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The Protein Kinase Encoded by the *Akt* Proto-Oncogene Is a Target of the PDGF-Activated Phosphatidylinositol 3-Kinase

Thomas F. Franke,* Sung-Il Yang,†‡ Tung O. Chan,* Ketaki Datta,* Andrius Kazlauskas,§ Deborah K. Morrison,† David R. Kaplan,† and Philip N. Tsichlis*

*Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111
†Eukaryotic Signal Transduction Section
ABL–Basic Research Program
National Cancer Institute
Frederick Cancer Research Facility
and Development Center
Frederick, Maryland 21702
§National Jewish Center for Immunology
and Respiratory Medicine
Denver, Colorado 80206

Summary

The serine/threonine protein kinase encoded by the *Akt* proto-oncogene is catalytically inactive in serum-starved primary and immortalized fibroblasts. Here we show that Akt and the Akt-related kinase AKT2 are activated by PDGF. The activation was rapid and specific, and it was abrogated by mutations in the Akt Pleckstrin homology (PH) domain. The Akt activation was also shown to depend on PDGFRβ tyrosines Y740 and Y751, which bind phosphatidylinositol 3-kinase (PI 3-kinase) upon phosphorylation. Moreover, Akt activation was blocked by the PI 3-kinase–specific inhibitor wortmannin and the dominant inhibitory N17Ras. Conversely, Akt activity was induced following the addition of phosphatidylinositol-3-phosphate to Akt immunoprecipitates from serum-starved cells in vitro. These results identify Akt as a novel target of PI 3-kinase and suggest that the Akt PH domain may be a mediator of PI 3-kinase signaling.

Introduction

Following ligand binding, tyrosine kinase receptors undergo dimerization leading to the activation of their intrinsic tyrosine kinase activity and autophosphorylation. The tyrosine-phosphorylated regions of the receptor, in turn, function as high affinity binding sites for a series of Src homology 2 (SH2) domain– and phosphotyrosine-binding (PTB) domain–containing proteins (Cohen et al., 1995; Pawson, 1995), which, upon phosphorylation, transduce receptor-generated signals (Heldin, 1995). The receptor binding of these signaling proteins is site specific (Cohen et al., 1995; Pawson, 1995). Mutation of individual receptor autophosphorylation sites prevents the transduction of signals mediated by the molecules that bind to these sites (Valius and Kazlauskas, 1993). On the basis of these observations, it has been proposed that the unique specificity of individual receptors in a given cell type is determined by the sum of the activities of the signaling proteins that interact with the receptor in its tyrosine-phosphorylated form. Accordingly, the interaction of overlapping subsets of signaling proteins with different activated receptors initiates distinct, receptor-specific signaling cascades (Heldin, 1995).

Mitogenic signals are transmitted from the activated tyrosine kinase receptors through several known pathways. One such pathway involves the activation of protein kinase C (PKC) (Divecha and Irvine, 1995); another, the activation of the mitogen-activated protein kinases (MAPK or ERK [for extracellular signal–regulated protein kinase]) (Marshall, 1995); a third, the phosphorylation and activation of the $p70^{S6k}$ kinase (Downward, 1994); and a fourth, the tyrosine phosphorylation and nuclear translocation of a family of cytoplasmic transcription factors called STATs (for signal transducer and activator of transcription) (Heldin, 1995). Triggering these pathways depends on direct protein–protein interactions, posttranslational modifications, and the induction of second messenger molecules (Marshall, 1995; Divecha and Irvine, 1995). The synthesis of one class of such molecules involves the phosphorylation of phosphatidylinositol and its D-4- and D-5-phosphorylated derivatives in the D-3 position, in a reaction catalyzed by the phosphatidylinositol 3-kinase (PI 3-kinase) (Kapeller and Cantley, 1994). Recent studies have shown that the PI 3-kinase exhibits both lipid and protein kinase activities (Kapeller and Cantley, 1994), activates the $p70^{S6k}$ (Downward, 1994), and in many cell types, plays a critical role in mitogenic or other signaling (Kapeller and Cantley, 1994).

Akt (or c-*akt*), the cellular homolog of the viral oncogene v-*akt*, encodes a serine/threonine protein kinase that is ubiquitously expressed (Bellacosa et al., 1993) and whose catalytic domain is closely related to the catalytic domain of all the members of the PKC family (Bellacosa et al., 1991). Akt, however, differs from other PKC family members in that it contains an N-terminal domain (amino acids 1–147) (Akt homology [AH] domain) (Bellacosa et al., 1991, 1993; Franke et al., 1994), part of which (amino acids 1–106) is related to the Pleckstrin homology (PH) domain, present in a large number of signaling molecules (Cohen et al., 1995). Recent studies from this laboratory have shown that the AH domain of Akt is a domain of protein–protein interaction that mediates the formation of Akt multimeric complexes and that the formation of these complexes is associated with protein kinase activation (Datta et al., 1995). Probing DNA from vertebrate and invertebrate species with an AH/PH domain probe revealed that Akt is a member of a conserved family of kinases (Cheng et al., 1992; Franke et al., 1994). In addition to *Akt*, this family includes the human *AKT1/RACα* and *AKT2/RACβ* (Cheng et al., 1992), the Drosophila melanogaster *Dakt1* (Franke et al., 1994), and at least two Caenorhab-

‡Present address: Korea Research Institute of Chemical Technology, Taejon, Korea 305–606.

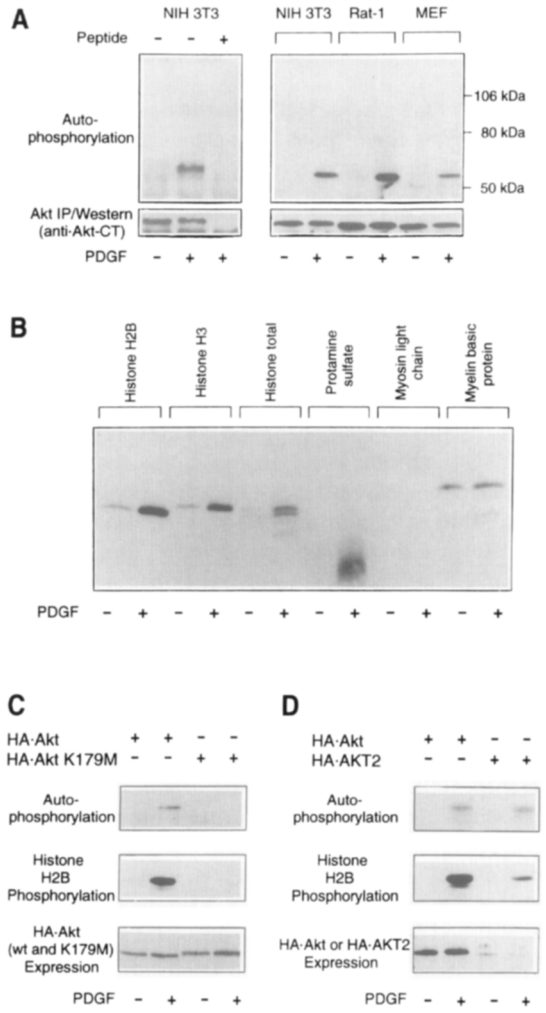

Figure 1. The Akt Kinase Is Activated by PDGF: Identification of Akt Substrates

(A) In vitro kinase assays of Akt immunoprecipitated, in the presence or absence of immunizing peptide (1 μM), from lysates of serum-starved or serum-starved and PDGF-stimulated (50 ng/ml PDGF-AB for 10 min) NIH 3T3 cells (left) or NIH 3T3, Rat-1, and MEF cells (right). Immunoprecipitations were carried out with the anti-Akt-CT antibody. The lower part of both panels shows an anti-Akt-CT Western blot of the immunoprecipitates used in the experiment.

