AKT2, a Member of the Protein Kinase B Family, Is Activated by Growth Factors, v-Ha-ras, and v-src through Phosphatidylinositol 3-Kinase in Human Ovarian Epithelial Cancer Cells


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Abstract

Three members have been identified in the protein kinase B (PKB) family, i.e., Akt/PKBα, AKT2/PKBβ, and AKT3/PKBγ. Previous studies have demonstrated that only AKT2 is predominantly involved in human malignancies and has oncogenic activity. However, the mechanism of transforming activity of AKT2 is still not well understood. Here, we demonstrate the activation of AKT2 with several growth factors, including epidermal growth factor, insulin-like growth factor I, insulin-like growth factor II, basic fibroblast growth factor, platelet-derived growth factor, and insulin, in human ovarian epithelial cancer cells. The kinase activity and the phosphorylation of AKT2 were induced by the growth factors and blocked by the phosphatidylinositol (PI) 3-kinase inhibitor, wortmannin, and dominant-negative Ras (N17Ras). Moreover, the activated Ras and v-Src, two proteins that transduce growth factor-generated signals, also activated AKT2, and this activation was not significantly enhanced by growth factor stimulation but was abrogated by wortmannin. These results indicate that AKT2 is a downstream target of PI 3-kinase and that Ras and Src function upstream of PI 3-kinase and mediate the activation of AKT2 by growth factors. The findings also provide further evidence that AKT2, in cooperation with Ras and Src, is important in the development of some human malignancies.

Introduction

PKB is a subfamily of the second messenger-regulated serine/threonine protein kinases characterized by an NH2-terminal PH domain, a central kinase domain, and a serine/threonine-rich C-terminal region (1, 2). Three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, have been identified to date in this family (1–5). However, only AKT2 has been implicated in several types of human malignancy. In particular, alterations of AKT2 have been detected in 10–20% of ovarian carcinomas and pancreatic cancers (2, 6). Overexpression of AKT2 in NIH 3T3 cells resulted in a transformed phenotype (7). Moreover, antisense of AKT2 can significantly inhibit expression of AKT2 in NIH 3T3 cells resulted in a transformed phenotype (8). A previous study showed that Akt is nononcogenic in nude mice when overexpressed in a nontumorigenic rat T-cell lymphoma cell line (9). Therefore, AKT2 appears to play a more important role than Akt and AKT3 in malignant transformation. However, the mechanism by which AKT2 contributes to cell transformation is unclear.

Recent studies demonstrated that Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (10–12). Growth factor-induced Akt activation is mediated by PI 3-kinase (10, 11, 13), whereas activation of Akt by cellular stress appears to be through a different signaling pathway that could be p38/HOG (12). Activation of Akt by growth factors depends on the integrity of the PH domain (10), which binds to the products of PI 3-kinase, phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate (14, 15). Akt activation also requires phosphorylation on both Thr-308 and Ser-473, and the phosphorylation of these two residues was blocked by wortmannin (16). Three downstream targets of Akt have been identified, one of which is GSK3; Akt phosphorylates GSK3 at the same serine residue which is targeted by insulin, leading to inactivation of GSK3 and stimulation of glycogen synthesis (17). There is also evidence that Akt induces p70S6k, but the connection is likely to be indirect (10). More recently, Akt was shown to phosphorylate BAD at Ser-136 both in vitro and in vivo, to reduce binding ability of BAD to Bcl-xL, resulting in suppression of apoptosis and promoting cell survival (18).

In this report, we present evidence that AKT2 is rapidly activated by mitogenic growth factors, including EGF, IGF1, IGFII, bFGF, PDGF, and insulin, through PI 3-kinase in human ovarian cancer cells. Activated Ras and Src also induced AKT2 kinase activity in a PI 3-kinase-dependent manner. Moreover, dominant-negative Ras (N17Ras) blocked the growth factor-induced AKT2 activity.

Materials and Methods

Cell Lines, Transfection, and Stimulation. Human ovarian epithelial cancer cell lines, A2780, OVCAR-3, SKOV-3, OVCAR-5, and OVCAR-10, were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 0.5 × 105 cells/dish. Following incubation overnight, the cells were transfected with 8 μg of DNA per dish using SuperFect Transfection Reagent (Qiagen), according to the protocol suggested by the manufacturer. After 36 h, the cells were serum-starved overnight and stimulated with growth factors (50 ng/ml EGF, 100 ng/ml IGF1, 100 ng/ml PDGF, 100 ng/ml bFGF, and 100 nm insulin) for 10 min.

