Akt Is a Direct Target of the Phosphatidylinositol 3-Kinase

ACTIVATION BY GROWTH FACTORS, v-src and v-Ha-ras, IN Sf9 AND MAMMALIAN CELLS*

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Ketaki Datta, Alfonso Bellacosa†, Tung O. Chan, and Philip N. Tsichlis‡
From the Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The Akt protooncogene encodes a serine-threonine protein kinase which is activated by growth factor-generated signals that are transduced via the phosphatidylinositol 3-kinase (PI3-K). Earlier studies suggested that the activation of Akt by PI3-K may be mediated by the binding of D3-phosphorylated phosphoinositides to the Akt pleckstrin homology (PH) domain. On the basis of these studies, it was hypothesized that Akt is a direct PI3-K target. To test this hypothesis, we reconstituted the pathway of Akt activation in baculovirus-infected Sf9 cells. The results showed that Akt, which is normally catalytically inactive in these cells, was activated when coexpressed with the activated PI3-K. Moreover, they showed that activated forms of c-Ha-ras (v-Ha-ras) and c-src (v-src or srcY527F), two molecules that transduce growth factor-generated signals, also activate Akt in a PI3-K-dependent manner in Sf9 as well as NIH 3T3 cells. The activation of Akt by both growth factors and v-ras and v-src (or srcY527F) depends on the integrity of the Akt PH domain and carboxyl-terminal tail. These results show that Akt activation via the PI3-K can be faithfully reproduced in baculovirus-infected Sf9 cells. The same results support the hypothesis that Akt is a direct target of the PI3-K and identify cytoplasmic signaling molecules that may contribute to the transduction of PI3-K/Akt activation signals.

c-akt, the cellular homolog of the viral protooncogene v-akt (1), encodes a serine-threonine protein kinase which is highly related to protein kinase C and contains an amino-terminal PH domain (1–3). Akt is activated by serum and a variety of growth factors sharing the ability to activate the PI3-K, such as PDGF, epidermal growth factor, bovine fibroblast growth factor, insulin (4–7), insulin-like growth factor-1,2 and interleukin-2.3 Activation of Akt by growth factors depends on the integrity of the PH domain (6) and is blocked by wortmannin (4–7), a powerful PI3-K inhibitor. In vitro incubation of inactive Akt immunoprecipitated from serum-starved NIH 3T3 cells, with enzymatically synthesized D3-phosphorylated phosphoinositides (PPIs), activated Akt in a dose-dependent manner (6). This suggested that the modulation of the Akt catalytic activity is mediated by binding of PI3-K-generated PPIs to the Akt PH domain. The preceding results were interpreted to indicate that Akt is a direct target of the PI3-K. However, this conclusion was challenged by a report showing that the activation of Akt by insulin, contrary to its activation by PDGF, was unaffected by PH domain mutations (7). To test the hypothesis that Akt is a direct target of the activated PI3-K, we reconstituted the pathway of Akt activation by infecting Sf9 cells with combinations of Akt-, p85-, p110-, and PDGFRβ-expressing baculoviruses. The results reported here support the hypothesis that the Akt activation by PI3-K is direct.

The activation of the PI3-K by growth factor-generated signals is mediated by intracellular signaling molecules. One such molecule, the cytoplasmic tyrosine kinase c-src binds p85, the regulatory subunit of the PI3-K (8), while another, c-Ha-ras, binds and activates p110, the PI3-K catalytic subunit (9). Our earlier studies had indeed shown that the activation of Akt by PDGF was inhibited by the dominant negative mutant RasN17 suggesting that Ras contributes to the transduction of PDGF-induced signals that activate Akt (6). To determine whether activated forms of c-src and c-Ha-ras activate Akt and whether their potential contributions to the activation of Akt were dependent on the PI3-K, we examined their role in Akt activation in Sf9 and NIH 3T3 cells. The data presented in this report show that v-src (or srcY527F) and v-Ha-ras or the combination of the two activate Akt in a PI3-K-dependent manner in both Sf9 and mammalian cells. The activation of Akt by both growth factors and v-src (or srcY527F) and/or v-Ha-ras depends on the Akt PH domain and carboxyl-terminal tail.