(B) Substrate specificity of Akt. In vitro phosphorylation of a panel of exogenous substrates (500 ng per reaction).

(C) Kinase-defective Akt does not respond to PDGF. In vitro kinase assays of Akt immunoprecipitated with the anti-HA monoclonal antibody 12CA5 from NIH 3T3 cells transiently transfected with HA–Akt or HA–Akt K179M. The exogenous substrate, histone H2B, was used at a concentration of 0.1 mg/ml. The expression of the wild-type and mutant Akt proteins was monitored by probing a Western blot of the immunoprecipitates with the polyclonal anti-HA antibody.

(D) Activation of AKT2 by PDGF. In vitro kinase assays of Akt and AKT2 immunoprecipitated with the anti-HA monoclonal antibody from NIH 3T3 cells transiently transfected with HA–Akt or HA–AKT2. Protein expression was monitored by probing a Western blot of the immunoprecipitates with the anti-Akt-CT antibody.

ditis elegans genes (Waterston et al., 1992). Of these genes, *AKT2/RACβ* is amplified in approximately 10% of human ovarian neoplasms (Cheng et al., 1992).

In this paper, we present evidence that Akt is activated rapidly and specifically following exposure of primary mouse embryo fibroblasts (MEFs), NIH 3T3 cells, and Rat-1 cells to platelet-derived growth factor (PDGF). The related kinases AKT2 and Dakt1 are also activated by PDGF. Genetic, pharmacological, and biochemical studies revealed that the transduction of PDGF-generated Akt activation signals depends on the activation of the PI 3-kinase and on the Akt AH/PH domain. In addition to defining a signal that activates the Akt kinase, these data also provide a function for the PI 3-kinase-generated D-3-phosphorylated phosphoinositides and suggest that the Akt AH/PH domain may be a mediator of PI 3-kinase signaling.

Results

PDGF Induces Akt Kinase Activity

Previous studies had shown that the murine serine/threonine protein kinase Akt is ubiquitously expressed (Bellacosa et al., 1993). However, Akt immunoprecipitated from serum-starved immortalized mouse and rat embryo fibroblasts (NIH 3T3 and Rat-1 cells) as well as primary MEFs is catalytically inactive. Since PDGF is one of the major mitogens in serum (Stiles et al., 1979), we examined whether PDGF induced Akt protein kinase activity in serum-starved cells. NIH 3T3 cells were exposed to PDGF-AB (50 ng/ml) following 24 hr of serum starvation. After 10 min, the cells were lysed, and Akt was immunoprecipitated with a polyclonal anti-Akt antiserum raised against a peptide composed of the 15 C-terminal amino acids of Akt (anti-Akt-CT). In parallel reactions, the immunoprecipitation of Akt was competed by excess immunizing peptide (1 μM). Akt kinase activity was induced rapidly, following exposure to PDGF (Figure 1A, left). Moreover, Akt was activated by PDGF not only in NIH 3T3 but also in Rat-1 and MEF cells by using a similar protocol (Figure 1A, right).

The end point in the assays, shown in Figure 1A, was the autophosphorylation of Akt. To identify a suitable exogenous substrate, we carried out in vitro kinase reactions of Akt immunoprecipitated from serum-starved or serum-starved and PDGF-stimulated NIH 3T3 cells by use of the panel of proteins shown in Figure 1B. Among six proteins compared in this experiment, histone H2B was the most sensitive and specific Akt substrate.

The PDGF-induced kinase activity could be due to the activation of Akt or to the activation of a coprecipitating protein kinase. To distinguish between these possibilities, we constructed a kinase-defective Akt mutant in which the lysine of the ATP-binding site at position 179 (Bellacosa et al., 1993) was replaced by a methionine (Akt K179M). Expression constructs of wild-type and kinase-defective Akt carrying a hemagglutinin (HA)-derived N-terminal epitope tag were transiently transfected into NIH 3T3 cells. The wild-type and the kinase-defective mutant Akt proteins were subsequently immunoprecipitated from serum-starved, transiently transfected cells before and after PDGF stimulation. PDGF activated only the wild-type Akt kinase. These results confirmed that the PDGF-induced protein kinase activity associated with the Akt immunoprecipitates was due to the activation of Akt.

To determine whether the human Akt-related kinase

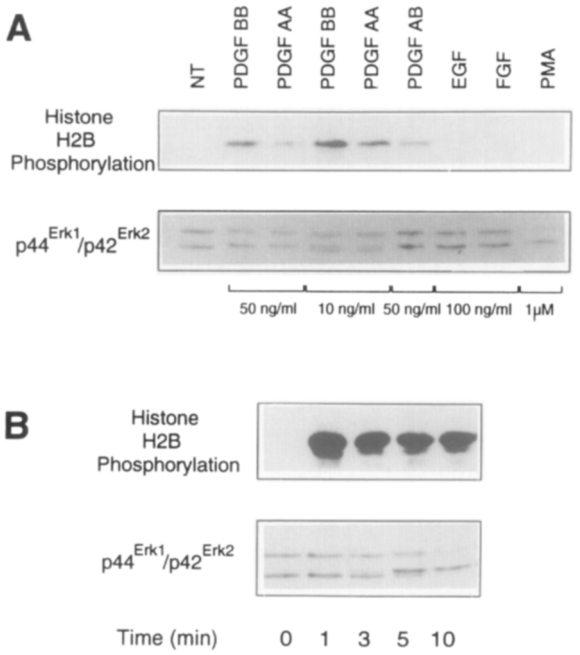

Figure 2. Akt Is Activated Rapidly and Specifically by PDGF

(A) The activation of Akt by PDGF is growth factor-specific. NIH 3T3 cells were serum-starved overnight and then exposed for 10 min to a panel of growth factors as indicated.

(B) Time course of Akt activation by PDGF in serum-starved (time 0) or serum-starved and PDGF-stimulated NIH 3T3 cells. The lower parts of both panels show Western blots of total cell lysates, probed with the anti-Erk antibody.

AKT2 was also activated by PDGF, we transiently transfected NIH 3T3 cells with HA epitope–tagged *AKT2* expression constructs. AKT2 immunoprecipitated from the transfected cells with the anti-HA monoclonal antibody 12CA5 was activated by PDGF (Figure 1D). The expression of the transiently transfected HA–*AKT2* construct was confirmed by probing a Western blot of the anti-HA immunoprecipitates with the anti-Akt-CT antibody (Figure 1D). In similar experiments, the closest homolog of Akt in humans, AKT1, and the Drosophila homolog Dakt1 were also activated by PDGF (data not shown). Moreover, the protein encoded by the viral oncogene v-*akt* was shown to exhibit basal kinase activity and enhanced responsiveness to PDGF stimulation (T. F. F. and P. N. T., unpublished data).

