Autophosphorylation of Akt at Threonine 72 and Serine 246

A POTENTIAL MECHANISM OF REGULATION OF Akt KINASE ACTIVITY

Xinquan Li,† Yang Lu,† Weidong Jin,† Ke Liang,† Gordon B. Mills‡, and Zhen Fan†‡

From the Departments of †Experimental Therapeutics and ‡Molecular Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Activation of the serine/threonine protein kinase Akt is a multi-step process. We here propose that the kinase activity of Akt is regulated via autophosphorylation in trans at two putative sites (threonine 72 and serine 246) that lie in the characteristic Akt substrate motif (RXRXXS/T). Incubation of Akt immunoprecipitated from transfected cells with a pre-activated Akt recombinant protein and γ-32P-labeled ATP led to marked incorporation of radioactivity in wild-type Akt but not Akt/T72A/S246A mutant. Western blot analysis using a phosphorylated Akt substrate-specific antibody of Akt immunoprecipitated from transfected cells confirmed the autophosphorylation of wild-type Akt but not Akt/T72A/S246A mutant in insulin-like growth factor-1 (IGF-1)-stimulated cells. Autophosphorylation of Akt on Thr-72 and Ser-246 appeared to require prior phosphorylation of Akt on Thr-308 and Ser-473. Compared with wild-type Akt, Akt/T72A/S246A mutant exhibited markedly reduced basal Akt kinase activity and response to cellular stimulation by insulin-like growth factor-1, and also conferred less cellular resistance to doxorubicin-induced apoptosis. The findings from these pilot studies suggest that Akt regulates its kinase activity through autophosphorylation. Further investigation of this potential novel regulatory mechanism by which Akt performs its cellular functions is warranted.

The proto-oncogene Akt/PKB, which encodes a serine/threonine protein kinase, is frequently expressed, and its product is highly activated in a variety of human cancers, including cancer of the breast (1, 2), ovary (2, 3), prostate (2, 3), lung (4), thyroid (5), pancreas (6, 7), and myeloma (8). Extensive biochemical studies have shown that activation of phosphatidylinositol 3-kinase leads to increased intracellular levels of 3-phosphorylated phosphatidylinositols, which recruit Akt through the pleckstrin homology (PH) domain of the molecule to the plasma membrane (9–11), where Akt undergoes conformational changes and is activated after phosphorylation at both the Thr-308 residue in its activation loop and the Ser-473 residue at the hydrophobic carboxyl-terminal (13–15). The bona fide kinase or kinases responsible for Ser-473 phosphorylation remain unknown despite reports that some candidate kinases, such as mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) (16, 17), integrin-linked kinase (18), DNA-dependent protein kinase (19), and rictor-mammalian target of rapamycin complex (20), induced Ser-473 phosphorylation in vitro studies. Besides these two critical sites (Thr-308 and Ser-473), residues serine 124 and threonine 450 were found to be constitutively phosphorylated (16), and tyrosine residues 315 and 326 phosphorylated by Src kinase may also be involved in the regulation of Akt activity (21).

Because Akt is a serine/threonine kinase, we wondered whether its activity is regulated by autophosphorylation, which is a common regulatory mechanism among protein kinases (22). Earlier studies have suggested that the Ser-473 of Akt may be phosphorylated via autophosphorylation (23); however, it is arguable because the site does not concur with the well described phosphorylation motif (Arg-Xaa-Arg-Xaa-Xaa-Thr, or RXRXXS/T) that is characteristic of Akt substrates (24). Using in vitro kinase assays and cell-based assays with transfection, we here report that Akt can indeed phosphorylate itself in vitro at two putative sites (threonine 72 and serine 246), located in the PH domain and the kinase domain of the molecule, respectively, that fall into the Akt substrate phosphorylation motif. The autophosphorylation of Akt may play a role in the transduction of the signals that activate Akt, because experimental point mutation of the two sites to alanine (Akt/T72A/S246A) markedly reduced basal Akt kinase activity and the response to cellular stimulation by insulin-like growth factor 1 (IGF-1) when expressed in cells. Furthermore, expression of the Akt/T72A/S246A mutant was less effective than wild-type Akt expression in protecting cells from doxorubicin-induced apoptosis. Our results from these pilot studies suggest a potential novel regulatory mechanism through which the signals are augmented after Akt activation. In-depth exploration of this mechanism is warranted, because its elucidation may be crucial for successful development of new drugs that inhibit the function of Akt for treating human diseases such as cancer.

