal constitutive activity of PDK-1 is sufficient to mediate translocation of GLUT4 when overexpressed at high levels. However, this seems less plausible because wortmannin pretreatment of cells overexpressing PDK1-WT completely blocked the translocation of GLUT4 in the absence of insulin without blocking the basal kinase activity of PDK-1. Thus, the effects of PDK-1 on translocation of GLUT4 depend, at least in part, on activity of PI3K. The fact that the kinase-inactive mutant of PDK-1 (PDK1-K114A) had no effect on translocation of GLUT4 suggests that catalytic activity of PDK-1 is necessary for its actions to mediate recruitment of GLUT4. However, PDK1-K114A did not block insulin-stimulated translocation of GLUT4 even when overexpressed at high

levels. Moreover, coexpression of PDK1-K114A with wild-type Akt (a known downstream effector of PDK-1) did not interfere with effects of overexpressed wild-type Akt to mediate recruitment of GLUT4. Thus, while PDK1-K114A is inactive, it does not inhibit endogenous PDK-1 under our experimental conditions. Overexpression of the S244A mutant yielded results that were similar to overexpression of PDK1-K114A suggesting that the Ser^{244} is critical for PDK-1 activity. There are conflicting reports in the literature regarding the inhibitory ability of kinase-inactive mutants of PDK-1. Some studies find that these mutants can inhibit endogenous PDK-1 activity (*15*, *31*, *40*) while others do not (*22*, *33*). It is possible that differences in cellular context resulting from the use of different cell types and experimental conditions may explain, in part, these conflicting findings. We also coexpressed PDK1-WT with a mutant Akt (Akt-AA) missing the regulatory phosphorylation sites that are targets of PDK-1 and -2. This mutant has been shown to function in a dominant inhibitory manner in other contexts (*41*) and causes a small inhibition of insulin-stimulated translocation of GLUT4 when overexpressed in rat adipose cells (*25*). This is similar to what is observed with overexpression of kinase-inactive Akt (*4*). Interestingly, coexpression of Akt-AA partially inhibited the effects of PDK1-WT to recruit GLUT4 to the cell surface, consistent with the fact that Akt is downstream from PDK-1. However, the Akt-AA mutant did not completely inhibit an insulin response in either the presence or absence of PDK1-WT. Our results are in agreement with previous studies using different Akt mutants that suggest Akt may play a partial role in mediating the effects of insulin on translocation of GLUT4 (*4*, *25*, *42*). The partial inhibition of PDK-1 effects caused by coexpression of Akt-AA implies that either the mutant is not completely effective at inhibiting endogenous Akt or that other effectors downstream of PDK-1 may also contribute to insulin-stimulated translocation of GLUT4. Indeed, recent studies have suggested that the atypical PKC-ζ and -λ (also substrates for PDK-1) may play significant roles in this process (*25*, *41*, *43*, *44*).

Conclusions. We conclude that PDK-1 has basal kinase activity that can be stimulated further by insulin in a PI3K-dependent manner in intact cells. Importantly, insulin-stimulated phosphorylation of PDK-1 is correlated with its activity, suggesting a general mechanism for upstream inputs to regulate PDK-1 activity. Ser^{244} is a critical autophosphorylation site in the activation loop of PDK-1 that is necessary for insulin-stimulated activation. Finally, insulin-regulated PDK-1 activity may play an important role in mediating metabolic effects of insulin by propagating signaling from PI3K to downstream effectors such as Akt, PKC-ζ, and PKC-λ.