Akt Kinase Activity Is Induced Rapidly and Specifically by PDGF

To determine the specificity of the PDGF-induced activation of Akt, we carried out in vitro kinase assays using Akt immunoprecipitates from serum-starved NIH 3T3 cells before and after exposure to PDGF-AA, PDGF-BB, PDGF-AB, epidermal growth factor (EGF), fibroblast growth factor (FGF), and phorbol-12-myristate-13-acetate (PMA). The results (Figure 2A) revealed that the PDGF-induced activation of Akt is highly specific, in that it is induced by all three isoforms of PDGF but not by the other tested factors. The failure of EGF, FGF, and PMA to induce the activation of Akt could be due to the failure of the NIH 3T3 cells to respond to these factors. To test this possibility, we examined the SDS–polyacrylamide gel electrophoresis migration of the MAP kinases p44^{Erk1} and p42^{Erk2}, in the same lysates used for the in vitro kinase assays in Figure 2A, by Western blot analysis using a polyclonal anti-Erk antiserum. EGF, FGF, and PMA each induced MAPK size shifts diagnostic of MAPK activation (Figure 2A) (Marshall, 1995). Therefore, the failure of these factors to induce Akt activation was not due to their inability to stimulate the NIH 3T3 cells.

Subsequently, we examined the kinetics of Akt activation following exposure of serum-starved NIH 3T3 cells to PDGF at room temperature and at 4°C. Figure 2B shows the time course of Akt activation at room temperature. Akt was activated within 1 min, with maximum activity reached within 3 min of exposure to PDGF. A delay in Akt activation (3 min) was observed when the stimulation was carried out at 4°C (data not shown). Western blot analyses of the cell lysates in Figure 2B, using the anti-Erk antiserum, showed that the PDGF-induced MAPK size shift was delayed relative to the PDGF-induced activation of Akt. Despite the rapidity of activation of Akt, however, immunoprecipitations of the PDGF receptor (PDGFR) or of Akt from lysates of PDGF-stimulated NIH 3T3 cells failed to show a direct association between the two molecules (data not shown).

The PDGF-Induced Activation of Akt Is AH/PH Domain Dependent

Given that the AH/PH domain is a characteristic feature of all the Akt family members (Cheng et al., 1992; Franke et al., 1994) and that all the known members of this family are activated by PDGF (this paper), we examined whether the AH/PH domain may be mediating PDGF-induced Akt signals. Constructs of two mutants, one carrying an arginine to cysteine mutation at amino acid 25 (HA–Akt R25C) and another carrying a deletion of amino acids 11–35 (HA–Akt Δ11–35), were transiently transfected in NIH 3T3 cells. In vitro kinase assays of the two mutants immunoprecipitated from lysates of serum-starved or serum-starved and PDGF-stimulated cells revealed that both fail to respond

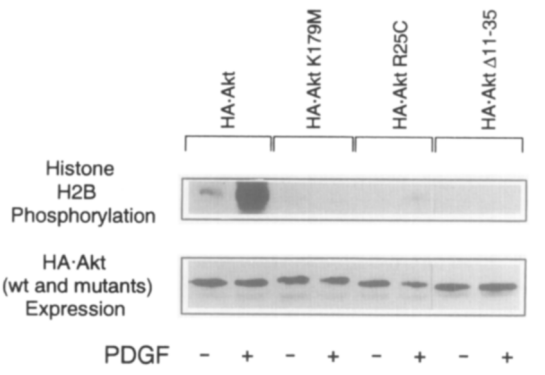

Figure 3. Mutations in the AH/PH Domain of Akt Abolish Akt Activation

In vitro kinase assays of Akt immunoprecipitated with the monoclonal anti-HA antibody from NIH 3T3 cells transiently transfected with the indicated HA-tagged Akt constructs. Expression of the transfected constructs was assessed by probing a Western blot of the immunoprecipitates with the anti-HA antiserum.

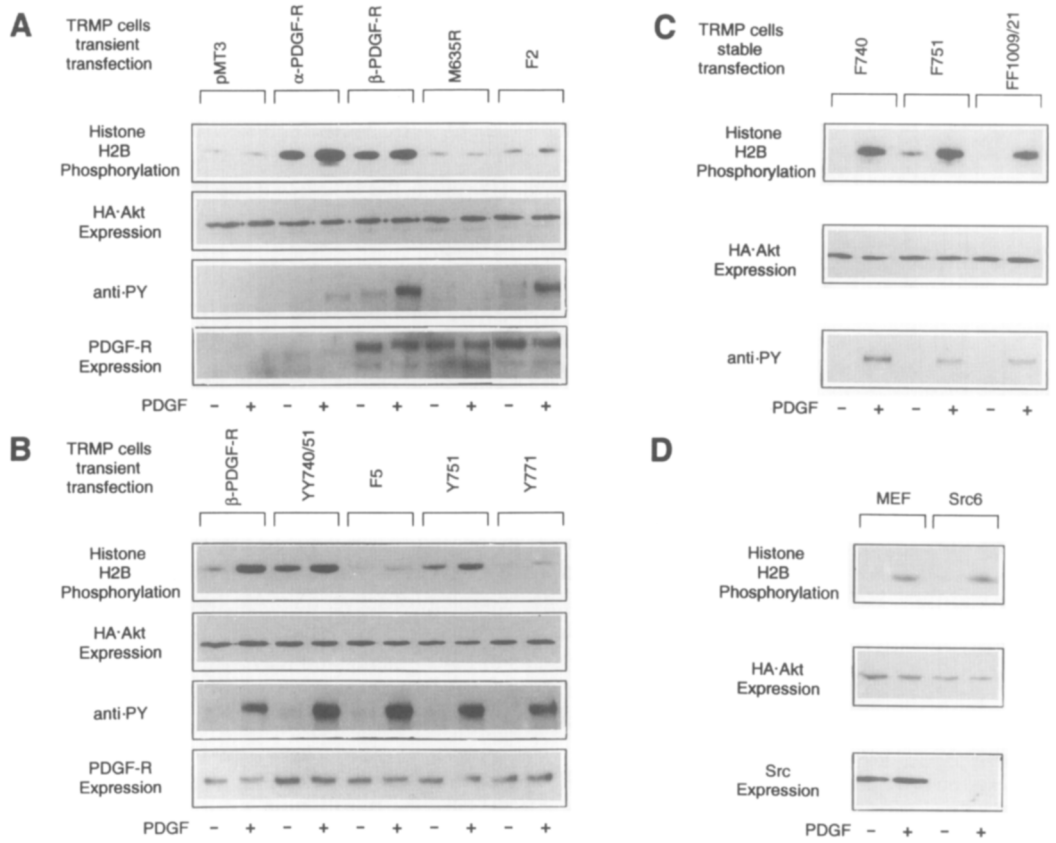

Figure 4. Akt Activation Is Mediated by Y740 and Y751 of the PDGFRβ

(A) Y740 and Y751 of the PDGFRβ (β-PDGF-R) are required for Akt activation. In vitro kinase assays of Akt immunoprecipitated with the anti-HA monoclonal antibody from TRMP cells transiently transfected with HA–Akt and pMT3 vector DNA, or the indicated pMT3-based PDGF receptor constructs. The cells were stimulated with PDGF-AB (400 ng/ml for 5 min) following overnight serum starvation.