EXPERIMENTAL PROCEDURES

Cells and cDNA Constructs—MCF7 human breast cancer cells were originally obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% CO₂ and 95% air. Human Akt1 cDNA was obtained from MCF7 cells by reverse transcriptase-polymerase chain reaction with primers described previously (25) and cloned into the pcDNA3.1-HisC expression vector (Invitrogen). Constructs containing various point mutations (T72A/S246A, T308A/S473A, and T308D/S473D) of Akt1 were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the wild-type Akt1 expression construct as the polymerase chain reaction template. All of the point mutations were verified by DNA sequence analysis.

Expression of the constructs was conducted with the use of a transient transfection procedure by exposing the MCF7 cells to a mixture of the construct and the FuGENE 6 transfection reagent (Roche Diagnos-
Akt Autophosphorylation at Thr-72 and Ser-246

tics, Minneapolis, NM) for 16 h. The cells were cultured in regular medium for an additional 24 h for sufficient expression of the transfected constructs before any further experiments were conducted.

Antibodies and Other Reagents—Antibodies directed against total Akt protein, phosphorylated Akt substrate (PAS), phosphorylated glycogen synthase kinase 3 (GSK3α/β (S21/9), poly(ADP-ribose) polymerase (PARP), and cleaved PARP were obtained from Cell Signaling Technology (Beverly, MA). The anti-HisG tag monoclonal antibody was obtained from Upstate Biotechnology (Charlottesville, VA), and the anti-HA tag monoclonal antibody was from Invitrogen. Protein A-conjugated Sepharose beads were purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma (St. Louis, MO).

Immunoprecipitation and Immunoblotting Analysis—Lysates from cultured cells were prepared with a lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100. The lysates were cleared by centrifugation, and the supernatants were collected. For immunoprecipitation, equal amounts of lysate protein were incubated with specific anti-tag (HisG or HA) antibody (1 μg per 500 μg of protein) for 2 h and then incubated for 1 h with the protein A-conjugated Sepharose beads. The beads were washed three times with the lysis buffer, and the immunoprecipitates were solubilized by boiling the beads in Laemmlı SDS buffer at 100 °C for 5 min. For immunoblotting analysis, immunoprecipitates or lysates with equal amounts of protein were boiled in the Laemmlı SDS buffer, separated by SDS-PAGE, and electrotransferred to nitrocellulose membranes. The membranes were incubated with specific primary antibodies and then subjected to further incubation with horseradish peroxidase-conjugated secondary antibodies. The signals were developed by using the enhanced chemiluminescence detection kit (Amersham Biosciences).

Radioactive and Nonradioactive Akt Kinase Assays—A radioactive Akt kinase assay was performed by in vitro incubation of a pre-activated Akt1 recombinant protein (0.5 μg/assay, Upstate Biotechnology) with 0.8 μl (20 μCi)/assay of γ-[32P]-labeled ATP (specific activity = 3000 Ci/mmol, PerkinElmer Life Sciences) that was titrated with increasing amounts of unlabeled ATP in a kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 10 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM Na₃VO₄) for 30 min at 30 °C with a total volume of 30 μl/assay. Alternatively, the wild-type Akt protein or various Akt mutants immunoprecipitated by corresponding anti-tag antibodies from MCF7 cells transiently transfected with various Akt constructs were used as the substrates for the pre-activated Akt recombinant protein by incubating them in vitro in the presence of [γ-32P]ATP. This procedure was followed by thorough washing of the immunoprecipitation beads to maximally remove the added pre-activated Akt recombinant protein and residual [γ-32P]ATP. After each reaction, the products were separated by SDS-PAGE, and the gels were dried and subjected to autoradiography. The results were analyzed with a PhosphorImager or quantitated densitometrically, and the ratios of autophosphorylated Akt to the Akt proteins immunoprecipitated were determined to normalize for any potential differences in each sample caused by immunoprecipitation.

A nonradioactive Akt in vitro kinase assay was performed by incubating a GSK3 fusion protein (used as the substrate, 1 μg/assay, Cell Signaling Technology) with the Akt proteins (used as the kinase) immunoprecipitated with corresponding anti-tag antibodies from the lysates of the cells transfected with various Akt expression constructs in the presence of 33.3 μM unlabeled ATP for 30 min at 30 °C in a total volume of 30 μl/assay. The products of the kinase reaction were resolved by SDS-PAGE followed by Western blot analysis with the phosphorylated GSK3β (Ser-21/9)-specific antibody.

