Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 12, Issue of March 24, pp. 8271–8274, 2000
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Akt/Protein Kinase B Is Regulated by Autophosphorylation at the Hypothetical PDK-2 Site*

(Received for publication, October 28, 1999, and in revised form, January 27, 2000)

Alex Toker‡§ and Alexandra C. Newton¶‖

From the ¶Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0640 and the ‡Signal Transduction Group, Boston Biomedical Research Institute, Boston, Massachusetts 02114

The function of Akt (protein kinase B) is regulated by phosphorylation on two sites conserved within the AGC kinase family: the activation loop (Thr-308) in the kinase core and a hydrophobic phosphorylation site on the carboxyl terminus (Ser-473). Thr-308 is phosphorylated by the phosphoinositide-dependent kinase-1, (PDK-1), whereas the mechanism of phosphorylation of the hydrophobic site, tentatively referred to as the PDK-2 site, is unknown. Here we report that phosphorylation of the hydrophobic motif requires catalytically competent Akt. First we show that a kinase-inactive construct of Akt fails to incorporate phosphate at Ser-473 following IGF-1 stimulation *in vivo* but does incorporate phosphate at Thr-308 and a second carboxyl-terminal site, Thr-450; this ligand triggers the phosphorylation of both sites in wild-type enzyme. Neither does a catalytically inactive construct in which phosphorylation at the activation loop is blocked, T308A, become phosphorylated on the hydrophobic site in response to stimulation. Second, we show that Akt autophosphorylates on the hydrophobic site *in vitro*: phosphorylation of the activation loop by PDK-1 triggers the phosphorylation of the hydrophobic site in kinase-active, but not thermally inactivated, Akt α. Thus, Akt is regulated by autophosphorylation at the Ser-473 hydrophobic site.

Phosphorylation exquisitely regulates a plethora of cellular functions, including the function of the protein kinases themselves. Many members of this superfamily are controlled by two phosphorylation switches, one on a segment near the entrance to the active site and a second on the carboxyl terminus. Phosphorylation at one or both positions appears to be required to correctly align residues to create a catalytically competent conformation. For kinases such as protein kinase A and the conventional protein kinase Cs (PKC),[1] phosphorylation of these positions is constitutive and part of the processing of the enzyme (1). In contrast, phosphorylation of kinases such as p70 S6 kinase is tightly regulated by specific stimuli (2). The carboxyl-terminal switch contains two motifs: a turn motif, typically rich in Pro, which, in the case of protein kinase A, is positioned at the apex of a tight turn (Ser-338) (3), and a hydrophobic motif (F*XX*S/T*X*F) first identified in protein kinase C and p70 S6 kinase (4, 5).

The serine/threonine kinase, Akt (protein kinase B) transduces signals that result in phosphoinositide-3-kinase activation, (6–13). The membrane-localized enzyme becomes rapidly phosphorylated at two positions, the activation loop (Thr-308) and the hydrophobic phosphorylation motif (Ser-473). The activation loop reaction is catalyzed by the recently discovered PDK-1 (14–16). The phosphorylation of the hydrophobic motif is tightly coupled to that of the activation loop; however, the mechanism of this phosphorylation is unknown. The mitogen sensitivity of this site, like that of the activation loop, has led to the proposal that it, too, is phosphorylated by an agonist-sensitive kinase, tentatively named PDK-2 (14). Nonetheless, such a kinase has remained refractory to molecular or biochemical identification (17). In this regard, protein kinase C βII has recently been shown to be regulated by autophosphorylation, and not by a heterologous kinase, at its hydrophobic phosphorylation motif (19, 20). In addition to the mitogen-sensitive sites, ^{32}P labeling studies have revealed that Akt is constitutively phosphorylated at Thr-450 *in vivo* (18). This position corresponds to the turn motif in the protein kinase Cs as it has the consensus motif T/SP*X*D (21).