Expression Constructs. HA epitope-tagged AKT2 was prepared by ligating a HA epitope encoding oligonucleotide to the AKT2 cDNA in-frame with the initiator methionine in the mammalian expression PcDNA3 (Invitrogen). The HA-ΔN4AKT2 and HA-AKT2AC constructs (lacking the NH2-terminal 117 or C-terminal 70 amino acids, respectively) were created by PCR using a 5′ oligonucleotide encoding amino acids 118–126 or a 3′ primer corresponding to amino acids 405–411. The resulting PCR products were digested with BamHI and XbaI and subcloned into the polylinker of the vector PcDNA3. The v-src construct was described elsewhere (13). The CMV6-v-Ha-ras and CMV5-N17Ras were gifts from Jonathan Chernoff and Philip Tsichlis (Fox Chase Cancer Center).
showed that AKT2 was activated about 30-fold with EGF, IGF1, or insulin and 18-fold with PDGF, IGFII, or bFGF in A2780 cells. A very recent report also observed activation of AKT2 by these growth factors in other cell types (19).

In addition, endogenous AKT2 kinase activity was examined in five ovarian cancer cell lines, one (OVCAR-3) of which exhibited amplification and overexpression of AKT2 (2). After serum starvation for 24 h and stimulation with a growth factor for 10 min, AKT2 was immunoprecipitated with a polyclonal anti-AKT2 antiserum raised against a helix region of the protein, which does not cross-react with Akt and AKT3 (7). Elevated AKT2 activity was observed in all five cell lines after growth factor stimulation. The highest level of AKT2 activation was observed with EGF in OVCAR-3 and IGF1 in A2780 (Fig. 1B), which may be due to a different expression level of the receptors in these cell lines (20).

We have also observed three different mobility forms of AKT2 by immunoblotting analysis, termed a, b, and c (Fig. 1A and Fig. 2). The a and b forms were predominantly detected in the untreated cells. After growth factor stimulation, the slower migration forms c, which was believed to be a phosphorylated form (21), became more prominent, followed by a decreased amount of form a. These data suggested that AKT2 activation may be modulated by a reversible phosphorylation process.

To determine whether PI 3-kinase mediates the activation of AKT2 by growth factors, we examined the effects of the PI 3-kinase inhibitor, wortmannin, on the growth factor-induced AKT2 activation. Because wortmannin at nanomolar concentrations is known to inhibit only the PI 3-kinase whereas at micromolar concentrations it also inhibits protein kinases, the cells transfected with HA-AKT2 were pretreated with increasing concentrations of wortmannin (0.1–200 nM) for 30 min before EGF stimulation. Wortmannin abrogated AKT2 activation in vivo at concentrations similar to the ones inhibiting the PI 3-kinase (data not shown; Ref. 22). Growth factor-induced kinase activity and phosphorylation of AKT2 were completely inhibited by wortmannin at the 100-nM concentration (Fig. 2), indicating that growth factor-induced AKT2 activation is mediated by PI 3-kinase.

Activation of AKT2 by v-Ha-ras and v-src in a PI 3-Kinase-dependent Manner. Previous investigations revealed that the activation of the PI 3-kinase by growth factor-generated signals is mediated by several intracellular signaling proteins. One such protein, Ras, binds and activates p110, the PI 3-kinase catalytic subunit (23). Src, another such protein, binds to the proline-rich region of p85, the regulatory subunit of the PI 3-kinase, and contributes to transduction...
To further determine the effects of Ras on AKT2 activation, we examined kinase activity of AKT2, before and after growth factor stimulation, in A2780 cells after cotransfection of dominant-negative mutant Ras (N17Ras) and HA-AKT2. Repeated experiments revealed that N17Ras partially inhibits growth factor-induced AKT2 activation (Fig. 3B).

Deletion of PH-Domain and C-terminal Region Affects AKT2 Activation. Our previous study demonstrated that the C-terminal region of AKT2 is essential for its transforming activity (7). To assess the role of the C-terminal (amino acids 411-481) and the NH2-terminal PH (amino acids 1-117) domains in AKT2 activation, we expressed HA epitope-tagged deletion constructs (HA-ΔNAKT2 and HA-AKT2ΔC) of AKT2 in A2780 cells and examined the kinase activity of HA-ΔNAKT2 and HA-AKT2ΔC before and after growth factor stimulation. Expression and migration of the proteins were confirmed by Western blot analysis with a monoclonal anti-HA antibody (data not shown). In vitro kinase assays revealed that both AKT2 mutant events failed to respond to growth factor stimulation (Fig. 4), indicating that both regions are required for AKT2 activation.

