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FAST TRACK

Translocation and Activation of AKT2 in Response to Stimulation by Insulin

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Abstract The *AKT2* oncogene encodes a protein-serine/threonine kinase that was recently shown to be activated by a variety of growth factors. In addition, we previously showed that AKT2 is abundant in brown fat and skeletal muscle, tissues that are highly insulin responsive and that play a role in glucose metabolism. In this study, we demonstrate that AKT2 is activated in response to stimulation by insulin in a dose- and time-dependent manner in human ovarian carcinoma cells and that activation of AKT2 is abolished in cells pretreated with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase). Activation of AKT2 is manifested by changes in its phosphorylation state. Immunofluorescence experiments demonstrate that AKT2 is translocated to the plasma membrane after insulin stimulation, and this translocation is abolished by wortmannin. Both wild-type AKT2 activated by insulin and constitutively active AKT2, which has been targeted to the membrane by the addition of a myristoylation signal, were found to inactivate glycogen synthase kinase-3 (GSK-3) in vitro. GSK-3 was not inactivated by a catalytically inactive AKT2 mutant. Collectively, these data indicate that activation of AKT2 by insulin is mediated by PI 3-kinase and that GSK-3 is a downstream target of AKT2, suggesting a potentially important role of AKT2 in glycogen synthesis and other GSK-3 signaling pathways. J. Cell. Biochem. 70:433–441, 1998.

Key words: AKT2; serine-threonine kinase; oncogene; insulin; phosphatidylinositol 3-kinase

AKT2 and *AKT1* are human cellular homologues of the viral oncogene v-*akt* [Cheng et al., 1992; Staal et al., 1988]. AKT2 (also known as PKBβ) and AKT1 (PKBα) are serine/threonine kinases related to protein kinase A and protein kinase C [Cheng et al., 1992; Jones et al., 1991]. The N-terminus of AKT2 and AKT1 contains a pleckstrin homology (PH) domain, a region shared by a large family of proteins with diverse functions, especially molecules involved in signal transduction pathways [Haslam et al., 1993; Mayer et al., 1993].

AKT1 has been shown to be amplified in only a single human gastric carcinoma [Staal, 1987], whereas AKT2 has been implicated in a significant number of human tumors. Amplification and/or overexpression of *AKT2* has been observed in 10–20% of human ovarian [Bellacosa et al., 1995; Cheng et al., 1992] and pancreatic carcinomas [Cheng et al., 1996; Miwa et al., 1996]. In ovarian cancer, alterations of *AKT2* appear to be especially frequent in undifferentiated tumors and are associated with a poor prognosis [Bellacosa et al., 1995]. Overexpression of AKT2 is sufficient to transform NIH 3T3 cells, and the oncogenic activity of AKT2 is abolished by truncation of its 70-amino acid, proline-rich C-terminus [Cheng et al., 1997]. In addition, *AKT2* antisense RNA inhibits the tumorigenic phenotype of human pancreatic cancer cells exhibiting amplification and overexpression of AKT2 [Cheng et al., 1997].

We previously reported in situ hybridization analysis of endogenous *Akt2* mRNA expression during mouse embryonic development [Altomare et al., 1998]. These studies showed that, in comparison with *Akt*, *Akt2* is preferentially transcribed in tissues targeted by insulin, i.e., brown fat, skeletal muscle and liver. Furthermore, Northern blot analyses of *Akt2* and *Akt* in mouse 3T3-L1 pre-adipocytes and C2C12 myoblasts demonstrated that *Akt2*, but not *Akt*, is increased in differentiating fat and muscle cells in vitro. Similarly, we previously have shown elevated *Akt2* transcript levels in differentiat-

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ing *Myod*-transformed 10T½ fibroblasts [Altomare et al., 1995], whereas *Akt* expression remained unchanged in differentiating *Myod*-transformed 10T½ cells [Altomare et al., 1998]. The high expression of *Akt2,* as compared with *Akt,* in mature fat and skeletal muscle suggests that Akt2 may be the physiological mediator of insulin signaling in these tissues. Akt2 may mediate some of the effects of insulin by influencing glucose uptake, membrane translocation of the glucose transporter 4, and cellular differentiation, as is the case with Akt [Kohn et al., 1996].

In contrast to the vast literature concerning AKT1/Akt activation and cellular signaling [Hemmings, 1997; Testa and Bellacosa, 1997], systematic studies on the role of AKT2 in mitogenic signaling have been sparse [Meier et al., 1997], despite the relevance of AKT2 alterations to human malignancy. Moreover, studies of AKT2 signaling in human tumor cells have not been reported to date. As an initial step to understand the biochemical implications of AKT2 perturbations in human cancer, we have examined the mechanism of activation of AKT2 in a human ovarian carcinoma cell line. We demonstrate marked activation of AKT2 in response to insulin stimulation via phosphatidylinositol 3-kinase (PI 3-kinase), and the activation of AKT2 is shown to be dose- and time-dependent. Immunofluorescence experiments demonstrate that transfected, wild-type AKT2 is translocated to the plasma membrane after insulin stimulation. Furthermore, we demonstrate that activated AKT2 inhibits glycogen synthase kinase-3 (GSK-3), implicating GSK-3 as a downstream target of AKT2 and suggesting that AKT2 may play an important role in glycogen synthesis and other GSK-3 signaling pathways in vivo.