This contribution addresses the mechanism of phosphorylation of the carboxyl-terminal sites of Akt. Specifically, we ask which sites depend on the intrinsic catalytic activity of the enzyme in order to dissect the contribution of autophosphorylation *versus* heterologous phosphorylation in the regulation of Akt.

MATERIALS AND METHODS

Dipalmitoylphosphatidylinositol tris-3,4,5-phosphate (PtdIns-3,4,5-P_3) was obtained from Matreya, palmitoyl oleoyl phosphatidylcholine (PC), and bovine brain L-α-phosphatidylserine (PS) were obtained from Avanti Polar Lipids. Microcystin and peroxidase-conjugated goat anti-rabbit antibodies were from Calbiochem. Protein kinase C βII (rat) was purified from the baculovirus expression system as described (22). Baculoviral expressed, homogeneously pure mouse Akt α was a generous gift from Dr. Frank Sicheri, Samuel Lunenefeld Research Institute. An antibody that recognizes the phosphorylated Thr-641 in the turn motif of protein kinase C βII (23) was provided by David Sweatt (Baylor College of Medicine). Antibodies, which recognize phosphorylated Thr-308 or phosphorylated Ser-473 were obtained from New England Biolabs. In one experiment, an antibody that recognizes the phosphorylated hydrophobic motif of Akt and all the protein kinase C isozymes was used (New England Biolabs, PAN-PKC antibody). The catalytic subunit of protein phosphatase 1 was a generous gift from Dr. Anna DePaoli-Roach (Indiana University). All other chemicals were reagent-grade.

cDNA Constructs—The murine Akt T308A and K179M mutants were a kind gift of Philip Tsichlis (Thomas Jefferson University). The Akt T450A mutant was made by polymerase chain reaction-based site-directed mutagenesis using the Pfu Quickchange methodology (Stratagene).

* This work was supported by National Institutes of Health Grants GM 43154 (to A. C. N.) and CA 75134 (to A. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§To whom correspondence can be addressed. Present address: Dept. of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave., RN-237, Boston, MA 02215. Tel.: 617-667-8535; Fax: 617-667-3616; E-mail: atoker@caregroup.harvard.edu.

‖ To whom correspondence can also be addressed: Dept. of Pharmacology, UCSD, La Jolla, CA 92093-0640 (Tel.: 858-534-4527; Fax: 858-534-6020; E-mail: anewton@ucsd.edu).

[1] The abbreviations used are: PKC, protein kinase C; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; PDK-1, phosphoinositide-dependent kinase 1; PIF, PDK-1 interacting fragment; PC, phosphatidylcholine; PS, phosphatidylserine; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PP1, protein phos- phatase 1; HA, hemagglutinin.

Cell Culture—Human embryonic kidney HEK 293E cells and HepG2 cells expressing the β subunit of the wild-type platelet-derived growth factor-receptor (β-PDGF-R, a kind gift of Andrius Kazlauskas, Harvard Medical School) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum.

Phosphatase Treatment of Akt—Purified Akt (80 nM) was incubated with the catalytic subunit of protein phosphatase 1, in the presence of lipid vesicles, as described in the legend to Fig. 1. Incubation mixtures included PC vesicles (100 μM total lipid) containing no other lipid, 10 mol % PtdIns-3,4,5-P_3, or 10 mol % PtdIns-3,4,5-P_3 and 45 mol % PS. Microcystin (4 μM) was included in some experiments. Protein kinase C βII (45 nM) was treated similarly except that lipid was presented in the form of sonicated dispersions of phosphatidylserine (140 μM) and diacylglycerol (4 μM) and 200 nM $CaCl_2$ was present. Reactions were quenched by addition of SDS-PAGE sample buffer and samples analyzed by SDS-PAGE (7.5% polyacrylamide) followed by silver staining or Western blot analysis.