EXPERIMENTAL PROCEDURES

Insect Cells, Culture Conditions, and Baculoviruses—Sf9 cells derived from Spodoptera frugiperda were obtained from the ATCC (Rockville, MD). The cells were grown in Grace’s insect medium supplemented with yeastolate, lactalbumin, glutamine, 10% fetal bovine serum, penicillin (30 units/ml), streptomycin (30 μg/ml), and kanamycin (60 μg/ml). Expression constructs of wild type and mutant Akt, v-src, v-Ha-ras, and N17Ras in the pVL1392 vector (Pharmingen) were recombined into the Autographa californica strain of the nuclear polyhedrosis virus (AcNPV) using the Baculogold transfection kit and following procedures suggested by the supplier (Pharmingen). Baculovirus recombinants carrying the PI3-K regulatory and catalytic subunit genes p85 and p110 and the PDGFRβ were kindly provided by G. Panayotou (Ludwig Institute, London, UK). Viral stocks were amplified to achieve titers higher than 106 plaque-forming units/ml.

Baculovirus infections were carried out in 60-mm Petri dishes using 2 × 106 cells and a multiplicity of infection of 10. Twenty-four hours later, the cells were serum-starved overnight. The next day the cells

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‡ To whom correspondence should be addressed: Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Tel.: 215-728-3635; Fax: 215-728-2741.

1 The abbreviations used are: PH, pleckstrin homology; PI3-K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PPI, phosphorylated phosphoinositides; PDGFRβ, platelet-derived growth factor receptor β; D-PBS, Dulbecco’s phosphate-buffered saline; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium.

2 A. Bellacosa and P. N. Tsichlis, unpublished data.

3 N. N. Ahmed, T. O. Chan, A. Bellacosa, and P. N. Tsichlis manufactured script in preparation.

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were washed in D-PBS and harvested. Cells treated with wortmannin (Sigma) were exposed to 200 nM concentration of the drug for 30 min prior to harvesting.

Mammalian Cell Culture, Expression Constructs, and Transfections—NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and kanamycin (100 μg/ml).

The CMV6 expression constructs of wild type and mutant Akt tagged at their amino terminus with a hemagglutinin (HA) epitope tag (HA-Akt, HA-AktR25C, HA-AktΔ11–35, and HA-Akt K179M) were described previously (6). The mutant HA-AktΔ11–60 is a HA epitope-tagged Akt mutant with a deletion of the PH domain amino acids 11 to 60. The expression construct MT-Akt was generated by substituting the HA epitope tag with a 12-amino acid c-myc epitope tag (10). The c-myc epitope-tagged carboxyl-terminal deletion mutant (Δ431–480) has been described previously (10).

To generate a mammalian expression construct of the activated c-src (srcY527F), mutant DNA was transferred from the pUC18-based construct psrc527 (provided by R. Davis, University of Massachusetts, Worcester, MA) into CMV6. The v-Ha-ras, c-Ha-ras, and N17Ras expression constructs were described elsewhere (11).

Transient transfections of NIH 3T3 cells seeded in 60-mm Petri dishes at 0.5 × 10⁵ cells/dish were carried out using 4 μg of DNA and LipofectAMINE (Life Technologies, Inc.) according to the protocol suggested by the manufacturer. Twenty-four hours later, cells were washed once with D-PBS, and then cultured in serum-free Dulbecco's modified Eagle's medium overnight. The next day, the cells were stimulated with PDGF-AB (Life Technologies, Inc.) (50 ng/ml) as indicated. Cells treated with Akt or its mutants were exposed to 200 nM concentration of the drug for 30 min prior to their treatment with PDGF-AB.

Western Blotting—Protein expression was determined by probing Western blots of total cell lysates with the appropriate antibodies. Alternatively, Western blots of immunoprecipitates used for in vitro kinase assays were probed with the same antibodies. In both cases, cells were lysed using a Nonidet P-40 (Nonidet P-40) lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 1 mM EDTA). The expression of Akt or its mutants was determined using the anti-Akt-CT antibody (6, 10). PI3-K (p85) and PDGFRβ were detected using anti-p85 or anti-PDGFRβ polyclonal antibodies from Upstate Biotechnology, Inc. (UBI). Expression of srcY527F in NIH 3T3 or v-src in Sf9 cells was monitored using the anti-v-src monoclonal antibody 327 from Oncogene Science. Expression of v-Ha-ras was monitored using an anti-v-Ha-ras monoclonal antibody from Transduction Laboratories. Detection of antigen-bound antibody was carried out using enhanced chemiluminescence (ECL, Amersham) as described previously (10).