MATERIALS AND METHODS

Materials

Restriction enzymes and other modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Life Technologies Inc. (Gaithersburg, MD); protein A- and protein G-agarose beads and LipofectAMINE were from Life Technologies; histone H2B was from Boehringer Mannheim (Indianapolis, IN); wortmannin and ATP were from Sigma Chemical Co. (St. Louis, MO); [γ-^{32}P]ATP (111 TBq/mmol) and the enhanced chemiluminescence (ECL) Western blotting analysis system were from Amersham Life Science (Arlington Heights, IL); anti-HA monoclonal antibody (HA.11) was from Babco (Richmond, CA); Cy3-conjugated goat anti-mouse antibody was from Jackson Immuno Research Lab., Inc. (West Grove, PA); GSK-3β monoclonal antibody was from Transduction Laboratories (Lexington, KY); P81 paper, phospho-GS peptide 2, protein phosphatase type-2A (PP2A) and GSK-3β isoform were from Upstate Biochemicals (Lake Placid, NY).

Plasmid Constructs

Polymerase chain reaction (PCR) was employed to construct hemagglutinin (HA) epitope-tagged human AKT2 using pcDNA3 as an expression vector. The DNA sequences corresponding to an HA epitope tag (amino acid sequence; YPYDVPDYAS) was inserted after the first methionine of human AKT2 and mouse Akt to construct HA-AKT2 and HA-Akt, respectively. Constitutively active HA-AKT2 was created by adding a double-stranded DNA fragment corresponding to a myristoylation signal (amino acid sequence; MGSSKSKPKDPSQ-RGGEF) at the 5′-end of a cDNA encoding HA-AKT2 (myrHA-AKT2). For catalytically inactive HA-AKT2 (HA-AKT2E299K), a glutamine at 299 of human AKT2 was changed to a lysine using PCR. The integrity of each construct was confirmed by nucleotide sequence analysis.

Immune-Complex Kinase Assays

After transfection with HA-AKT2 or HA-Akt, cells were serum-starved for 18–24 h in serum-free RPMI1640 medium. At the end of the incubation, cells were treated with or without wortmannin for 20 min before insulin stimulation for various times, as indicated in the figure legends. The cells were washed twice with ice cold PBS and then lysed at 4°C in lysis buffer (50 mM Tris-HCl [pH 7.5], 137 mM NaCl, 10% [v/v] glycerol, 1 mM EDTA, 1 mM PMSF, 2 μg of aprotinin per ml, 2 μM leupeptin, 0.1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 50 mM NaF, and 10 mM sodium pyrophosphate). Lysates were centrifuged for 5 min at 18,400 *g* at 4°C and precleared by mixing with protein A–protein G (1:1) agarose beads at 4°C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated

with the anti-HA monoclonal antibody, and protein A–protein G (1:1) agarose beads were used to precipitate immunocomplexes. After extensive washing, the beads and immunocomplexes were equilibrated with kinase buffer (20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [pH 7.4], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, and 1 mM EDTA). The AKT2 and Akt kinase assays were carried out at room temperature for 25 min in the presence of 5 μM [γ-^{32}P]ATP (300 GBq/mmol) in kinase buffer using histone H2B as a substrate. The reactions were terminated by the addition of 2× Laemmli sample loading buffer and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Fuji BAS 1000 image analyzing system. For the GSK-3β kinase assay, the beads were washed with kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$ and 1 mM DTT) and preincubated with GSK-3β isoform with 10 μM ATP for 10 min at room temperature. The reactions were initiated by adding [γ-^{32}P]ATP (74 GBq/mmol) and Phospho-GS peptide and then incubated for 20 min at 30°C. The reactions were stopped by spotting a 10-μl aliquot of each reaction mixture onto a piece of P81 paper. Incorporation of ^{32}P into the phospho-GS peptide was assayed by scintillation counting. The proteins immunoprecipitated for kinase assays were separated by SDS-PAGE and then assayed by immunoblotting.

Immunofluorescence Analysis

Cells were fixed on poly-D-lysine-coated microscope slides with 3.5% paraformaldehyde for 5 min, washed, and permeabilized for 5 min with 0.1% Triton X-100 in KB buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl and 1% bovine serum albumin [BSA]), and washed with KB buffer. Mouse monoclonal anti-HA antibody was used as a primary antibody. The cells were washed thoroughly with KB, and Cy3-conjugated goat anti-mouse antibody was used as the secondary antibody. After washing with KB buffer, the cells were counterstained with the nuclear stain diamidino-2-phenylindole (DAPI). The Cy3 and DAPI images were captured separately using a cooled CCD camera from Photometrics (Tucson, AZ), pseudo-colored and then merged using Oncor Image software, version 1.6.