Mammalian Cell Transfections—HEK 293E and HepG2 cells were transiently transfected either by the calcium-phosphate procedure (24) or by the LipofectAMINE procedure (Life Technologies, Inc.) as described previously (25). Cells were serum-starved for 24 h in Dulbecco's modified Eagle's medium with no added serum. Cells were stimulated with either IGF-1 (20 ng ml^{-1}, Life Technologies, Inc.) or with PDGF-BB (50 ng ml^{-1}, Life Technologies, Inc.), typically for 10 min. Immunoprecipitated Akt proteins were assayed for protein kinase activity using histone H2B as substrate, as described by Franke *et al.* (26).

In Vitro Phosphorylation of Akt—A highly purified insect cell-expressed recombinant Akt preparation was used to detect phosphorylation of relevant sites by PDK-1 *in vitro*. Recombinant human PDK-1 was expressed as a fusion protein in insect cells. For *in vitro* assays, 0.1 μg of each protein was mixed in a nonradioactive kinase assay reaction (using 80 μM [ATP]) either in the presence or absence of PS:PC:PtdIns-3,4,5-P_3 vesicles (see above).

RESULTS AND DISCUSSION

Carboxyl Terminus of Akt Contains Two Conserved Phosphorylation Sites—The carboxyl terminus of Akt is phosphorylated at two positions: Thr-450 is constitutively phosphorylated (18, 27) and Ser-473 is phosphorylated in a mitogen-sensitive manner (18). Alignment of the carboxyl-terminal residues of Akt isozymes with those of protein kinase C βII revealed the Thr-450 corresponds to the turn motif described for the protein kinase Cs (Fig. 1*A*). The mechanism of phosphorylation of both positions is autophosphorylation for protein kinase C (19). Given the high sequence similarity between these two kinases, we explored whether such a mechanism accounted for the phosphorylation of the corresponding sites in Akt.

First, we tested whether unstimulated Akt is phosphorylated on the turn motif. Homogeneously pure Akt from the baculoviral expression system was used for this study; mass spectrometric analysis revealed there was no phosphate on either the activation loop or the hydrophobic site.[2] Pure Akt was treated with the catalytic subunit of protein phosphatase 1 (PP1), a phosphatase that dephosphorylates the turn motif of protein kinase C (4). The silver stain in Fig. 1*B* shows that untreated Akt migrated as a major band with an apparent molecular mass of 64 KDa, with a minor (<10%) faster migrating species (apparent molecular mass of 62 kDa). Treatment with PP1 resulted in disappearance of the upper band and accumulation of the faster migrating species. Samples from this experiment were also probed with an antibody that recognizes the phosphorylated, but not unphosphorylated, turn motif of protein kinase C (see "Materials and Methods"). Fig. 1*B*, *lower panel*, shows that this antibody cross-reacted with untreated Akt (*lane 1*). However, it did not react with the faster migrating species resulting from phosphatase treatment. These data reveal that unstimulated Akt is almost quantitatively phosphorylated on the turn motif. For comparison, *lanes 6* and *7* show the migration of purified, baculoviral-expressed protein kinase C βII before (*lane 6*) and after treatment with PP1 (*lane 7*). The