Immunoprecipitation: in Vitro Kinase Assay for Akt—Transiently transfected NIH 3T3 cells or infected Sf9 cells were lysed in Nonidet P-40 lysis buffer at 48 h following transfection or infection, respectively. Lysates were clarified by centrifugation at 10,000 × g for 10 min and precleared with 20 μl of a 50% suspension of protein A-protein G (1:1) agarose (Life Technologies, Inc.) at 4°C for 20 min. The beads were removed by centrifugation at 10,000 × g for 10 min, and immunoprecipitations were carried out by incubating the precleared lysates with the appropriate antibodies (anti-Akt-CT (dilution 1/500), anti-c-myc monoclonal 9E10 (dilution 1/10000) or anti-HA monoclonal 12CA5 (dilution 1/5000) and 40 μl of protein A-protein G agarose beads for 2 h at 4°C. Immunoprecipitates were washed three times with the lysis buffer and once with water, and once with Akt kinase buffer (20 mM Hapes, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂). Kinase assays were carried out as described previously (6, 10). Histone H2B (Boehringer) was used as the exogenous substrate (6). The anti-HA monoclonal antibody was obtained from BAbCo. The anti-c-myc monoclonal 9E10 was harvested from ascites induced in mice inoculated intraperitoneally with the corresponding hybridoma obtained from ATCC.

RESULTS

Akt Is a Direct Target of the PI3-K—To determine whether Akt is a direct target of the PI3-K, we expressed Akt in Sf9 cells and we examined its activity in the presence or absence of activators. Immunoprecipitates of wild type and mutant Akt from Akt-expressing Sf9 cells were catalytically inactive. Coexpression of Akt with p85, the regulatory subunit of the PI3-K, had little if any effect. However, coexpression of Akt with p110, the catalytic subunit of the PI3-K, or coexpression of Akt with p110 and p85 induced moderate Akt activation. Coexpression of PDGFRβ with Akt, p85, and p110 boosted further the Akt catalytic activity (Fig. 1A). Treatment of Sf9 cells coexpressing Akt, p85, p110, and PDGFRβ with nanomolar concentrations of wortmannin (200 nM) inhibited the activation of Akt (Fig. 1B). The effect of the PDGFRβ on the activity of Akt, in the absence of PDGF, was hypothesized to be due to the overexpression and spontaneous dimerization and activation of the receptor (12). This was confirmed by probing Western blots of total lysates of PDGFRβ expressing Sf9 cells with the antiphosphotyrosine monoclonal antibody 4G10 (UBI). The results showed that the overexpressed receptor is tyrosine-phosphorylated in the absence of growth factor stimulation (Fig. 2). Overall, the results of these experiments revealed that the PI3-K-dependent activation of Akt by PDGF can be reproduced in baculovirus-infected Sf9 cells and provided additional independent evidence that Akt is a direct target of the PI3-K.

The PI3-K-dependent Activation of Akt Is Inhibited by Mutations in the PH Domain and by a Partial Deletion of the Carboxyl-terminal Tail—Our earlier studies had shown that the PDGF-induced activation of Akt in NIH 3T3 cells depends on the integrity of the Akt PH domain (6). This, combined with the activation of Akt by in vitro incubation with enzymatically synthesized PPIs, suggested that Akt is activated as a result of the interaction of these PPIs with the PH domain (6). However, studies by others on the activation of Akt by insulin suggested that the PH domain may not be required for Akt activation (7). Because of these conflicting data, we re-examined the role of the PH domain on the activation of Akt. To this end, Sf9 cells were infected with p85, p110, and wild type or mutant Akt baculovirus constructs. The Akt mutants included the PH domain mutants Akt Δ11–35, Akt R25C, and Akt Δ11–60 and the kinase-deficient mutant Akt K179M. All the Akt baculovirus constructs were HA-tagged at their amino terminus. The baculovirus-infected cells were then stimulated by coexpression of the PDGFRβ. Some of the cultures expressing wild type Akt
were treated with wortmannin (200 nM) for 30 min prior to lysis. In vitro kinase assays of Akt immunoprecipitated from cells starved of serum overnight confirmed that Akt is activated by PDGFRβ-generated signals and that its activation is inhibited by wortmannin. However, the Akt mutants Δ11–35 and R25C, as well as the kinase-deficient mutant K179M, failed to respond to the PDGFRβ (Fig. 3). These findings confirmed that the activation of Akt by PI3-K-mediated signals is PH domain-dependent. One of the PH domain mutants examined in this experiment, Δ11–60, was active in the absence of PI3-K-transduced Akt activation signals. This mutant failed to respond to the PDGFRβ, but was still sensitive to wortmannin (Fig. 3).