RESULTS

Dose- and Time-Dependent Activation of AKT2 by Insulin

The human ovarian carcinoma cell line A2780 was used to investigate the possible involvement of human AKT2 in insulin signaling. A2780 cells were transfected with a cDNA encoding a HA epitope-tagged wild-type human AKT2 (HA-AKT2) and stimulated with insulin following serum-starvation (Fig. 1). In vitro immune-complex kinase assays were performed by using histone H2B as substrate. Activation of AKT2 by insulin occurred in a dose- and time-dependent manner, and coincided with an increase in a slower mobility HA-AKT2 band on SDS-PAGE (indicated as band c in Fig. 1, top). The highest levels of activation were observed after stimulation with insulin at a concentration of ≥100 nM. AKT2 activation occurred within 2 min of stimulation, and AKT2 remained in its activated state for at least 60 min.

As a control experiment, vector alone was transfected into cells, and immunoprecipitation was performed followed by Western blot analysis with an anti-HA antibody. HA-AKT2 bands a, b, and c were not detected in the control experiments (data not shown; see Fig. 5). A slower mobility band c on SDS-PAGE is likely to be a phosphorylated form of HA-AKT2. In order to demonstrate that the decrease in the mobility of AKT2 resulted from phosphorylation, we pre-incubated immunoprecipitated HA-AKT2 from insulin-treated cells with protein phosphatase type-2A (PP2A) (Fig. 2, lane 3). This treatment resulted in a 90–100% reduction of AKT2 protein kinase activity with a concomitant change in its pattern of electrophoretic mobility, i.e., from a slower (indicated as bands b and c in Fig. 2) to a faster mobility form (band a in Fig. 2).

Further studies were conducted with a catalytically inactive AKT2 construct (HA-AKT2E299K), described under Materials and Methods. As shown in Figure 2, upon insulin stimulation HA-AKT2E299K exhibited a mobility shift similar to that observed with wild type HA-AKT2, although the mutated AKT2 remained kinase inactive. These data suggest that alteration of the mobility concomitant with AKT2 activation results from *trans*-phosphorylation.

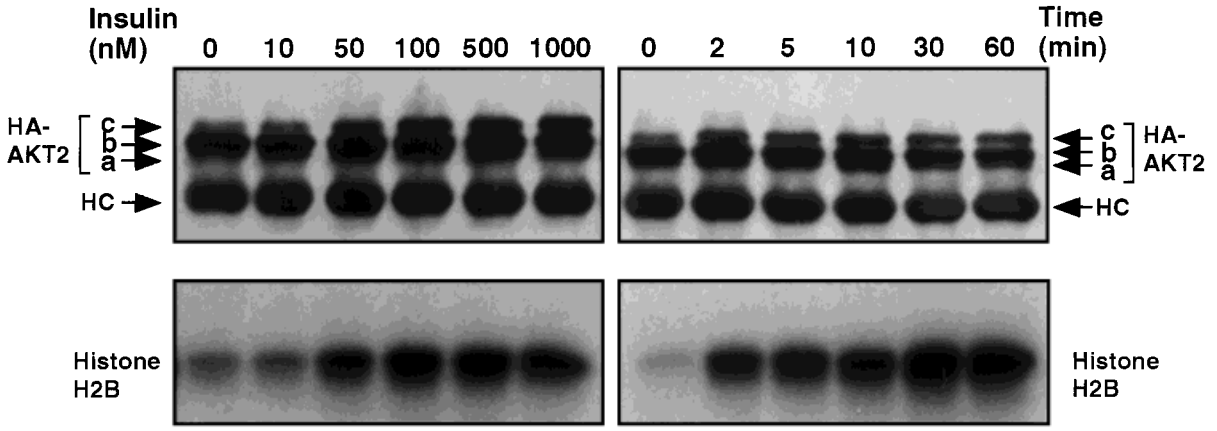

Fig. 1. Activation of AKT2 by insulin in a dose- and time-dependent manner. An HA-AKT2 expression plasmid was transfected into A2780 cells. The cells were serum-starved for 18 h, followed by stimulation with increasing concentrations of insulin for 20 min (**left**) or with 100 nM insulin for various times (**right**). Expressed HA-AKT2 was immunoprecipitated with an anti-HA antibody and kinase assays were performed using histone H2B as substrate (see Materials and Methods). **Top:** Western blot analysis of immunoprecipitates using anti-HA antibody. **Bottom:** Corresponding AKT2 kinase activity. *Arrows* a, b, c, different phosphorylation states of AKT2 as described under Results. HC, mouse immunoglobulin G heavy chain.