Cell Apoptosis Assays—An apoptosis enzyme-linked immunosorbent assay kit (Roche Diagnostics) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) in the untreated and treated MCF7 cells expressing various Akt constructs. This colorimetric enzyme immunoassay was performed as we reported previously (26). Induction of apoptosis was further documented by detection of PARP cleavage in Western blotting analysis with an antibody that recognizes the cleaved PARP fragment.

RESULTS

To determine whether Akt can autophosphorylate, we first performed an in vitro kinase assay using recombinant purified Akt1. Incubation of the pre-activated form of Akt1 recombinant protein with [γ-32P]ATP for 30 min in vitro resulted in marked radioactive phosphate incorporation into Akt (Fig. 1A). The level of Akt-associated radioactivity could be reduced by titration of [γ-32P]ATP with increasing amounts of unlabeled ATP added to the incubation mixture. This result strongly suggests that the Akt protein was autophosphorylated in vitro. To determine whether Akt autophosphorylation could occur between two Akt molecules (in trans), we modified the in vitro kinase assay by incubating the pre-activated Akt recombinant protein with a tagged-Akt protein that was immunoprecipitated with an anti-HisG tag antibody from MCF7 breast cancer cells transiently transfected with an Akt expression construct. After a 30-min kinase reaction in the presence of [γ-32P]ATP, the immunoprecipitates were thoroughly washed to remove the pre-activated Akt recombinant protein and any residual [γ-32P]ATP. The immunoprecipitates were then resolved by SDS-PAGE, and the gel was dried and subjected to autoradiography. The controls for the experiment included immunoprecipitation with an anti-HisG antibody from the same cells transfected with the backbone expression vector pcDNA3.1 and a mock immunoprecipitation with an anti-mouse immunoglobulin G antibody from cells transfected with the HisG-tagged Akt expression construct. Compared with the controls, which retained a basal level of 32P radioactivity at the migrating position of the Akt protein after extensive washing of the protein A-Sepharose beads, the immunoprecipitates pulled down from His-G-tagged Akt-transfected cells by the anti-His-G antibody showed a statistically significant higher level of 32P radioactivity incorporation (p < 0.05) (Fig. 1B, upper gel, lane 4 versus lanes 2 or S). An accompanying Western blot analysis of the immunoprecipitates showed that His-G-tagged Akt was successfully pulled down from lysates of the cells transfected with the His-G-Akt expression construct by the antibody (Fig. 1B, lower gel). In these experiments, it is unlikely that kinases other than the pre-activated Akt, which may be co-immunoprecipitated in the Akt immunoprecipitates, phosphorylated the immunoprecipitated Akt, because no phosphorylation was detected in the Akt immunoprecipitates when the pre-activated Akt was not added.