(B) Y740 and Y751 in the context of the F5 mutant are sufficient for Akt activation. In vitro kinase assays of Akt immunoprecipitated with the anti-HA monoclonal antibody from TRMP cells transiently transfected with HA–Akt and the indicated PDGFRβ constructs. The cells were stimulated with PDGF-BB (400 ng/ml) following overnight serum starvation.

(C) Tyrosine to phenylalanine mutations at position 740 (F740) or 751 (F751) of the PDGFRβ do not abolish Akt activation. In vitro kinase assays of Akt immunoprecipitated with the anti-HA monoclonal antibody from TRMP cells stably transfected with PDGFRβ mutants, as indicated, and supertransfected transiently with HA–Akt. The cells were stimulated with PDGF-BB (400 ng/ml) following overnight serum starvation.

(D) Src is not required for Akt activation. In vitro kinase assays of Akt immunoprecipitated with the anti-HA monoclonal antibody from primary MEFs and SV40-transformed MEFs from *Src* knockout mice (Src6), transiently transfected with HA–Akt. Following overnight serum starvation, the cells were stimulated with PDGF-AB (50 ng/ml).

to PDGF stimulation (Figure 3). The R25C mutation was tested because a comparable mutation (R28C) in the PH domain of Bruton's tyrosine kinase (Btk) had been shown to be responsible for the induction of X-linked immunodeficiency in CBA/N mice (Rawlings et al., 1993). To determine whether these AH/PH mutations affected the basal kinase activity of Akt, we overexpressed the wild-type and mutant proteins in COS1 cells, and following serum starvation, we examined their kinase activity. The results (data not shown) revealed that the mutant proteins continued to exhibit kinase activity. Therefore, the AH/PH mutations did not affect the ability of these proteins to function as protein kinases.

The Activation of Akt by PDGF Requires Signals That Depend upon the PDGFRβ Tyrosines 740 and 751

To define the signaling pathway responsible for the PDGF-induced activation of Akt, we cotransfected canine kidney epithelial TRMP cells, which do not express PDGFR (Valius and Kazlauskas, 1993), with wild-type or mutant PDGFR and HA epitope–tagged Akt constructs. Following PDGF stimulation, Akt was immunoprecipitated from the transfected cells by use of the monoclonal anti–HA tag antibody. In vitro kinase assays of the Akt immunoprecipitates, shown in Figure 4A, revealed that both the α and the β subunits of the human PDGFR (Heldin, 1995) transduce Akt activation signals. However, the kinase-defective PDGFRβ mutant M635R and another mutant, whose two tyrosine phosphorylation sites Y740 and Y751 had been mutated into phenylalanines (F2), failed to transmit PDGF signals leading to Akt activation. The phosphorylation of the PDGFRβ tyrosine Y751 generates a high affinity binding site for Nck (Heldin, 1995), while the phosphorylation of tyrosines Y740 and Y751 generates a high affinity binding site for the p85 regulatory subunit of the PI 3-kinase

(Heldin, 1995). These data suggested, therefore, that the transduction of PDGF-induced Akt activation signals depends on the interaction of the activated PDGFR with PI 3-kinase, Nck, or both.

To confirm and extend these results, we repeated the experiment in Figure 4A but used the wild-type PDGFRβ; a PDGFRβ mutant whose tyrosine phosphorylation sites Y740, Y751, Y771, Y1009, and Y1021 had been mutated into phenylalanines (F5 mutant) (Valius and Kazlauskas, 1993); and three F5 phenylalanine to tyrosine add-back revertants (YY740/51, Y751, and Y771). The tyrosine phosphorylation sites Y771, Y1009, and Y1021 direct the binding of rasGAP, Syp, and phospholipase C (PLC)-γ1 to PDGFRβ, respectively (Heldin, 1995). The results (Figure 4B) revealed that full activation of Akt required tyrosine residues at both the 740 and 751 positions. The phenylalanine to tyrosine mutation at position 751 could partially reconstitute the ability of the receptor to activate Akt following PDGF stimulation. Finally, the Y771 revertant was identical to the F5 mutant with regard to its inability to activate Akt. Both the HA–Akt and the PDGFR constructs were expressed in the transfected cells. Moreover, both the α and β subunits of the PDGFR as well as all the mutants of the PDGFRβ, with the exception of M635R, responded to PDGF stimulation with tyrosine autophosphorylation (Figures 4A and 4B). Therefore, the failure of some of the mutant receptors to activate Akt was not due to lack of expression or to lack of PDGF receptor activity.

The results of the experiment in Figure 4B were confirmed by additional experiments utilizing TRMP cells stably transfected with expression constructs of PDGFRβ mutants in which the tyrosines Y740, Y751, or Y1009 and Y1021 had been changed into phenylalanines. The end point in these experiments was the activation of the Akt protein kinase encoded by a transiently transfected HA–Akt expression construct. The results (Figure 4C) showed that PDGFRβ constructs with tyrosine to phenylalanine mutations at position 740 or 751 encoded proteins that were able to transmit PDGF-induced Akt activation signals. TRMP cells stably transfected with PDGFRβ constructs, carrying tyrosine to phenylalanine mutations at positions Y1009 and Y1021, were also shown to respond to PDGF with full activation of the Akt kinase. These results confirmed the results of the preceding experiments. Moreover, they suggested that the phosphotyrosine phosphatase Syp and PLC-γ1, which bind to tyrosines Y1009 and Y1021, respectively (Heldin, 1995), are not involved in the activation of Akt. Finally, given that the mutant F751 retains the ability to transduce Akt activation signals, these data suggested that it is unlikely for Nck, which binds to phosphorylated Y751 (Heldin, 1995), to contribute to this process.

In addition to the PDGFRβ tyrosines mutated in the F5 mutant, other tyrosines involved in PDGF signaling are the tyrosines at positions 579 and 581. These tyrosines define the high affinity binding site for Src (Heldin, 1995). To determine whether Src is involved in the PDGF-induced activation of Akt, we could not use phenylalanine to tyrosine receptor mutants at this site, because these mutants lack tyrosine kinase activity (Heldin, 1995). To address this question, we used a large T antigen–immortalized mouse embryo fibroblast cell line derived from *Src* knockout mice (Soriano et al., 1991). PDGF stimulation induced full activation of the Akt kinase (Figure 4D). Although it may be possible that other Src-like kinases can substitute for Src in these cells (Heldin, 1995), these data suggest that it is unlikely for Src to play an important role in Akt activation. The data shown in Figure 4 therefore collectively suggest that the major factor responsible for the PDGF-induced activation of Akt is the activation of the PI 3-kinase.