REFERENCES

1. Vanhaesebroeck, B., and Alessi, D. R. (2000) *Biochem. J. 346* (Part 3), 561−576.
2. Toker, A., and Newton, A. C. (2000) *Cell 103*, 185−188.
3. Quon, M. J., Chen, H., Ing, B. L., Liu, M. L., Zarnowski, M. J., Yonezawa, K., Kasuga, M., Cushman, S. W., and Taylor, S. I. (1995) *Mol. Cell. Biol. 15*, 5403−5411.
4. Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) *Mol. Endocrinol. 11*, 1881−1890.
5. Bandyopadhyay, G., Standaert, M. L., Sajan, M. P., Karnitz, L. M., Cong, L., Quon, M. J., and Farese, R. V. (1999) *Mol. Endocrinol. 13*, 1766−1772.
6. Zeng, G., Nystrom, F. H., Ravichandran, L. V., Cong, L. N., Kirby, M., Mostowski, H., and Quon, M. J. (2000) *Circulation 101*, 1539−1545.
7. Nystrom, F. H., and Quon, M. J. (1999) *Cell. Signalling 11*, 563−574.
8. Montagnani, M., and Quon, M. J. (2000) *Diabetes Obes. Metab. 2*, 285−292.
9. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. (1999) *Annu. Rev. Biochem. 68*, 965−1014.
10. Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998) *Curr. Biol. 8*, 684−691.
11. Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R., and Lucocq, J. (1999) *Biochem. J. 337*, 575−583.
12. Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A., and Bownes, M. (1997) *Curr. Biol. 7*, 776−789.
13. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) *Science 277*, 567−570.
14. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr. Biol. 8*, 69−81.
15. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) *Science 279*, 707−710.
16. Kobayashi, T., and Cohen, P. (1999) *Biochem. J. 339*, 319−328.
17. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) *Science 281*, 2042−2045.
18. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) *J. Biol. Chem. 271*, 21920−21926.
19. Prasad, N., Topping, R. S., Zhou, D., and Decker, S. J. (2000) *Biochemistry 39*, 6929−6935.
20. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol. 7*, 261−269.
21. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) *Biochem. J. 342*, 287−292.
22. Yamada, T., Katagiri, H., Asano, T., Inukai, K., Tsuru, M., Kodama, T., Kikuchi, M., and Oka, Y. (2001) *J. Biol. Chem. 276*, 5339−5345.
23. Bram, R. J., Hung, D. T., Martin, P. K., Schreiber, S. L., and Crabtree, G. R. (1993) *Mol. Cell. Biol. 13*, 4760−4769.
24. Dong, L. Q., Zhang, R., Langlais, P., He, H., Clark, M., Zhu, L., and Liu, F. (1999) *J. Biol. Chem. 274*, 8117−8122.
25. Standaert, M. L., Bandyopadhyay, G., Sajan, M. P., Cong, L., Quon, M. J., and Farese, R. V. (1999) *J. Biol. Chem. 274*, 14074−14078.
26. Quon, M. J., Guerre-Millo, M., Zarnowski, M. J., Butte, A. J., Em, M., Cushman, S. W., and Taylor, S. I. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5587−5591.
27. Kadowaki, T., Bevins, C. L., Cama, A., Ojamaa, K., Marcus-Samuels, B., Kadowaki, H., Beitz, L., McKeon, C., and Taylor, S. I. (1988) *Science 240*, 787−790.
28. Chen, H., Wertheimer, S. J., Lin, C. H., Katz, S. L., Amrein, K. E., Burn, P., and Quon, M. J. (1997) *J. Biol. Chem. 272*, 8026−8031.
29. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) *Biochem. J. 342*, 287−292.
30. Zeng, G., and Quon, M. J. (1996) *J. Clin. Invest. 98*, 894−898.
31. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) *Curr. Biol. 8*, 1069−1077.
32. Kim, S., Jee, K., Kim, D., Koh, H., and Chung, J. (2001) *J. Biol. Chem.* (in press).
33. Filippa, N., Sable, C. L., Hemmings, B. A., and Van Obberghen, E. (2000) *Mol. Cell. Biol. 20*, 5712−5721.

34. Wick, M. J., Dong, L. Q., Riojas, R. A., Ramos, F. J., and Liu, F. (2000) *J. Biol. Chem. 275*, 40400−40406.
35. King, C. C., Zenke, F. T., Dawson, P. E., Dutil, E. M., Newton, A. C., Hemmings, B. A., and Bokoch, G. M. (2000) *J. Biol. Chem. 275*, 18108−18113.
36. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) *Curr. Biol. 9*, 393−404.
37. Toker, A., and Newton, A. C. (2000) *J. Biol. Chem. 275*, 8271−8274.
38. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D. R. (2000) *Curr. Biol. 10*, 439−448.
39. Grillo, S., Gremeaux, T., Le Marchand-Brustel, Y., and Tanti, J. (1999) *FEBS Lett. 461*, 277−279.
40. Bertrand, L., Alessi, D. R., Deprez, J., Deak, M., Viaene, E., Rider, M. H., and Hue, L. (1999) *J. Biol. Chem. 274*, 30927−30933.
41. Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakaue, H., Hino, Y., Miyake, K., Sano, W., Akimoto, K., Ohno, S., and Kasuga, M. (1998) *Mol. Cell. Biol. 18*, 6971−6982.
42. Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R., and Klip, A. (1999) *Mol. Cell. Biol. 19*, 4008−4018.
43. Bandyopadhyay, G., Standaert, M. L., Galloway, L., Moscat, J., and Farese, R. V. (1997) *Endocrinology 138*, 4721−4731.
44. Bandyopadhyay, G., Standaert, M. L., Kikkawa, U., Ono, Y., Moscat, J., and Farese, R. V. (1999) *Biochem. J. 337*, 461−470.

BI010743C