Previous studies identified a consensus motif (RXRXX[S/T]) that Akt phosphorylates (24). Phosphorylation of this consensus motif can be detected with specific antibodies that recognize the motif when phosphorylated, which in fact has been used for identifying novel substrates of Akt (27). Using Western blot analysis with a PAS-specific antibody, we detected a phosphorylated protein band in the lysates of MCF7 cells transiently transfected with the His-G tagged Akt expression construct (described as...
FIGURE 1. Autophosphorylation of Akt1 at Thr-72 and Ser-246. A, a baculovirus-expressed, full-length human recombinant Akt1 protein that was pre-activated in vitro by MAPKAPK2 and phosphoinositide-dependent kinase 1 (Upstate) was incubated in a kinase buffer containing \( [\gamma-\text{P}]\text{ATP} \) and up to 100 μM unlabeled ATP for 30 min at 30 °C. The products were resolved by SDS-PAGE followed by autoradiography and quantitation with the use of phosphorimaging analysis. B, a His-G-tagged full-length human Akt1 construct or the pcDNA3.1 backbone expression vector was transiently transfected into MCF7 breast cancer cells overnight (16 h) with FuGENE 6 and then cultured for 24 h in regular culture medium. Cell lysates were prepared for immunoprecipitation (IP) with an anti-HisG antibody or anti-mouse immunoglobulin G antibody (Mock). The immunoprecipitates were then split into two equal parts; one part was used as substrates for an in vitro Akt kinase reaction by the pre-activated Akt protein described in A, and the other part was used for Western blot analysis with an Akt antibody. *, \( p < 0.05 \) versus column 2 (n = 3). C, the His-G-tagged Akt expression construct or pcDNA3.1 was transiently transfected into MCF7 cells as described in B. The cells were then left untreated or treated with 10 nM IGF-1 for 15 min before cell lysis and subsequent processing for immunoprecipitation with anti-HisG antibodies. Before SDS-PAGE, a duplicate set of the immunoprecipitates was incubated in vitro with 33.3 μM unlabeled ATP for 30 min. Western blot analysis was performed with PAS-specific antibody and an Akt antibody, respectively. D, schematic presentation of the Akt functional domains and putative phosphorylation sites. Akt is composed of an N-terminal PH domain followed by a kinase domain and a C-terminal regulatory tail. Two putative motifs characteristic of Akt substrates (RPRPNT and RERVFS) are located in the PH and kinase domains, respectively. E, constructs containing full-length wild-type Akt and Akt mutants (Akt/T72A, Akt/S246A, and Akt/T72A/S246A) were transiently transfected into MCF7 cells as described in B. Cell lysates were then prepared and processed for immunoprecipitation. The immunoprecipitates were equally split and used as the substrates for an in vitro Akt kinase reaction and for Western blot analysis with an Akt antibody, respectively as described in B, *, \( p < 0.05 \) and **, \( p < 0.01 \) versus column 2 (n = 3). F, the expression constructs described in E and pcDNA3.1 were transiently transfected into MCF7 cells. Cell lysates were prepared and processed for immunoprecipitation with anti-HisG antibody. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blot analysis with PAS-specific antibody and reblotted with an Akt antibody. G, Akt autophosphorylation sequence alignment of three human Akt isoforms and of Akt derived from mouse, rat, cow, and chicken (source from GenBank).
Akt Autophosphorylation at Thr-72 and Ser-246

in Fig. 1B) and stimulated with IGF-1 (Fig. 1C). Reprobing the same blot with an Akt antibody confirmed that the band detected by the anti-PAS antibody had the same migration position as Akt. We had a separate set of the immunoprecipitates that were incubated in vitro with unlabeled ATP for 30 min before further processing of the immunoprecipitates for SDS-PAGE and Western blot analysis (Fig. 1C, last two lanes). The signal detected by the PAS-specific antibody in the IGF-1-stimulated cells was higher than that in unstimulated cells (Fig. 1C). Taken together, our results support the observation that Akt may phosphorylate itself under certain circumstances.

An analysis of the amino acid sequence of Akt indicated that there are two sites in Akt that conform to the Akt substrate phosphorylation motif (RXRXX[S/T]): threonine 72 (RPRPNT) and serine 246 (RERVFS) (Fig. 1D). With this information, we performed serial site-directed point mutations of Akt to generate specific Akt mutants wherein Thr-72 and Ser-246 were replaced by alanine, respectively, and concurrently. Constructs containing the mutants (Akt/T72A, Akt/S246A, and Akt/T72A/S246A) and the wild-type Akt construct were transiently expressed in MCF7 cells. The expressed proteins were immunoprecipitated with the anti-HisG antibody and were used as substrates for an in vitro Akt kinase in a manner similar to that described for Fig. 1B. Fig. 1E shows that, compared with the phosphorylation level of the wild-type Akt, mutation at Thr-72 or Ser-246 led to a statistically significantly reduced level of phosphorylation of the protein after incubation with the pre-activated Akt recombinant protein (p < 0.05 for both mutations) and that simultaneous mutation at both sites further decreased the level of phosphorylation (p < 0.01). The decrease of autophosphorylation of the Akt mutants indicates that both Thr-72 and Ser-246 may serve as putative sites of Akt autophosphorylation. To further demonstrate phosphorylation at these sites, we subjected the immunoprecipitates of the constructs transiently expressed in MCF7 cells to Western blot analysis with the PAS-specific antibody and Akt antibody described for Fig. 1C. The analysis showed that the levels of expression of the wild-type protein and the mutants were comparable (Fig. 1F, lower gel). However, compared with the result with the wild-type Akt, which showed a distinct protein band detected by Western blot analysis with the PAS-specific antibody, the signal detectable by the antibody was markedly reduced in the immunoprecipitates pulled down from the cells transfected with either of the Akt single mutants (Akt/T72A or Akt/S246), and no signal was detected in the immunoprecipitates from the cells transfected with the Akt dual mutant (Akt/T72A/S246A) (Fig. 1F, upper gel).