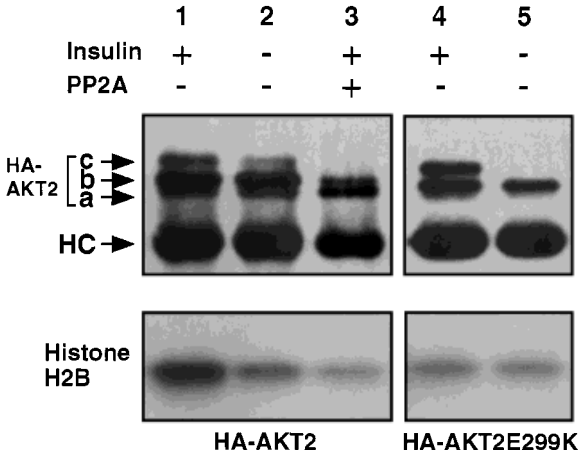

Fig. 2. Activation and phosphorylation of AKT2 after stimulation with insulin. A2780 cells transiently transfected with HA-AKT2 (**lanes 1–3**) or HA-AKT2E299K (**lanes 4 and 5**) expression plasmid were treated with (+) or without (−) insulin (100 nM, 10 min) after serum starvation for 18 h. After immunoprecipitations, the beads were treated with phosphatase buffer (**lanes 1 and 2**) or phosphatase buffer containing 0.1 U PP2A (**lane 3**) for 10 min at room temperature prior to the kinase assay. Protein kinase assays were performed as described under Materials and Methods. **Top:** Western blot analysis of immunoprecipitates using anti-HA antibody. **Bottom:** Corresponding AKT2 kinase activity. Abbreviations are as defined in Figure 1.

Wortmannin Inhibits AKT2 Activation In Vivo

We examined the effects of wortmannin on AKT2 activation in serum-starved A2780 cells with or without insulin stimulation. After transient transfection of HA-AKT2 and serum-starvation, in vitro kinase assays were performed on cells incubated with various concentrations of wortmannin for 20 min before insulin stimulation (Fig. 3). In control experiments, wortmannin was added directly either to the cell lysate or to the kinase assay reaction mixture containing insulin-stimulated, catalytically active HA-AKT2. Wortmannin did not directly affect the activity of AKT2 in vitro (Fig. 3, lanes 7 and 8), but it did inhibit AKT2 activation in vivo at concentrations of ≥50 nM. Concomitantly, the amount of hyperphosphorylated AKT2 was decreased, whereas the less phosphorylated a form of AKT2 was increased. Serum-starved cells not treated with insulin exhibited a basal level of hyperphosphorylated AKT2 (lane 2), which was completely abolished by wortmannin (lane 6). These and other data suggest that AKT2 activity is regulated by changes in its phosphorylation state via PI 3-kinase, phosphoinositide-dependent kinases [Alessi et al., 1997; Cohen et al., 1997], and perhaps other undefined kinase(s) and phosphatase(s).

Translocation of AKT2 After Insulin Stimulation

Akt family members from *Drosophila* and *Caenorhabditis elegans* to mammals possess a highly conserved PH domain at their N-terminus. PH domains have been identified in a large number of signaling and cytoskeletal molecules, many of which associate with membranes by binding to specific phospholipids [Saraste and Hyvonen, 1995]. The phospholipid product of PI 3-kinase, phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P_2), accumulates tran-

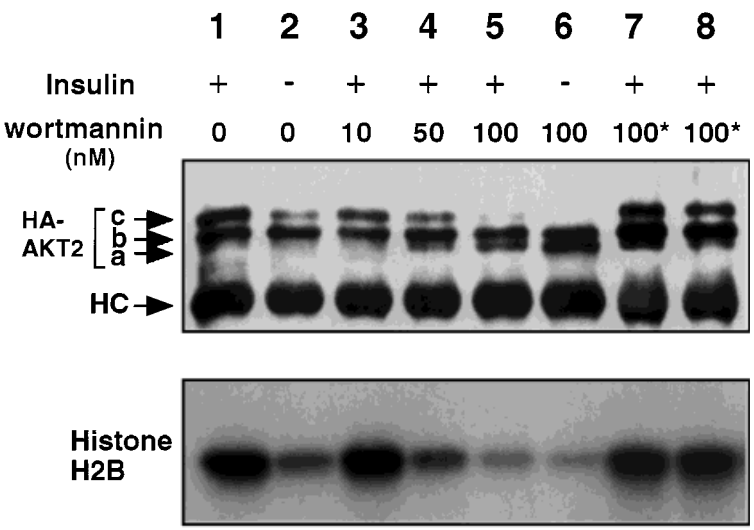

Fig. 3. Effect of wortmannin on activation of AKT2 by insulin stimulation. A2780 cells transfected with an HA-AKT2 expression plasmid were serum-starved, followed by treatment with various concentrations of wortmannin for 20 min and then incubated in the presence (+) or absence (−) of 100 nM insulin for 10 min. Expressed HA-AKT2 was immunoprecipitated with anti-HA antibody, and kinase assays were performed using histone H2B as substrate (see Materials and Methods). As a control (*), 100 nM wortmannin was added to the cell lysate (**lane 7**) or the kinase reaction mixture (**lane 8**). Western blot analysis, kinase assays. Abbreviations are as indicated in Figure 1.