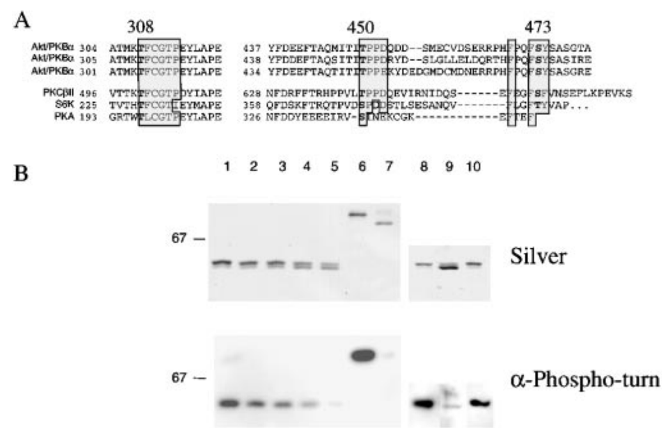

FIG. 1. *A*, alignment of activation loop and carboxyl-terminal sequences of Akt and related kinases showing conservation (*shaded areas*) of phosphorylation sites on the activation loop, turn motif, and hydrophobic motif. Phosphorylated residues are in *bold* with the numbering for Aktα indicated on top. Shown are the sequences for murine Akt α (29), human Akt β (30), human Akt γ (31), rat protein kinase C βII (32), rat S6 kinase (33), and murine protein kinase A (34). *B*, dephosphorylation of unstimulated Akt results in a specific shift in electrophoretic mobility and loss of immunoreactivity with phospho-turn motif antibody. Silver-stained gel (silver; *upper panel*) and corresponding Western blot probed with an antibody that specifically recognizes the phosphorylated turn motif in protein kinase C βII (P-turn motif; *lower panel*) showing Akt (0.15 μg) before (*lane 1*) and after treatment with the catalytic subunit of PP1 (4 μg ml^{-1}) for 5 (*lane 2*), 11 (*lane 3*), 30 (*lane 4*), or 60 (*lane 5*) min at 22 °C, in the presence of phosphatidylcholine vesicles (100 μM total lipid) containing 10 mol % PtdIns-3,4,5-P_3; protein kinase C βII (0.12 μg) was treated without (*lane 6*) or with (*lane 7*) the catalytic subunit of PP1 (2 μg ml^{-1}) for 60 min, 22 °C, in the presence of 200 μM Ca^{2+} and sonicated dispersions of phosphatidylserine (140 μM) and diacylglycerol (4 μM). Akt was also treated without (*lane 8*) or with (*lanes 9* and *10*) PP1 (2 μg ml^{-1}), and in the presence of microcystin (4 μM, *lane 10*), for 60 min at 22 °C.

shift in electrophoretic mobility following PP1 treatment arises from loss of phosphate at the turn motif and the hydrophobic motif; a smaller shift, similar to that observed for Akt above, results from loss of phosphate at the hydrophobic motif only (4). The increased electrophoretic mobility and loss of immunoreactivity with the phospho-specific antibody did not result from proteolysis: the presence of microcystin in the incubation medium (*lane 10*) prevented both the shift and loss of immunoreactivity revealing that phosphatase activity was required for these effects.

Characterization of Kinase-inactive Constructs of Akt—To address whether autophosphorylation or phosphorylation by an upstream kinase regulates the two carboxyl-terminal positions of Akt, we first examined the agonist-dependent protein kinase activities of wild-type and mutant Akt alleles *in vivo*. HA-tagged constructs of Akt were expressed in HepG2 cells expressing the wild-type β PDGF-R or in HEK 293E cells (18, 26). HEK 293E cells (Fig. 2*A*) or HepG2 cells (Fig. 2*B*) were stimulated for 10 min with IGF-1 or PDGF-BB, respectively, and Akt activity from HA immunoprecipitates was assayed using histone H2B as substrate. Fig. 2*A*, *lower panel*, shows that wild-type Akt was activated 12.5-fold by treatment of HEK 293 cells with IGF-1. Importantly, the activity of T450A was similarly (10.7-fold) stimulated by IGF-1. Identical results were obtained with PDGF-stimulation of HepG2 cells: PDGF induced an 8.3-fold increase in wild-type Akt activity and a 7.5-fold increase in the activity of the T450A mutant (Fig. 2*B*, *lower panel*). Thus, the effect of the T450A mutation in Akt was not restricted to one cell type alone. In contrast, neither the K179M (*lanes 8* and *9*) or the T308A (*lanes 4* and *5*) constructs had any detectable activity in the absence or presence of mitogens. These data reveal that negative charge at position 450

[2] F. Sicheri, personal communication.