To determine whether the two autophosphorylation sites that we identified in human Akt1 are widely conserved, we reviewed the relevant sequence data of Akt1 Thr-72 and Ser-246 among the human Akt isoforms as well as in mouse, rat, cow, chicken, Drosophila, and Caenorhabditis elegans. As indicated in Fig. 1G, the relevant motifs containing the two sites are similar among all three human Akt isoforms and are phylogenetically conserved across the species back to the birds. In human Akt2, Ser-246 is changed to Thr-248. In chicken Akt1 and human Akt3, the second Arg is substituted with an equivalent Lys, shown as RXXKAT; however, this remains an Akt phosphorylation consensus sequence. The two sites are not conserved in the more primitive organisms Drosophila or C. elegans (data not shown). This suggests that phosphorylation of these two sites (Thr-72 and Ser-246) is acquired during evolution in higher eukaryotes, and thus may not be as essential as the phosphorylation at Thr-308 and Ser-473 for the kinase activity of Akt.

We next determined whether Thr-308/Ser-473 phosphorylation is required for Akt as a substrate for phosphorylation at Thr-72 and Ser-246 by examining the autophosphorylation of Akt in which Thr-308 and Ser-473 were mutated to a nonphosphorylatable amino acid, alanine (Akt/T308A/S473A). The constructs expressing wild-type Akt and Akt/T308A/S473A mutant were transiently transfected in MCF7 cells, and the expressed proteins were immunoprecipitated and subsequently processed for in vitro phosphorylation with the procedure described for Fig. 1 (B and E). We found that wild-type Akt protein and Akt/T308A/S473A mutant protein were immunoprecipitated at comparable levels (Fig. 2A, lower gel). However, compared with the results for the cells transfected with the wild-type Akt, the Akt/T308A/S473A mutant was phosphorylated at a remarkably lower level (Fig. 2A, upper gel, p < 0.01). This result suggests that phosphorylation of Thr-308/Ser-473 is a prerequisite for the efficient autophosphorylation of Akt, at least in vitro.

To further assess the role of Thr-308 and Ser-473 phosphorylation in determining the level of autophosphorylation of Akt at the Thr-72 and Ser-246 residues, we used the same method to examine the autophosphorylation of Akt in which Thr-308 and Ser-473 were replaced with aspartic acid (T308D/S473D), which mimics the constitutive phosphorylation status of the two sites by introduction of a negative charge. Despite comparable levels of expression of wild-type Akt and Akt/T308D/S473D mutant when the constructs were transiently expressed in MCF7 cells (Fig. 2B, inset), the protein levels of Akt/T308D/S473D mutant were much lower when immunoprecipitated with the same anti-HisG antibody (Fig. 2B). We speculate that this effect may be caused by a conformational change of the protein after experimental mutation of T308D and S473D that resulted in the His tag epitope being less accessible to the antibody. However, although the levels of Akt/T308D/S473D protein immunoprecipitated with the anti-HisG antibody were much lower, an in vitro kinase assay showed marked autophosphorylation when the immunoprecipitates were incubated with the pre-activated Akt recombinant protein in the presence of [γ-32P]ATP (Fig. 2B). After normalization of Akt/T308D/S473D protein levels, autophosphorylation of Akt/T308D/S473D was statistically significantly higher than that of the wild-type Akt (p < 0.05), indicating the importance of Thr-308/Ser-473 phosphorylation in the modulation of Akt autophosphorylation.

For further evidence that phosphorylation at Thr-308 and Ser-473 of the protein contribute to Akt autophosphorylation, we conducted an additional in vitro Akt kinase assay compare the autophosphorylation levels of Akt immunoprecipitated from IGF-1-stimulated and unstimulated MCF7 cells. Similar to the results with Akt/T308D/S473D, we found that the level of autophosphorylation was higher in Akt immunoprecipitated from IGF-1-stimulated cells than that from unstimulated cells, although the difference was moderate (Fig. 2C). A caveat is that, in this experiment, the autophosphorylation of Akt was catalyzed primarily by the pre-activated Akt recombinant protein in vitro other than the endogenous Akt, because there were only minimal increases in Akt autophosphorylation levels when the Akt proteins immunoprecipitated from the IGF-1-stimulated or unstimulated cells were incubated with only [γ-32P]ATP in vitro (without addition of the pre-activated Akt recombinant protein). Taken together, these data suggest that Akt autophosphorylation is enhanced when Thr-308 and Ser-473 are already phosphorylated, presumably because phosphorylation of these two sites conferred a conformational change that increased access for cross-phosphorylation of Akt by another Akt molecule.