Since deletion of the carboxyl-terminal tails of several kinases, including Src (13) and Tpl-2,4 induces constitutive kinase activation, we examined the activity of a c-myc epitope-tagged mutant of Akt carrying a partial carboxyl-terminal tail deletion (Δ431–480). The mutant was expressed in S9 cells either alone or in combination with p85, p110, and PDGFRβ and was shown to be inactive both in the absence and in the presence of the activated PI3-K (Fig. 3). The same mutant also failed to be activated by PDGF when transfected transiently into NIH 3T3 cells (Fig. 4). Since Akt activation has been linked to phosphorylation (4, 5, 7), and the carboxyl-terminal tail of Akt contains several potential phosphorylation sites (1), we suggest that the tail may be the site of phosphorylation events critical for Akt activation.

**Activation of Akt by v-src and v-Ha-ras in S9 Cells**—The preceding data confirmed our earlier observations that Akt is an immediate target of the growth factor-activated PI3-K (6). The activation of the PI3-K by growth factors is mediated by several intracellular signaling molecules. Src, one of the molecules that may contribute to the transduction of PI3-K-activation signals, interacts, via its SH3 domain, with the proline-rich domain of p85, the regulatory subunit of the PI3-K (14). Ras, another such molecule, interacts directly with the PI3-K catalytic subunit p110 (9). To determine the biological outcome of these interactions, we first examined whether activated Src and Ha-ras activate the PI3-K and Akt in S9 cells. To this end, S9 cells were infected with HA-Akt, p85, and p110 baculovirus constructs and they were superinfected with activated src (v-src), activated Ha-ras (v-Ha-ras), or v-src plus v-Ha-ras baculovirus constructs. Subsequently, Akt was immunoprecipitated with the anti-Akt-CT antibody, and its activity was measured in vitro. The results (Fig. 5) confirmed that v-src, v-Ha-ras, and the combination of the two activate Akt in a PI3-K-dependent manner. The activation of Akt by v-src plus v-Ha-ras was partially inhibited by the dominant negative mutant N17Ras. Moreover, similarly to the activation of Akt by PDGFRβ, its activation by v-src and v-Ha-ras was also inhibited by mutations in the PH domain and by wortmannin (Fig. 6).

**Activation of Akt by Activated Src (Y527F) and v-Ha-ras in NIH 3T3 Cells**—The preceding data showed that Akt can be activated in baculovirus-infected S9 cells, both by growth factors and by v-src and/or v-Ha-ras in a PI3-K-dependent manner. The activation of Akt by v-src and/or v-Ha-ras in S9 cells predicted that Akt may also be activated by these molecules in mammalian cells. To test this hypothesis, NIH 3T3 cells were cotransfected with an MT-Akt expression construct and expression constructs of activated src (srcY527F) (8) and v-Ha-ras separately or in combination. Following overnight serum starvation, Akt was immunoprecipitated with the anti-c-myc mono-

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clonal antibody 9E10, and in vitro kinase assays were carried out on the immunoprecipitates. The results showed that Akt is activated by v-src, v-Ha-ras, or a combination of v-src and v-Ha-ras in Sf9 cells in a PI3-K-dependent manner. Upper panel, in vitro kinase assays of HA-Akt immunoprecipitated from lysates of Sf9 cells infected with HA-Akt, p85, p110 and v-src, v-Ha-ras, and N17-ras baculoviruses as indicated. Lower panel, Western blots of the cell lysates in the upper panel were probed with anti-Akt-CT, anti-p85, anti-v-src, and anti-Ha-ras antibodies.

**Fig. 5. Activation of Akt by v-src and v-Ha-ras in Sf9 cells.** Akt is activated by v-src, v-Ha-ras, or a combination of v-src and v-Ha-ras in Sf9 cells in a PI3-K-dependent manner. Upper panel, in vitro kinase assays of HA-Akt immunoprecipitated from lysates of Sf9 cells infected with HA-Akt, p85, p110 and v-src, v-Ha-ras, and N17-ras baculoviruses as indicated. Lower panel, Western blots of the cell lysates in the upper panel were probed with anti-Akt-CT, anti-p85, anti-v-src, and anti-Ha-ras antibodies.