siently upon growth factor stimulation and activates Akt by binding to the PH domain [Franke et al., 1997; Klippel et al., 1997]. It is likely that this transient accumulation of PtdIns-3,4-P_2 promotes the association of AKT2 with the plasma membrane. To address this issue, we performed immunofluorescence experiments using A2780 cells transiently transfected with wild type HA-AKT2. Transfected cells were treated with or without insulin for 5, 30, or 60 min in the presence or absence of wortmannin, and anti-HA antibody and anti-mouse Cy3-conjugated IgG were used as primary and secondary antibodies, respectively. As shown in Figure 4, in serum-starved cells, HA-AKT2 is predominantly localized in the cytoplasm. After insulin stimulation, a significant fraction of HA-AKT2 is translocated to the plasma membrane at all time points examined (Fig. 4D–F). No nuclear translocation was detected after insulin stimulation. Parental cells and cells transfected with vector alone exhibited little or no staining with the anti-HA antibody (data not shown). Furthermore, wortmannin abolished translocation of HA-AKT2 by insulin (Fig. 4C). These data indicate that AKT2 translocates to the plasma membrane by insulin stimulation. Furthermore, forced membrane translocation of HA-AKT2 by the addition of myristoylation signal (myrHA-AKT2) has constitutively high activity, even without insulin stimulation (data not shown). Taken together, these data indicate that the membrane translocation of AKT2 associates with AKT2 activation via PI 3-kinase in vivo.

Regulation of AKT2 and GSK-3 by Insulin Stimulation

GSK-3 regulates glycogen synthesis, one of the major metabolic processes stimulated by insulin. Glycogen synthesis can be inhibited by wortmannin and the unrelated PI 3-kinase inhibitor LY294002 [Cross et al., 1995]. GSK-3 inactivation subsequently results in the dephosphorylation and activation of downstream metabolic and gene-regulatory proteins [Welsh et al., 1996, 1997]. In vitro AKT2 kinase assays followed by GSK-3β kinase assays were performed to determine if AKT2 can regulate GSK-3 activity. As shown in Figure 5, catalytically active AKT2, like AKT1/Akt ([Cross et al., 1995]), inactivates GSK-3β (bars 3–6) and constitutively active AKT2 (myrHA-AKT2) was sufficient to inactivate GSK-3β in the absence of insulin stimulation (bars 7 and 8). Catalytically inactive HA-AKT2E299K was used as a control in a parallel experiment, and no decrease in GSK-3β activity was observed (bars 9 and 10). The latter experiment also indicates that the immunoprecipitation does not include significant contamination by other kinases that can phosphorylate phospho-GS peptide, the GSK-3β substrate.

DISCUSSION

Several lines of evidence implicate Akt family members in cell growth: (1) Akt has been shown to participate in growth factor maintenance of cell survival by transducing antiapoptotic and proliferative signals [Ahmed et al., 1997; Dudek