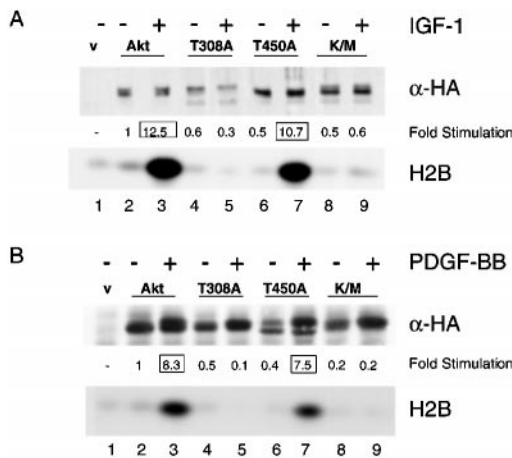

FIG. 2. **Phosphorylation of Thr-450 in Akt does not depend on the intrinsic catalytic activity of Akt.** *A*, HEK 293 cells transfected with the indicated Akt plasmids and left unstimulated (−) or stimulated (+) with 20 ng/ml IGF-1 for 10 min. *B*, HepG2 cells expressing the wild-type β PDGF-R were transfected with the indicated Akt plasmids and left unstimulated (−) or stimulated (+) with PDGF-BB (50 ng/ml) for 10 min. The *top panels* of *A* and *B* show an immunoblot of Akt from 5% of total cell lysates detected with anti-HA (*α-HA*) antibody. The *bottom panels* show an immune-complex kinase assay of Akt using histone H2B as substrate. Fold stimulation of Akt was quantitated on a Molecular Imager. The data are representative of four independent experiments.

does not influence either the basal or stimulated activity of Akt.

Thr-450 Is Phosphorylated in Kinase-inactive Constructs of Akt—Previous studies have indicated that the turn motif in Akt, Thr-450, is constitutively phosphorylated in serum-starved cells and that phosphorylation of this site does not contribute to the activity of the enzyme (18, 27). The mechanism of regulation of this site has not been examined to date. The Western blots in Fig. 2, *A* and *B*, show the expression of wild-type Akt, the activation loop mutant T308A, the turn motif mutant T450A, and the kinase-inactive mutant K179M in HEK 293E cells detected using anti-HA antibodies (*upper panels*). Wild-type Akt migrated as a doublet (*lanes 2* and *3*). The upper band comigrated with Akt that is phosphorylated on Thr-450 and was labeled with the phospho-turn motif antibody (Fig. 3, *α-P450*), whereas the lower band comigrated with completely dephosphorylated Akt (Fig. 1*B* and data not shown). Importantly, the T450A mutant (*lanes 6* and *7*) comigrated with the faster migrating wild-type species consistent with lack of negative charge at position 450 (see Fig. 1*B*). Similar to wild-type Akt, the kinase-inactive mutant K179M (*lanes 8* and *9*) migrated as a doublet, consistent with partial phosphorylation of Thr-450. Similarly, the activation loop mutant, T308A (*lanes 4* and *5*) also comigrated with wild-type enzyme, although it was enriched in upper band. This mutant was labeled with the anti-phospho-turn antibody (Fig. 3, *α-P450*). These data reveal that Thr-450 is phosphorylated by a reaction that depends neither on the catalytic competence of Akt nor on the phosphorylation state of Thr-308. These results indicate that both wild-type Akt as well as the T450A mutant retain full catalytic competency, whereas both T308A and K179M are completely inactive.

Phosphorylation of Ser-473 Requires the Intrinsic Catalytic Activity of Akt—We next explored whether phosphorylation of the hydrophobic site depended on the intrinsic catalytic activity of Akt. The Western blot in Fig. 3 shows immunoprecipitated Akt proteins from control and IGF-1-stimulated HEK 293 cells probed with an antibody that specifically labels phosphorylated Thr-473 (*α-P473*). This antibody strongly labeled wild-type Akt from stimulated (*lane 3*), but not unstimulated (*lane 2*), cells. Similarly, it labeled the T450A construct in stimulated (*lane 7*), but not unstimulated (*lane 6*), cells. For both wild-type and the