PDGF-Induced Akt Activation Is Inhibited by Wortmannin

The preceding results suggested that Akt activation is PI 3-kinase dependent. However, it is possible that Akt activation signals might be transduced by other proteins interacting with tyrosines Y740 or Y751 of the PDGFRβ. To address the role of the PI 3-kinase further, we examined the effects of the PI 3-kinase inhibitor wortmannin on the PDGF-induced Akt activation. Wortmannin at nanomolar concentrations is known to inhibit only the PI 3-kinase, whereas at micromolar concentrations, it also inhibits pro-

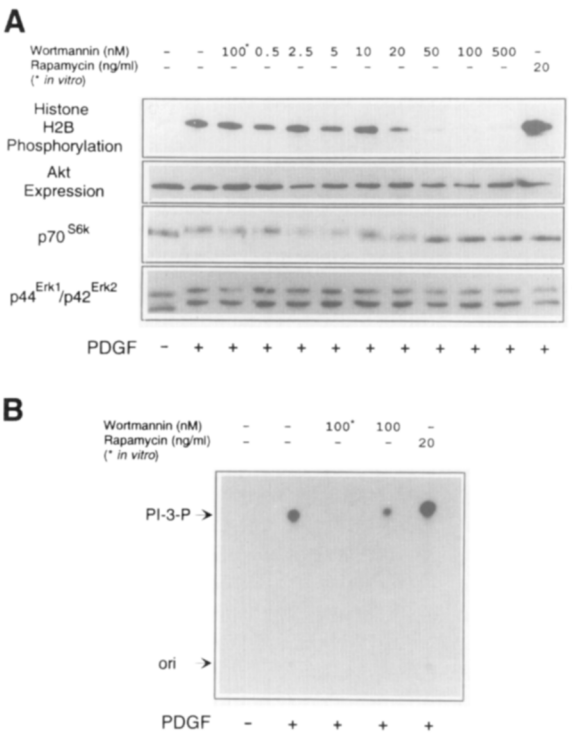

Figure 5. Wortmannin, but Not Rapamycin, Inhibits PDGF-Induced Akt Activation

(A) In vitro kinase assays of Akt immunoprecipitated from serum-starved NIH 3T3 cells treated with wortmannin or rapamycin dissolved in DMSO, or with DMSO only. Following treatment, all the cultures, with the exception of one that had been treated with DMSO only (first lane), were stimulated with PDGF-AB (50 ng/ml). Alternatively, wortmannin (100 nM) was added in vitro to lysates of NIH 3T3 cells stimulated with PDGF-AB (third lane, marked by asterisk). Anti-p70^{S6k} and anti-Erk antibodies were used to probe Western blots of NP-40 lysates from the same cells.

(B) PI 3-kinase assays were performed on NP-40 lysates from cells treated with wortmannin or rapamycin in parallel with the cells in (A).

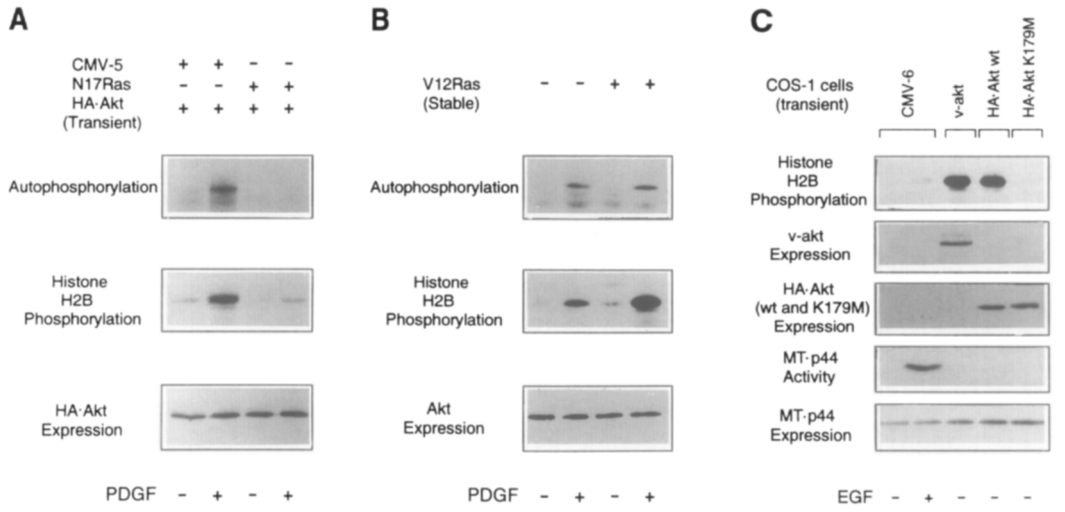

Figure 6. The Activation of Akt Is Inhibited by Dominant Inhibitory N17Ras, but Akt Fails to Activate the MAPK

(A) Dominant inhibitory N17Ras inhibits Akt activation. In vitro kinase assays of Akt immunoprecipitated with the monoclonal anti-HA antibody from NIH 3T3 cells transiently transfected with HA–Akt or HA–Akt and N17Ras in the expression vector pCMV-5.

(B) V12Ras does not activate Akt. In vitro kinase assays of Akt immunoprecipitated using the anti-Akt-CT antiserum from serum-starved or serum-starved and PDGF-treated fibroblasts or NIH 3T3 cells transformed by V12Ras.

(C) Akt does not induce MAPK activity. COS1 cells were transfected with Myc epitope–tagged MAPK (MT–p44) and v-akt or the indicated HA epitope–tagged Akt constructs. Akt and MAP kinase activities were examined in serum-starved or serum-starved and EGF-stimulated cells (100 ng/ml for 10 min).

tein kinases (Powis et al., 1994). NIH 3T3 cells were pretreated with increasing concentrations of wortmannin (0.5–500 nM) prior to PDGF stimulation. Alternatively, wortmannin was added in vitro to lysates of PDGF-stimulated NIH 3T3 cells (100 nM). Wortmannin inhibited Akt activation in vivo at concentrations similar to the ones inhibiting the PI 3-kinase (Powis et al., 1994). However, wortmannin failed to inhibit Akt in vitro (Figure 5A), suggesting that, at the concentrations used, it has no direct inhibitory effects on the activity of Akt.

The tyrosine kinase receptor–mediated activation of the PI 3-kinase leads to the activation of the $p70^{S6k}$ by a process that is inhibited not only by the PI 3-kinase inhibitor wortmannin but also by the macrolide antibiotic rapamycin (Downward, 1994). Rapamycin inactivates the PI 3-kinase-related protein RAFT1 (for rapamycin and FKBP12 target 1) by promoting its binding to FKBP12 (Sabatini et al., 1994). To determine whether Akt functions upstream or downstream of RAFT1 in the pathway leading to the activation of the $p70^{S6k}$, we examined the effects of rapamycin on the PDGF-induced activation of Akt. The results showed that rapamycin does not inhibit Akt (Figure 5A). Therefore, the activation of Akt is independent of RAFT1.

Subsequently, we examined the effects of wortmannin and rapamycin on the activity of the PI 3-kinase in the serum-starved or serum-starved and PDGF-stimulated NIH 3T3 cells utilized in the preceding experiment. PI 3-kinase assays carried out as described in the Experimental Procedures showed that wortmannin concentrations inhibiting the PDGF-induced activation of Akt also blocked the activity of the PI 3-kinase, whereas rapamycin had no effect on the activity of either (Figure 5B). Therefore, the activation of the PI 3-kinase and Akt by PDGF appear to be functionally linked.