Discussion

In this report, we demonstrate that AKT2 is activated and phosphorylated by a number of mitogenic stimuli in a pattern similar to Akt. Activation of AKT2 by these stimuli is mediated by PI 3-kinase. PI 3-kinase has been implicated in the regulation of several different cellular responses, including mitogenesis, membrane trafficking, and cell survival. Many types of extracellular signals, especially those involving activation of receptor protein tyrosine kinase, trigger the activation of PI 3-kinase. Oncogenic transformation or stimulation of cells with growth factors results in an increased level in phospholipid products of PI 3-kinase. Mutant alleles of certain oncogenes or PDGF receptor that fail to activate PI 3-kinase are also deficient in triggering oncogenic transformation or mitogenic responses, respectively. Recently, an oncogene, c-p3k, encoding a member of the catalytic subunit, p101, of PI 3-kinase, has been cloned and showed to transform cultured chicken embryo fibroblasts (25). These data suggest
that PI 3-kinase is important in regulating cell growth and cellular transformation. Three major targets of PI 3-kinase have been identified, i.e., p70s6k, JNK, and Akt (26). p70s6k mainly involves protein synthesis, whereas JNK and Akt transduce stress and growth factor-generated signals to regulate cell growth. We demonstrated previously that overexpression of AKT2 results in cell transformation (7). Here, we provide evidence that AKT2 is a downstream target of PI 3-kinase and mediates mitogenic signals to promote cell proliferation and cellular transformation.

We have also shown that active forms of Ras and Src activate AKT2. This activation was not further significantly enhanced by growth factor stimulation but was diminished by wortmannin. Furthermore, growth factor-induced AKT2 activity was partially blocked by dominant-negative Ras (N17Ras). These data suggest that Ras and Src function upstream of PI 3-kinase to mediate AKT2 activation by growth factor stimulation. It has been reported that Ras and Src directly bind to and activate PI 3-kinase (23, 24). In NIH 3T3 cells, activation of PI 3-kinase is required for efficient transformation by Ras (27). Because AKT2 is a downstream target of PI 3-kinase, AKT2 would mediate transforming signals of Ras and Src. It has been suggested that alteration of only one oncprotein in a signaling pathway is sufficient to activate or inactivate this pathway. In fact, we demonstrated previously that overexpression of AKT2 in NIH 3T3 cells is oncogenic, suggesting that alteration of AKT2 alone, within the Ras/Src-PI 3-kinase pathway, is sufficient in malignant transformation (7).

The fact that the level of AKT2 activation by active Ras is significantly higher than that induced by growth factors or Src and that this activation is only partially abolished by wortmannin (Fig. 3A) suggests that activation of AKT2 mediated by Ras may also involve another signaling pathway. Alternatively, the other possibility is that Ras could activate an isoform of PI 3-kinase that is resistant to PI 3-kinase inhibitors. It is known that at least four different PI 3-kinase family members interact with Ras, although none of them are known to be resistant to wortmannin (27). However, a novel PI 3-kinase isoform has been found to be approximately 50 times less sensitive to wortmannin than pi100a (28). Moreover, a recently identified chicken p110 catalytic subunit (c-p3k), a homologue of p110alpha, was shown to have PI 3-kinase activity and activate Akt in transformed cultured chicken embryo fibroblasts (25). It would be interesting to examine whether c-p3k activates AKT2 and is sensitive to PI 3-kinase inhibitors.

We have demonstrated previously that the oncogenic activity of AKT2 is abolished by truncation of 70 amino acids at the C-terminus (7). In this report, we show that both this region and the PH domain are important for AKT2 activation. Previous studies on Akt demonstrated that the products of PI 3-kinase bind to the PH domain, recruit Akt to the cell membrane, and thus activate Akt. Due to high homology (72%) of this region between Akt and AKT2 (2), the PH domain of AKT2 may function similarly to that of Akt. A very recent study identified that phosphorylation on Thr-309 and Ser-474 of AKT2, the latter located within the C-terminal region, is required for its activation (19). We have recently expressed a mutant HA-AKT2S474A in A2780 ovarian cancer cells and found that it failed to respond to growth factor stimulation (data not shown), suggesting that Ser-474 is essential for activation of AKT2. In addition, we have demonstrated that phosphorylation of AKT2 induced by growth factors, v-raf, and v-src is PI 3-kinase dependent, indicating that PI 3-kinase is required for both phosphorylation and activation of AKT2.

In summary, the data presented in this report showed that activation and phosphorylation of AKT2 by growth factors is mediated by Ras, Src, and PI 3-kinase in human ovarian cancer cells. Ras and Src function upstream of PI 3-kinase. AKT2 acts as downstream target of PI 3-kinase and transduces growth factor-generated mitogenic signals. AKT2 activation may be regulated by its reversible phosphorylation and requires the PH domain and the C-terminal tail to be intact. These results provide the basis for the further understanding of how AKT2 contributes to the control of cell proliferation and malignant transformation. Future work will hopefully clarify the difference between Akt and AKT2 signaling, which confer more malignant potential to AKT2 in contrast to Akt.

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References


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