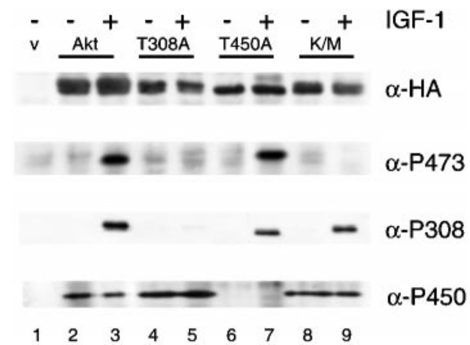

FIG. 3. Phosphorylation of Thr-473 *in vivo* requires the intrinsic catalytic activity of Akt. HEK 293 cells were transfected with the indicated Akt plasmid DNA's, serum-starved, and stimulated for 10 min with IGF-1 (20 ng/ml). The *top panel* shows 5% of total cell lysate probed with the anti-HA (*α-HA*) antibody. Phosphorylation of Akt was detected by immunoprecipitation with HA antibody, followed by immunoblotting with the anti-Thr-308(P) (*α-P308*), anti-Ser-473(P) (*α-P473*), and anti-phospho-turn (*α-P450*) antibodies. The immunoblots are representative of three independent experiments.

T450A construct, IGF-1 stimulation resulted in a 13–15-fold increase in immunoreactivity. In contrast, IGF-1 treatment had no detectable effect on the immunoreactivity of the two catalytically inactive constructs, T308A (*lanes 4* and *5*) or K179M (*lanes 8* and *9*). Based on the sensitivity of the detection method, any phosphorylation of Ser-473 would be less than 5% that observed for wild-type enzyme. Thus, phosphorylation of Ser-473 requires the catalytic competence of Akt.

One possibility is that Thr-473 phosphorylation requires phosphorylation of Thr-308. Thus, we addressed the phosphorylation state of the activation loop in the kinase-inactive constructs using an antibody that specifically labels phosphorylated Thr-308 (*α-P308*). Fig. 3, *lower panel*, shows that IGF-1 caused a marked increase in the phosphorylation of the activation loop of wild-type enzyme (*lane 3*), T450A (*lane 7*), but not T308A (*lane 5*), which has an alanine at the phosphoacceptor position. Importantly, the K179M mutant was phosphorylated on the activation loop to comparable levels as wild-type enzyme following IGF-1 stimulation. Thus, despite having a phosphate on the activation loop, the K179M mutant was unable to incorporate phosphate at position Ser-473. Identical results were obtained upon expression of kinase-inactive mutants in PDGF-stimulated HepG2 cells (data not shown). These data reveal that phosphorylation of the hydrophobic site requires the intrinsic catalytic competence of Akt.

Phosphorylation at the Activation Loop Triggers Autophosphorylation of Ser-473—We next tested whether the phosphorylation of Thr-308 by PDK-1 *in vitro* triggered the autophosphorylation of pure Akt. Homogeneously pure Akt and PDK-1 were incubated with Mg^{2+}/ATP, in the presence of PS:PC: PtdIns-3,4,5-P_3 vesicles. The electrophoretic mobility and phosphorylation of specific residues was assessed by immunoblotting with either anti-HA, anti-P308, or anti-P473 antibodies. Two forms of Akt were used for this experiment: untreated Akt or Akt that had been thermally inactivated by sonication. Fig. 4*A*, *middle panel*, shows that both forms of Akt were phosphorylated on Thr-308, with maximal phosphorylation catalyzed within 5 min. Thus, the untreated and thermally inactivated forms of Akt were equally good substrates for PDK-1, indicating that the sonication had not grossly perturbed the structure of the kinase. Analysis with the P473 antibody revealed incubation with Mg^{2+}/ATP resulted in phosphorylation of Ser-473. The kinetics of phosphorylation mirrored those of Thr-308, with maximal phosphorylation observed within the first 5 min of the reaction. Note that a mobility shift to a slower migrating