PDGF-Induced Activation of Akt Is Inhibited by N17Ras

GTP-bound c-Ha-Ras interacts with the catalytic subunit of the PI 3-kinase and contributes to its activation (Rodriguez-Viciana et al., 1994). In accordance with this, the dominant negative mutant of c-Ha-Ras, N17Ras, blocks the receptor-mediated activation of the PI 3-kinase (Rodriguez-Viciana et al., 1994). To test whether Akt is a downstream target of the PI 3-kinase, we also examined whether its activation by PDGF was inhibited by the dominant negative mutant of c-Ha-Ras, N17Ras. To this end, we examined the kinase activity of HA epitope–tagged Akt before and after PDGF stimulation following cotransfection of N17Ras and HA–Akt. N17Ras partially blocks the PDGF-induced activation of Akt (Figure 6A). To determine whether c-Ha-Ras was sufficient to induce Akt activation, we examined the kinase activity of the endogenous Akt protein, before and after PDGF stimulation, in NIH 3T3 cells stably transfected with a construct of the activated form of c-Ha-Ras, V12Ras. V12Ras was not sufficient to activate Akt in the absence of PDGF (Figure 6B). This result may be due to the activation of the PI 3-kinase by V12Ras at levels below the threshold necessary for Akt activation (Rodriguez-Viciana et al., 1994). Alternatively, it may be due to the fact that the activation of Akt by PDGF may be mediated by both Ras-dependent and Ras-independent signals.

The Ras-dependent activation of Akt shown in Figure 6A suggested that Akt may be a member of a signaling pathway that activates the MAP kinase (Marshall, 1995). To address this question, we tested the ability of overexpressed, enzymatically active v-akt and HA–Akt to activate MAPK in COS1 cells. EGF stimulation was used as a positive control, and the kinase-defective mutant of Akt (HA–

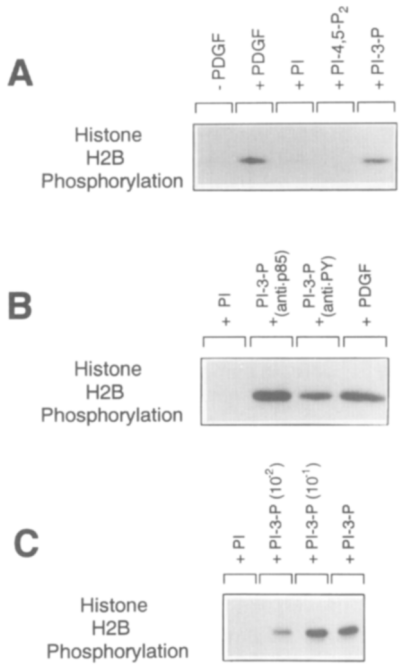

Figure 7. In Vitro Activation of Akt Activity by PIP

(A) Akt immunoprecipitated from lysates prepared from serum-starved or serum-starved and PDGF-stimulated NIH 3T3 cells is activated by PIP (PI-3-P) but not PI or PIP_2 (PI-4,5-P_2).

(B) Activation of Akt by PIP generated by PI 3-kinase immunoprecipitated either with anti-PI 3-kinase or with anti-PY.

(C) Akt activity is induced by PIP in a dose-dependent manner. Increasing concentrations of enzymatically produced PIP were added to Akt from serum-starved NIH 3T3 cells. PIP was diluted in PI carrier. Akt activity in the presence of PIP plus PI carrier relative to its activity in the presence of PI only was as follows: PI plus 10^{-2} PIP, 2.51×; PI plus 10^{-1} PIP, 4.54×; PIP, 4.49×.

Akt K179M) was used as a negative control. Even though v-akt and HA–Akt were active as determined by the ability of the immunoprecipitated proteins to phosphorylate histone H2B, they both failed to activate MAPK (Figure 6C). Oncogenic v-raf and V12Ras induced MAP kinase activity in parallel experiments (data not shown). Furthermore, MAPK was not activated in NIH 3T3 cells expressing v-akt (data not shown).

Akt Is Activated by PIP In Vitro

The data presented in this manuscript indicate that the PDGF-induced activation of Akt depends upon the integrity of its AH/PH domain, and upon the activation of the PI 3-kinase. Moreover, recent studies have shown that phosphatidylinositol 4,5-bisphosphate (PIP_2, also referred to as PI-4,5-P_2) binds to the PH domain of pleckstrin (Cohen et al., 1995). This observation, combined with our genetic and pharmacological data, suggested that Akt may be activated through the interaction between its AH/PH domain and PI 3-kinase-generated D-3-phosphorylated phosphoinositides. To test this hypothesis, we carried out in vitro kinase assays of Akt immunoprecipitated from serum-starved NIH 3T3 cells, in the presence of phosphatidylinositol (PI), PIP_2, and enzymatically synthesized phosphatidylinositol 3-phosphate (PIP, also referred to as PI-3-P) (Experimental Procedures). While PI or PIP_2 appeared to suppress Akt activity, PIP activated the Akt kinase in vitro (Figure 7A; Table 1). To confirm that the enzyme immunoprecipitated from PDGF-stimulated cells was the PI 3-kinase, the experiment in Figure 7A was repeated using anti-phosphotyrosine (anti-PY) or anti-p85 immunoprecipitates. The proteins immunoprecipitated with both antibodies phosphorylated PI (data not shown), and the products of both reactions induced Akt activation (Figure 7B). Serial dilutions of enzymatically produced PIP (using PI as a carrier) induced the activation of Akt in a dose-dependent manner (Figure 7C; Table 1). Owing to the suppression of Akt activity by PI, however, the induction of Akt activation by PIP appeared to be partially masked. Perhaps because of this and variations in the stoichiometry of phosphorylation of PI to PIP between experiments, the ability of different preparations of PIP to activate Akt in vitro varied (Table 1).

Table 1. Induction of Akt Activation by Phosphorylated PI Using PI as a Carrier

Phospholipid Added In Vitro	Fold Activation
Mock treated	1.58 ± 0.56
PI	1
Undiluted PIP	4.37 ± 2.36
PI plus 10^{-1} PIP	2.57 ± 1.32
PI plus 10^{-2} PIP	1.54 ± 0.56
PI plus 10^{-3} PIP	1.34 ± 0.01
PI plus 10^{-4} PIP	1.05 ± 0.04

Akt was immunoprecipitated from serum-starved NIH 3T3 cells by use of anti-Akt-CT. After washing, immunoprecipitates were divided equally and treated with 30 mM HEPES (pH 7.4) (mock), sonicated PI, or PIP as indicated. PIP generated enzymatically, using PI as the substrate, was used either directly or after dilution in carrier PI as indicated. Mean and standard deviation values were calculated on the basis of five experiments, with the exception of the 10^{-3} and 10^{-4} values, which were calculated on the basis of the results of three experiments. In all experiments PI was given a value of 1. Quantitation of experiments was performed with a Phosphorimager.