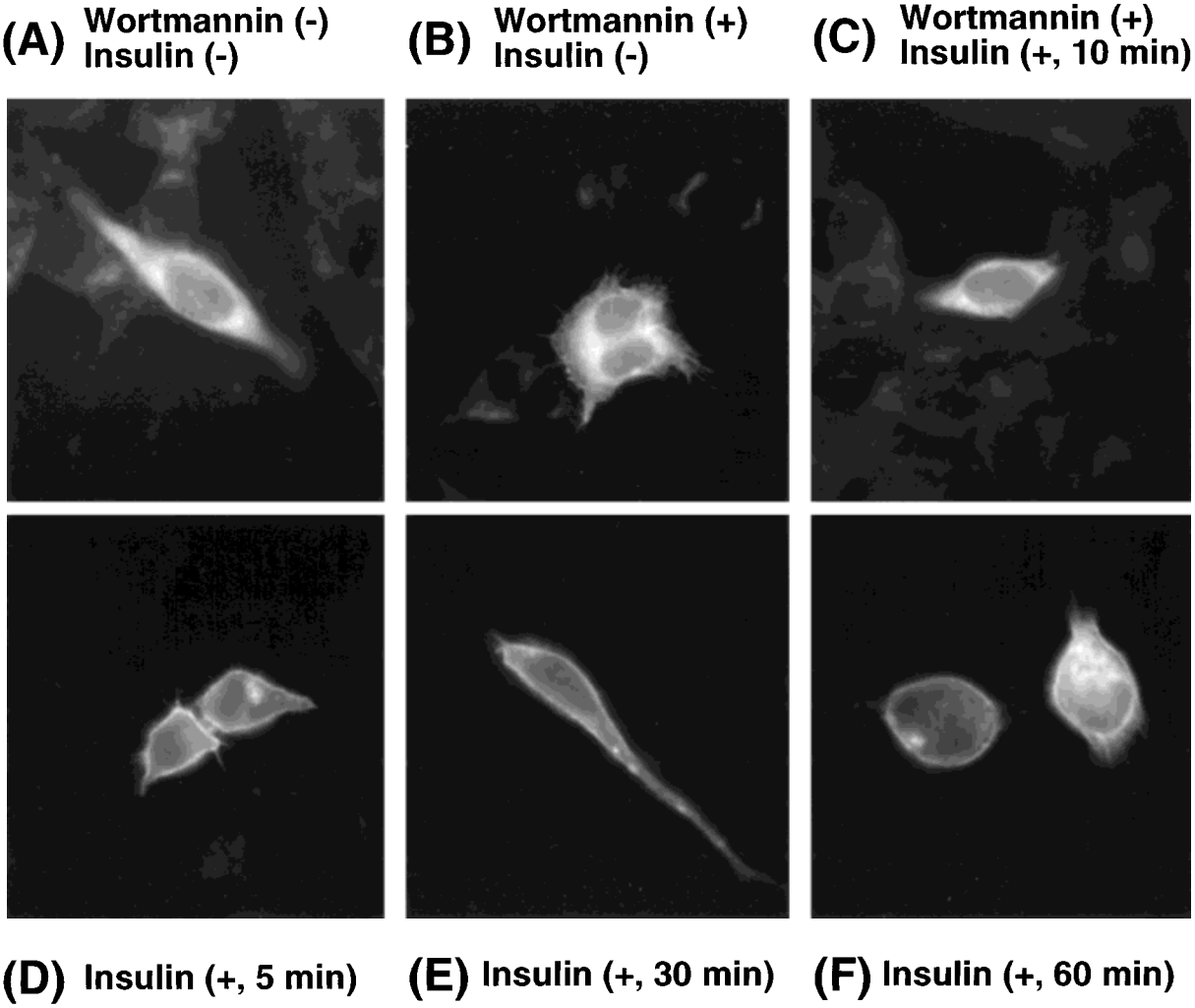

Fig. 4. AKT2 translocates to the plasma membrane after insulin stimulation. A2780 cells were transfected with HA-tagged wild-type AKT2 on poly-D-lysine-coated microscope slides. Cells were serum-starved for 24 h and then treated for the indicated time with (+) or without (−) 100 nM insulin. Untreated cells **(A)**. Cells treated with wortmannin (100 nM, 20 min) alone **(B)** or followed by insulin stimulation **(C).** Immunofluorescence experiments were performed as described under Materials and Methods.

et al., 1997]; (2) the products of the v-*akt* oncogene and experimentally overexpressed AKT2 are capable of transforming murine cells [Ahmed et al., 1993; Cheng et al., 1997]; (3) amplification and overexpression of *AKT2* is a recurrent finding in human ovarian and pancreatic carcinomas [Bellacosa et al., 1995; Cheng et al., 1992; Cheng et al., 1996; Miwa et al., 1996]; and (4) AKT2 antisense RNA inhibits tumorigenicity of pancreatic carcinoma cells overexpressing *AKT2* [Cheng et al., 1996].

The data presented here demonstrate that AKT2 is phosphorylated and activated by insulin stimulation in a dose- and time-dependent manner in an ovarian carcinoma cell line. Furthermore, we showed that PP2A inactivates AKT2 completely in vitro (Fig. 2) and that wortmannin inhibits activation of AKT2 in vivo (Fig. 3). Collectively, our findings suggest that the activation and conformation of AKT2 is regulated by a reversible phosphorylation mechanism that involves signaling molecules in the PI 3-kinase pathway, including PI 3-kinase, phosphoinositide-dependent kinases [Alessi et al., 1997; Cohen et al., 1997], and other undefined kinase(s) and phosphatase(s). Upon insulin stimulation, the catalytically inactive mutant HA-AKT2E299K exhibited a similar mobility shift as HA-AKT2 on SDS-PAGE, suggesting that HA-AKT2E299K may act as a useful dominant-negative mutant. The amino acid sequence of this region is highly conserved in