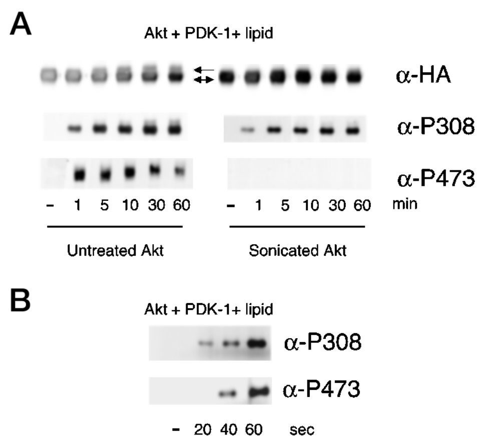

FIG. 4. **Phosphorylation of Ser-473 *in vitro* requires prior phosphorylation of the activation loop and requires the intrinsic catalytic activity of Akt.** *A*, Western blot showing native Akt (*left panels*) or Akt thermally inactivated by sonication (*right panels*) incubated with Mg^{2+}/ATP in the presence of PS:PC:PtdIns-3,4,5-P_3 vesicles and PDK-1 for the indicated times. The immunoblot was probed with antibody to the HA tag (*α-HA*), the phosphorylated activation loop (*α-P308*), or the phosphorylated hydrophobic site (*α-P473*; New England Biolabs PAN antibody that recognizes the hydrophobic phosphorylation motif in all the protein kinase Cs as well as Akt). −, no ATP added. *B*, Western blot showing Akt incubated with Mg^{2+}/ATP in the presence of PS:PC:PtdIns-3,4,5-P_3 vesicles and PDK-1 for the indicated times. The immunoblots were probed with the antibodies described in the legend to *A*. The immunoblots are representative of two independent experiments performed in triplicate.

species (*single arrow*) lagged behind the phosphorylation of Thr-308/Ser-473; this shift likely represents additional autophosphorylations that follow the two priming events. A more detailed analysis of the initial phosphorylation (Fig. 4*C*) revealed that phosphorylation of Ser-473 closely followed that of Thr-308, with Thr-308 being the first phosphorylation event (compare 20-s time points). Fig. 4*B*, *left panels*, shows that the phosphorylation of Ser-473 was abolished in the thermally inactivated sample. This reveals that the Ser-473 phosphorylation requires the activity of Akt and cannot result from contaminating kinases or from phosphorylation by PDK-1, as suggested recently (28).

Conclusions—The hydrophobic site has generally been accepted to be regulated by a heterologous kinase, referred to as PDK-2 (14) or Ser-473 kinase (9). Interestingly, a yeast two-hybrid screen led to the identification of the carboxyl terminus of the protein kinase C-related kinase, PRK2, as a module interacting with PDK-1 (28). *In vitro* studies revealed that a peptide based on this sequence, PIF (PDK-1 interacting fragment), promoted the incorporation of phosphate onto Ser-473 of Akt in the presence of PDK-1. This led to the proposal that PDK-2 is actually PDK-1 which undergoes a dramatic, and unprecedented, switch in substrate specificity through its interaction with PIF. Because this study did not examine whether kinase-inactive constructs of Akt are phosphorylated by PIF-bound PDK-1, it did not address whether autophosphorylation was promoted by binding of PDK-1 to PIF. One possibility is that PDK-1 binding to Akt sterically or conformationally blocks the hydrophobic site, thus preventing autophosphorylation. Autophosphorylation may be triggered by release of PDK-1 from Akt, an event that may be promoted by PIF.

Taken together with previous studies, our data support the following, alternative model for the regulation of Akt. In unstimulated cells, Akt is present in the cytosol in a conformation that blocks access of PDK-1 to the activation loop. Specifically, the PH domain masks Thr-308. Mitogen stimulation results in the membrane recruitment of Akt, unmasking of the activation loop, and allowing the phosphorylation of Thr-308 by PDK-1. The phosphorylation by PDK-1 renders Akt catalytically competent and causes the autophosphorylation of Ser-473 of the hydrophobic phosphorylation motif. This mechanism of regulation is similar to that recently elucidated for the regulation of the protein kinase C family of isozymes. Whether a hydrophobic site kinase, tentatively referred to as PDK-2, exists for other kinases such as p70 S6 kinase, or whether these kinases also autophosphorylate at the hydrophobic site, remains to be determined.