Discussion

In this paper, we have shown that the product of the c-*akt* proto-oncogene is specifically activated by PDGF. To determine the mechanism for PDGF-induced Akt activation, we expressed the wild-type PDGFR and a series of PDGFRβ tyrosine mutants in PDGFR-negative TRMP cells. Akt activity was examined in these cells before and after PDGF stimulation. The results showed that tyrosines Y740 and Y751, which upon autophosphorylation form a high affinity binding site for the p85 regulatory subunit of the PI 3-kinase (Heldin, 1995), are required for PDGF-induced Akt activation. These results suggested that Akt may be a downstream target of the PDGF-activated PI 3-kinase. Additional studies supporting this hypothesis showed that the activation of Akt by PDGF is abrogated by the PI 3-kinase inhibitor wortmannin (Powis et al., 1994) and by the dominant negative mutant of c-Ha-Ras, N17Ras. However, the activation of Akt was not abrogated by rapamycin (Figure 8), a macrolide antibiotic that inacti-

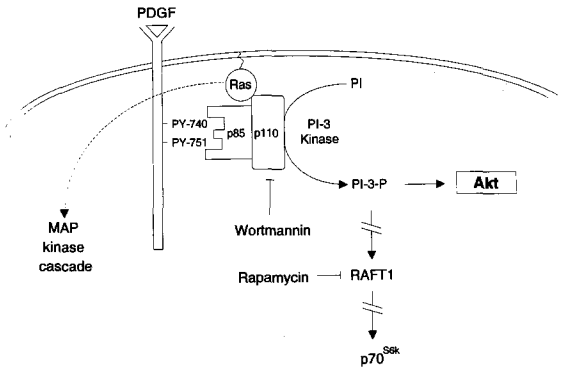

Figure 8. A Model for Akt Activation by PDGF

PDGF- and Ras-dependent activation of the PI 3-kinase induces PIP (PI-3-P), which activates Akt perhaps via binding to the Akt PH domain (see Discussion for details).

vates the PI 3-kinase homolog RAFT1 by promoting its interaction with FKBP12 (Sabatini et al., 1994). Mutational analysis of Akt revealed that the AH/PH domain is required for its PI 3-kinase-dependent activation. These results, combined, suggested that the PDGF-mediated activation of Akt may be due to conformational changes induced by the interaction of Akt with phosphoinositides phosphorylated in the D-3 position by the PDGF-activated PI 3-kinase. The ability of D-3-phosphorylated phosphoinositides to activate Akt was confirmed by in vitro kinase assays that examined the activity of Akt immunoprecipitated from serum-starved NIH 3T3 cells in the presence or absence of exogenously added phosphoinositides. The activation of Akt and perhaps other members of the Akt family of kinases via their interaction with this class of phospholipids is particularly interesting in light of the relationship of the Akt and PKC kinase families (Bellacosa et al., 1991). These findings, therefore, reinforce the concept that the two families are ancestrally related and that they may have been evolutionarily separated to optimize their response to different second messenger lipids.

The N-terminal 147 amino acids of Akt define a domain that is conserved among Akt molecules (the AH domain) (Franke et al., 1994) and whose 106 N-terminal amino acids exhibit homology with the PH domain (Cohen et al., 1995). Recently, the solution structure of the PH domains of pleckstrin and β-spectrin and the crystal structure of the PH domain of dynamin were determined (Cohen et al., 1995). All three structures revealed that the N-terminal portion of the PH domain forms a hydrophobic pocket consisting of seven antiparallel β sheets, whereas its C-terminal portion forms an amphipathic α helix that may cap the pocket. This structure suggested that PH domains may interact with lipids and perhaps other hydrophobic ligands. Support for this hypothesis was provided by experiments showing that PIP_2 binds the PH domain of pleckstrin (Cohen et al., 1995) and by earlier studies suggesting that the PH domain of PLC-δ_1 may be involved in the binding of the enzyme to lipid bilayers containing PIP_2 (Cifuentes et al., 1994). Viewed in the context of these observations, our data suggest that the PI 3-kinase-dependent activation of Akt may be due to the direct interaction of PIP with the hydrophobic pocket of the Akt AH/PH domain. We cannot, however, exclude the possibility that minor phospholipid products of the PI 3-kinase or of other enzymes coimmunoprecipitated with the PI 3-kinase contribute to the activation of Akt in our assay system. To confirm these results, it will therefore be necessary to use purified or synthetic phospholipids as they become available (Divecha and Irvine, 1995).

Our earlier studies had shown that Akt forms multimeric complexes through the interaction between AH domains and that the formation of such complexes may be associated with the activation of the Akt kinase (Datta et al., 1995). The PH domain alone fails to form complexes, suggesting that the interaction between Akt molecules depends on the integrity of the entire AH domain. Additional studies from other laboratories support the concept that PH domains are involved in protein–protein interactions (Touhara et al., 1994; Yao et al., 1994). These findings, combined with the results presented in this manuscript, suggest that PH domains may exert a dual function. Thus, Akt may interact with other proteins via its AH/PH domain, bringing it in close proximity to D-3-phosphorylated phosphoinositides and other hydrophobic molecules.

The specificity of Akt activation by PDGF was somewhat unexpected in view of the findings suggesting that Akt is activated as a result of the interaction of D-3-phosphorylated phosphoinositides with the AH/PH domain. Indeed, D-3-phosphorylated phosphoinositides are induced not only by PDGF but also by growth factors (Kapeller and Cantley, 1994) that do not activate Akt. This could be owing to the relatively higher levels of D-3-phosphorylated phosphoinositides generated by PDGF than by other growth factors, or to differences in the composition or subcellular distribution of phosphoinositides observed in cells treated with different factors (Kapeller and Cantley, 1994). Alternatively, different growth factors may activate PI 3-kinase enzymes that may vary with regard to their enzymatic properties, regulation, and subcellular distribution (Kapeller and Cantley, 1994). In addition, Akt activation may require the contribution of multiple signaling pathways. Thus, whereas the dominant inhibitory N17Ras inhibits Akt activation by PDGF, the activated V12Ras is not sufficient to stimulate Akt. While we cannot exclude the possibility that V12Ras fails to stimulate Akt because it is a weak activator of the PI 3-kinase (Rodriguez-Viciana et al., 1994), these results suggest that other signals initiated by PDGF may act in concert with Ras to induce Akt activity.

The specificity of Akt activation by PDGF suggests that Akt may transduce signals that distinguish PDGF from other growth factors. Therefore, the characterization of the signals transduced by activated Akt may provide significant insight into the specificity of the cellular response to growth factors. Our data to date indicate that Akt fails to activate the MAP kinase cascade despite the fact that its activation depends, at least in part, on Ras-mediated signals. This suggests, therefore, that Akt functions in a Ras-dependent pathway that is independent of the MAP kinases. One such pathway involving MEKK and the JNK kinases was indeed recently described (Marshall, 1995).

The data presented in this report also show that PDGF

activates not only the rodent Akt but also the human AKT2. In addition, data not shown indicate that PDGF activates all the known members of the Akt family of kinases, including the human AKT1 and the Drosophila Dakt1. AKT2 has been previously shown to be amplified and overexpressed in about 10% of ovarian neoplasms (Cheng et al., 1992). Moreover, it has been shown that the expression of PDGFRα is frequently elevated in ovarian tumors and that enhanced expression of PDGFRα is associated with poor prognosis (Henriksen et al., 1993). Our data therefore indicate the need for future studies to address the combined effects of Akt and PDGFR overexpression on the induction, progression, and prognosis of human ovarian tumors.

Overall, the studies presented in this report show that the PH domain is required for the enzymatic activation of Akt in response to signals originating in the PDGFR. Moreover, they show that Akt activation may be mediated by second messenger molecules generated via the PDGF-induced activation of the PI 3-kinase. These findings define a specific signal that activates Akt, identify a target for the PI 3-kinase, and implicate the Akt AH/PH domain as a mediator of PI 3-kinase signaling.