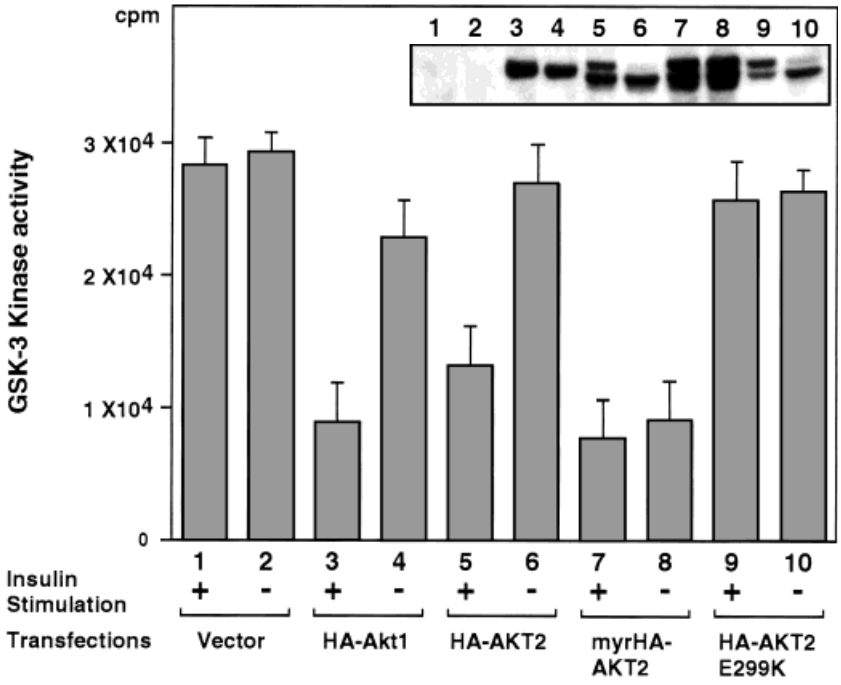

Fig. 5. Activation of AKT2 by insulin leads to the inactivation of GSK-3β. A2780 cells transfected with vector (pcDNA3) alone or the indicated AKT2 constructs were serum-starved for 24 h, followed by stimulation with (+) or without (−) 100 nM insulin for 10 min. Immunoprecipitation was performed using an anti-HA antibody, and GSK-3 kinase assays after Akt and AKT2 kinase assays were performed as described under Materials and Methods. The values shown represent means ± SE from two experiments. *Inset,* Western immunoblot analysis, using anti-HA antibody, of immunoprecipitated proteins used for kinase assays.

Akt and protein kinase C family members, as well as in the 90-kDa ribosomal S6 kinase (data not shown), suggesting that similar useful mutants of these other kinases could be generated. Further structural and biochemical analysis of AKT2E299K may facilitate the characterization of AKT2 function.

Our immunofluorescence experiments demonstrate that insulin stimulation induces AKT2 translocation to the plasma membrane. Although the function of the PH domain of AKT2 has not been characterized extensively, it is likely to be very similar to that of AKT1/Akt for several reasons: (1) the PH domains of Akt family members are highly conserved; (2) like AKT2, AKT1/Akt translocates to the plasma membrane and is activated after growth factor stimulation [Ahmed et al., 1997]; and (3) wortmannin inhibits both the translocation and activation of AKT2 and AKT1/Akt. Furthermore, in A2780 cells deprived of serum, HA-AKT2 was located primarily in the cytoplasm, although a small percentage (<10%) of cells exhibited HA-AKT2 in the nucleus as well (data not shown). Similar results have been obtained following serum deprivation in NIH 3T3 cells transfected with HA-Akt (A. Bellacosa, personal communication). After insulin stimulation, we observed translocation of HA-tagged wild-type AKT2 to the plasma membrane (Fig. 4). Meier et al. [1997] found that in REF52 cells most wild-type HA-AKT2 translocates to the nucleus within 20–30 min after stimulation with serum. With A2780 cells, we did not detect significant translocation of HA-AKT2 to the nucleus at any time point within the first hour after stimulation with insulin, insulin-like growth factor 1, or serum (Fig. 5, and data not shown). Likewise, in other experiments with REF52 cells (not shown), we observed translocation of HA-AKT2 to the plasma membrane but not the nucleus following serum stimulation. Other approaches, e.g., microinjection experiments, may be useful in resolving this discrepancy.

Because of their high degree of sequence and predicted structural homology, AKT2 and AKT1/Akt may participate in similar signal transduction cascades, such as the insulin/PI 3-kinase pathway. Insulin stimulates glycogen synthase by inhibiting GSK-3 and/or activating certain protein phosphatases. Our studies demonstrate that activation of AKT2 by insulin results in the inactivation of GSK-3β in vitro, suggesting that AKT2 is involved in glycogen synthase activation. GSK-3 is also involved in the regulation of several other intracellular signaling pathways, including control of transcription factors AP1 and CREB, and of the tumor suppressor gene product APC, wingless development in flies, and dorsoventral patterning in frogs [Cross et al., 1995]. Therefore, it is possible that AKT2 is

involved in multiple pathways and, thus, may play an important role through its phosphorylation of GSK-3. Given its elevated expression levels in highly insulin-responsive tissues [Altomare et al., 1998], AKT2 may be the prominent Akt family member involved in the transduction of insulin signals.