Acknowledgments—We thank Frank Sicheri and Kenji Miyata for providing the purified Akt, Anna DePaoli Roach for the gift of the catalytic subunit of protein phosphatase, David Sweatt for providing the antibody against phosphorylated Thr-641, Michael Comb (New England Biolabs) for the phospho-hydrophobic site antibody, and Carmen Baca (University of California at San Diego) and Wendy Chaplis (Boston Biomedical Research Institute) for excellent technical assistance.

REFERENCES

1. Newton, A. C. (1997) *Curr. Opin. Cell Biol.* **9,** 161–167
2. Chou, M. M., and Blenis, J. (1995) *Curr. Opin. Cell Biol.* **7,** 806–814
3. Taylor, S. S., and Radzio-Andzelm, E. (1994) *Structure* (*Lond.*) **2,** 345–355
4. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) *Curr. Biol.* **5,** 1394–1403
5. Pearson, R. B., Dennis, P. B., Han, J.-W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E. H., and Thomas, G. (1995) *EMBO J.* **14,** 5279–5287
6. Downward, J. (1998) *Curr. Opin. Cell Biol.* **10,** 262–267
7. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8,** 55–62
8. James, S. R., Downes, C. P., Gigg, R., Grove, S. J. A., Holmes, A. B., and Alessi, D. R. (1996) *Biochem. J.* **315,** 709–713
9. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 31515–31524
10. Frech, M., Andjelkovic, M., Ingley, E., Reddy, K. K., Falck, J. R., and Hemmings, B. A. (1997) *J. Biol. Chem.* **272,** 8474–8481
11. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) *Mol. Cell. Biol.* **17,** 338–344
12. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) *Science* **275,** 665–668
13. Watton, S. J., and Downward, J. (1999) *Curr. Biol.* **9,** 433–436
14. 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
15. 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
16. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998) *Science* **279,** 710–714
17. Peterson, R. T., and Schreiber, S. L. (1999) *Curr. Biol.* **9,** R521–R524
18. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551
19. Behn-Krappa, A., and Newton, A. C. (1999) *Curr. Biol.* **9,** 728–737
20. Dutil, E. M., Toker, A., and Newton, A. C. (1998) *Curr. Biol.* **8,** 1366–1375
21. Edwards, A. S., Faux, M. C., Scott, J. D., and Newton, A. C. (1999) *J. Biol. Chem.* **274,** 6461–6468
22. Orr, J. W., Keranen, L. M., and Newton, A. C. (1992) *J. Biol. Chem.* **267,** 15263–15266
23. Sweatt, J. D., Atkins, C. M., Johnson, J., English, J. D., Roberson, E. D., Chen, S. J., Newton, A., and Klann, E. (1998) *J. Neurochem.* **71,** 1075–1085
24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) *Current Protocols in Molecular Biology* (Chanda, V. B., ed) John Wiley & Sons, New York
25. 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
26. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81,** 727–736
27. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998) *Oncogene* **17,** 313–325
28. 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
29. Bellacosa, A., Franke, T. F., Gonzalez-Portal, M. E., Datta, K., Taguchi, T., Gardner, J., Cheng, J. Q., Testa, J. R., and Tsichlis, P. N. (1993) *Oncogene* **8,** 745–754
30. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9267–9271
31. Brodbeck, D., Cron, P., and Hemmings, B. A. (1999) *J. Biol. Chem.* **274,** 9133–9136
32. Ono, Y., Kurokawa, T., Kawahara, K., Nishimura, O., Marumoto, R., Igarashi, K., Sugino, Y., Kikkawa, U., Ogita, K., and Nishizuka, Y. (1986) *FEBS Lett.* **203,** 111–115
33. Kozma, S. C., Ferrari, S., Bassand, P., Siegmann, M., Totty, N., and Thomas, G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7365–7369
34. Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G., and McKnight, G. S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1300–1304