Experimental Procedures

Cells

NIH 3T3, Rat-1, and NIH 3T3 cells transformed by V12Ras (provided by F. McCormick) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, penicillin (10 IU/ml), and streptomycin (10 μg/ml). Untransfected canine kidney epithelial TRMP cells (provided by J. Cooper) and COS1 cells were cultured in the same medium supplemented with 10% fetal calf serum (FCS) and antibiotics. TRMP cells stably transfected with PDGFRβ mutant constructs (F740, F751, and FF1009/21) (also provided by J. Cooper) were maintained in the same media supplemented with G418 (200 μg/ml). MEFs and SV40 large T antigen–transformed embryo fibroblasts from *Src* knockout mice (Src6) (provided by P. Soriano) were grown in DMEM supplemented with 15% FCS and antibiotics. To carry out experiments requiring serum starvation, cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), following which they were cultured in serum-free DMEM overnight. The next day, cells were stimulated with growth factors at the concentrations indicated in the Results section and figure legends. PDGF-AA, PDGF-AB, PDGF-BB, and EGF were purchased from GIBCO BRL, FGF from Promega, PMA and wortmannin from Sigma, and rapamycin from Calbiochem.

Plasmids

R25C and K179M Akt mutants were generated by site-directed mutagenesis using the polymerase chain reaction (PCR) (Ito et al., 1991). The Δ11–35 deletion mutant of Akt was generated by overlap extension PCR (Ho et al., 1989). HA-tagged Akt proteins were expressed in various cell lines, as indicated in the Results section, by transient transfection of pCMV-6 (provided by J. Chernoff) constructs. HA–Akt was transferred to the pCMV-6 vector as a HindIII–EcoRI DNA fragment from preexisting pJ3Ω constructs (Datta et al., 1995). HA–Akt K179M, HA–Akt R25C, and HA–Akt Δ11–35 pCMV-6 constructs were generated by exchanging a 1.5 kbp XbaI–BamHI DNA fragment representing the entire Akt coding region in the HA–Akt construct with the corresponding mutant DNA fragments. The XbaI and BamHI sites in the mutant DNA fragments were generated by PCR. The HA–AKT2 construct was generated as described for HA–Akt. The v-akt construct in pJ3Ω has been described previously (Ahmed et al., 1993).

The SV-40-based vector pMT3 and expression constructs of PDGFRα, PDGFRβ, F5 PDGFRβ mutant (carrying tyrosine to phenylalanine mutations at positions 740, 751, 771, 1009, and 1021), and M635R kinase-defective PDGFRβ mutant in the pMT3 vector have been previously reported (Kazlauskas and Cooper, 1989; Valius and Kazlauskas, 1993). Also previously reported were the YY740/51 and Y771 revertants of the F5 mutant and the F2 mutant of the PDGFRβ (carrying tyrosine to phenylalanine mutations at positions 740 and 751) (Valius and Kazlauskas, 1993; Kazlauskas et al., 1992). The Y751 revertant of the F5 mutant was generated by PCR site-directed mutagenesis. All the mutants provided or generated in vectors other than pMT3 were transferred to the pMT3 vector, which was used in all the transfection experiments.

The Myc epitope–tagged Erk1 (MT–p44) and the N17Ras expression constructs were described elsewhere (Patriotis et al., 1994).

Transfections

Transient transfections of fibroblast cell lines and TRMP cells seeded in 10 cm petri dishes at a density of 10^6 cells per dish were carried out by using 10 μg of DNA per dish and lipofectamine (GIBCO BRL) according to the protocol suggested by the manufacturer. After 24 hr, the cells were serum-starved overnight. Later, 48 hours after transfection, cells were exposed for 5–10 min to PDGF. Transient transfections of COS1 cells were carried out by using the DEAE–dextran method as previously described (Datta et al., 1995).

Antibodies

The anti-Akt-CT antibody was raised against a peptide composed of the 15 C-terminal amino acids of Akt (RPHFPQFSYSASGTA). Anti-Myc tag (anti-MT) monoclonal antibody was prepared by centrifugation of ascites fluid arising in mice inoculated intraperitoneally with the antibody producing hybridoma 9E10 (American Type Culture Collection). Anti-HA monoclonal (12CA5) and polyclonal antibodies were purchased from BabCo (Berkeley Antibody Company). The rabbit anti-$p70^{S6k}$ polyclonal antibody was a gift from J. Blenis. The rabbit anti-p85 serum was provided by L. Cantley. The rabbit anti-Erk polyclonal antibody was purchased from Santa Cruz Biotechnology, Incorporated. The rabbit anti-PDGFRα/PDGFRβ polyclonal antibody and the mouse anti-PY monoclonal antibody (4G10) were obtained from Upstate Biochemicals, Incorporated. 4G10 antibody was also supplied by D. Morrison. The mouse anti-Src monoclonal antibody 327 was purchased from Oncogene Science.

Immunoblotting

To assess the level of protein expression, cell lysates were electrophoresed in SDS–polyacrylamide gel and then transferred onto Immobilon P membranes (Millipore). Western blots were probed with the appropriate antibodies. The anti-MT and anti-HA monoclonal antibodies were diluted at 1:2000. The remaining antibodies were diluted 1:1000. Horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibodies (Boehringer Mannheim) were diluted 1:20,000. The bound secondary antibodies were detected by enhanced chemiluminescence (Amersham).

Immunocomplex Protein Kinase Assays and Gel Kinase Assays

To assay for Akt protein kinase activity, cells were lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl [pH 7.4]) containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM $NaPP_i$, and 1 mM Na_3VO_4. Lysates were precleared by centrifugation and then preabsorbed with Protein A–Protein G (2:1) agarose slurry. Immunoprecipitation was carried out for 4 hr using anti-Akt-CT (1:500 dilution) or anti-HA (1:1000 dilution). Immunoprecipitates were washed three times with lysis buffer, once with water, and once with the Akt kinase buffer (20 mM HEPES–NaOH, 10 mM $MgCl_2$, 10 mM $MnCl_2$ [pH 7.4]). Kinase assays were carried out in Akt kinase buffer as previously described (Datta et al., 1995). Exogenous substrates were resuspended in water at a concentration of 5 mg/ml.

In gel kinase assays were carried out as previously described (Patriotis et al., 1994).

PI 3-Kinase Assays and Exposure of Akt Immunoprecipitates to Phosphorylated Phosphoinositides and Brain Extracts

PI 3-kinase was immunoprecipitated from NP-40 lysates of NIH 3T3 cells by use of the anti-PY monoclonal antibody 4G10 (1:1000 dilution). PI 3-kinase activity in the immunoprecipitates was measured as described elsewhere (Whitman et al., 1987).

PIP was produced preparatively by using anti-PY (1:1000 dilution) or anti-p85 immunoprecipitates (1:500 dilution) from PDGF-stimulated

NIH 3T3 cells and PI (Avanti Polar Lipids, Incorporated) as the substrate. PI was phosphorylated for 1 hr at room temperature. In vitro Akt protein kinase assays were carried out in the presence of PI, enzymatically produced PIP, or PIP_2 (Sigma). Before addition to the Akt immunoprecipitates at a final concentration of 0.02 mg/ml, the phospholipids were sonicated for 1 min in 30 mM HEPES–NaOH (pH 7.4) in a cup horn probe. For dose-response experiments, PIP was serially diluted in PI (total phospholipid concentration, 0.02 mg/ml) prior to sonication.

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