Finally, although the mechanism by which *AKT2* amplification contributes to the development of certain human cancers is unknown, evidence provided here and in other recent work [Meier et al., 1997] that AKT2 is a downstream target of PI 3-kinase activity may provide insight into this process. In addition to insulin, various growth factors have been shown to activate AKT2 [Meier et al., 1997]. Conceivably, constitutive overactivation of AKT2 by gene amplification and/or overexpression in human cancers could intensify signaling through this pathway or, alternatively, could act independently of these mitogenic signals and thereby contribute to the marked dysregulation of cell growth observed in these tumors [Ruggeri et al., 1998]. The fact that antisense *AKT2* can markedly diminish the tumorigenic and invasive potential of tumor cells exhibiting amplified/overexpressed *AKT2* [Cheng et al., 1996] lends additional support to this possibility.

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REFERENCES

Ahmed NN, Franke TF, Bellacosa A, Datta K, Gonzalez-Portal ME, Taguchi T, Testa JR, Tsichlis PN (1993): The proteins encoded by c-*akt* and v-*akt* differ in post-translational modification, subcellular localization and oncogenic potential. Oncogene 8:1957–1963.

Ahmed NN, Grimes HL, Bellacosa A, Chan TO, Tsichlis PN (1997): Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc Natl Acad Sci USA 94:3627–3632.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997): Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr Biol 7:261–269.

Altomare DA, Guo K, Cheng JQ, Sonoda G, Walsh K, Testa JR (1995): Cloning, chromosomal localization and expression analysis of the mouse *Akt2* oncogene. Oncogene 11:1055–1060.

Altomare DA, Lyons GE, Mitsuuchi Y, Cheng JQ, Testa JR (1998): *Akt2* mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. Oncogene 16:2407–2411.

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Benedetti Panici, P, Mancuso S, Neri G, Testa JR (1995): Molecular alterations of the *AKT2* oncogene in ovarian and breast carcinomas. Int J Cancer 64:280–285.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR (1992): *AKT2*, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci USA 89:9267–9271.

Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK, Testa JR (1996): Amplification of *AKT2* in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci USA 93:3636–3641.

Cheng JQ, Altomare DA, Klein MA, Lee WC, Kruh GD, Lissy NA, Testa JR (1997): Transforming activity and mitosis-related expression of the *AKT2* oncogene: Evidence suggesting a link between cell cycle regulation and oncogenesis. Oncogene 14:2793–2801.

Cohen P, Alessi DR, Cross DA (1997): PDK1, one of the missing links in insulin signal transduction? FEBS Lett 410:3–10.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995): Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785–789.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997): Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Franke TF, Kaplan DR, Cantley LC, Toker A (1997): Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 275:665–668.

Haslam RJ, Kolde HB, Hemmings BA (1993): Pleckstrin domain homology. Nature 36:309–310.

Hemmings BA (1997): Akt signaling: linking membrane events to life and death decisions. Science 275:628–630.

Jones PF, Jakubowicz T, Hemmings BA (1991): Molecular cloning of a second form of rac protein Chinese. Cell Reg 2:1001–1009.

Klippel A, Kavanaugh WM, Pot D, Williams LT (1997): A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. Mol Cell Biol 17:338–344.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA (1996): Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372–31378.

Mayer BJ, Ren R, Clark KL, Baltimore D (1993): A putative modular domain present in diverse signaling proteins. Cell 73:629–630.

Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA (1997): Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bβ. J Biol Chem 272: 30491–30497.

Miwa W, Yasuda J, Murakami Y, Yashima K, Sugano K, Sekine T, Kono A, Egawa S, Yamaguchi K, Hayashizaki Y, Sekiya T (1996): Isolation of DNA sequences amplified at chromosome 19q13.1–q13.2 including the AKT2 locus in human pancreatic cancer. Biochem Biophys Res Commun 225:968–974.

Ruggeri BA, Huang L, Wood M, Cheng JQ, Testa JR (1998): Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. Mol Carcinog 21:81–86.

Saraste M, Hyvonen M (1995): Pleckstrin homology domains: A fact file. Curr Opin Struct Biol 5:403–408.

Staal SP (1987): Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84:5034–5037.

Staal SP, Huebner K, Croce CM, Parsa NZ, Testa JR (1988): The AKT1 proto-oncogene maps to human chromosome 14, band q32. Genomics 2:96–98.

Testa J, Bellacosa A (1997): Membrane translocation and activation of the Akt kinase in growth factor-stimulated hematopoietic cells. Leukemia Res 21:1027–1031.

Welsh GI, Miyamoto S, Price NT, Safer B, Proud CG (1996): T-cell activation leads to rapid stimulation of translation initiation factor eIF2B and inactivation of glycogen synthase kinase-3. J Biol Chem 271:11410–11413.

Welsh GI, Stokes CM, Wang X, Sakaue H, Ogawa W, Kasuga M, Proud CG (1997): Activation of translation initiation factor eIF2B by insulin requires phosphatidyl inositol 3-kinase. FEBS Lett 410:418–422.