**DISCUSSION**

The data presented in this manuscript show that the activation of Akt via the PI3-K can be reproduced faithfully in Sf9 cells and provide support to the hypothesis that Akt is a direct target of the PI3-K. Moreover, they show that the activation of the PI3-K by both growth factors and intracellular signaling molecules is sufficient to activate Akt in both Sf9 and mammalian cells.

Earlier studies had shown that Akt activation by PDGF requires that the Akt protein has an intact PH domain (6). This observation combined with the in vitro activation of Akt by enzymatically synthesized PPIs was interpreted to suggest that Akt activation depends on the direct interaction between the Akt PH domain and PI3-K-generated PPIs (6). The validity of this hypothesis, however, was questioned because of independent studies showing that the PH domain is dispensable for Akt activation by insulin (7). Due to these conflicting data, we proceeded to re-evaluate the role of the PH domain in the PDGF-induced activation of Akt. The results reported here confirmed that only Akt with an intact PH domain can be fully activated and provided support to the hypothesis that Akt activation is induced by binding of PI3-K-generated PPIs to the PH domain. Alternatively, these mutations could lock Akt into a nonactivatable conformation. Such a conformational change could interfere with the ability of Akt to form dimers (10) or to interact with other proteins or it could block its stimulation-dependent translocation to the cell membrane. The differences between PDGF and insulin regarding the role of the PH domain in Akt activation could perhaps be due to differences in signaling between these factors.

Among the Akt PH domain mutants, particularly interesting was the mutant AktD11–60 which showed high constitutive kinase activity but failed to respond to PDGFRβ-generated signals. The high basal kinase activity of the AktD11–60 mutant suggests that mutations in the PH domain may either increase the affinity of the PH domain to activating PPIs or abrogate the binding of inhibitory PPIs. The former possibility appears to be supported by the fact that the kinase activity of
this mutant is partially inhibited by wortmannin. Alternatively, such mutations may lock the Akt protein in an active conformation perhaps because they may affect its ability to form dimers or to interact with other proteins.

The carboxyl-terminal tails of several kinases appear to play important regulatory roles. Thus, truncation of the carboxy-terminal tail of c-src (13) and Tpl-2 (15) or mutation of Tyr-527 of c-src into phenylalanine (16) activate these kinases constitutively. The data presented in this report show that deletion of the carboxy-terminal tail of Akt gives rise to a protein that is catalytically inactive and fails to respond to the activated PI3-K. Therefore, the Akt carboxyl-terminal tail may also play a regulatory role which is, however, qualitatively different from that of the c-src and Tpl-2 tails. The tail contribution to the regulation of the Akt kinase activity could be mediated through its interaction with residues in the kinase domain. Such interactions could maintain the protein in an activable conformation. A proline-rich region in the tail (amino acids 423–427) may provide the flexibility required for this function. Since the tail contains several potential phosphorylation sites, the proposed interaction between the tail and the kinase domain of Akt could be regulated by phosphorylation. Such carboxy-terminal tail phosphorylation events have been proposed to also contribute to the regulation of other kinases such as Raf-1 (17), p70S6K (18), and PKCβII (19).

The finding that Akt is activated directly by the PI3-K suggested that all growth factors and intracellular signaling molecules that activate the PI3-K may also activate Akt. Here we examined the potential role of two such signaling molecules, Ha-ras and Src, in Akt activation. c-Ha-ras is known to interact with, and to activate p110, the catalytic subunit of the PI3-K (9). Moreover, our earlier studies had shown that the PDGFinduced activation of Akt is inhibited by the dominant negative mutant RasN17 transiently transfected into NIH 3T3 cells (6). Src and other src-related kinases, on the other hand, are known to interact through their SH3 domains with a proline-rich region (amino acids 84–99) in the regulatory subunit of the PI3-K (14). Through this interaction, p85 appears to be phosphorylated on tyrosine residues and contributes to the activation of the PI3-K (14). The results of the experiments reported here confirmed that both v-src (or srcY527F) and v-Ha-ras, as well as the combination of the two, activate Akt in S9B and mammalian cells in a PI3-K-dependent manner.

In summary, the data presented in this report showed that the activation of Akt by the PI3-K can be reproduced in baculovirus-infected S9B cells and provided additional support to the hypothesis that Akt is a direct target of the PI3-K. The same data showed that Akt is activated by both growth factors and intracellular signaling molecules that transduce growth factor-generated signals that activate the PI3-K. Finally, both the PH domain and the carboxy-terminal tail of Akt play critical roles in regulating its activity.

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