A critical question is whether autophosphorylation of Akt at Thr-72 and Ser-246 plays a role in Akt activity. To answer this question, we explored potential differences in kinase activity between wild-type Akt and Akt mutants (Akt/T72A, Akt/S246A, and Akt/T72A/S246A) in response to stimulation with IGF-1 when the constructs were transiently expressed in MCF7 cells. With the use of GSK3 peptide as a...
substrate, all three Akt mutants exhibited remarkably reduced kinase activity in both IGF-1-stimulated and unstimulated cells compared with wild-type Akt (Fig. 3A). These results indicate that autophosphorylation of Akt at Thr-72 and Ser-246 is critical for the overall kinase activity of Akt.

Previous studies showed that the PH domain of Akt is important during the Akt activation process, because it directs the molecule to the membrane proximity for the required phosphorylation by upstream kinases, when there is an increased level of 3-phosphorylated phosphatidylinositols in cells after exposure to external stimuli that activate phosphatidylinositol 3-kinase. Our present observations suggest that the PH domain plays additional roles in the activity of Akt, because it harbors an important autophosphorylation site, Thr-72. To provide further insight into the role of the PH domain, we compared the kinase activity of a full-length Akt and a PH domain-deleted Akt, both of which were constructed to contain an N-terminal myristoylation sequence that preserves the potential for membrane anchorage for constitutive phosphorylation and activation without the PH domain (28, 29). Vectors without any insert (backbone) or constructed with full-length wild-type Akt were used as controls. Cell lysates were prepared and processed for immunoprecipitation with corresponding anti-tag antibodies. The immunoprecipitates were then equally split and used as the substrates for an in vitro Akt kinase reaction by the pre-activated Akt protein as described for Fig. 1A and for Western blot analysis with an Akt antibody, respectively. 

![FIGURE 2. Requirement of Akt Thr-308 and Ser-473 phosphorylation for Akt autophosphorylation. A, constructs containing wild-type Akt or Akt/T308A/S473A mutant were transiently transfected into MCF7 cells with procedures similar to that described for Fig. 1A and for Western blot analysis with an Akt antibody, respectively. **, p < 0.01 versus column 4 (n = 3). B, constructs containing wild-type Akt or Akt/T308D/S473D mutant were transiently transfected into MCF7 cells as described for Fig. 1B. Cell lysates were prepared and assayed for protein expression by Western blot analysis with anti-HisG and anti-β-actin antibodies (inset). The same lysates were also processed for immunoprecipitation with anti-HisG antibody. The immunoprecipitates were then equally split and used as the substrates for an in vitro Akt kinase reaction by the pre-activated Akt described in A and for Western blot analysis with an Akt antibody. The results of two independent experiments (EXP1 and EXP2) (representing a total of four repetitions) are shown. *, p < 0.05 versus column 4 (n = 4). C, a construct containing a HisG tag wild-type Akt was transiently transfected into MCF7 cells as described for Fig. 1C. Before lysis, the cells were either untreated or treated with 10 nM IGF-1 for 15 min. Cell lysates were prepared and subjected to immunoprecipitation with anti-HisG antibody or a mock immunoglobulin G antibody. The immunoprecipitates were then equally split and used as the substrates for an in vitro Akt kinase reaction by the pre-activated Akt protein described in A and for Western blot analysis with an Akt antibody.](image-url)
doxorubicin treatment. We used two independent assays for measuring the levels of apoptosis in the cells after treatment, an enzyme-linked immunosorbent assay that quantitatively measures the levels of cytoplasmic histone-associated DNA fragments in apoptotic cells and a Western blot analysis that detects the levels of cleavage of PARP during apoptosis. Compared with the cells transfected with the control vector, cells that were transfected with and expressed wild-type Akt markedly decreased the level of doxorubicin-induced apoptosis in MCF7 cells; however, expression of the Akt mutants, particularly Akt/T72A/S246A, had statistically significant less effect on reducing the levels of apoptosis by doxorubicin in the cells (p < 0.01) (Fig. 4A). These results are consistent with the findings shown in Fig. 3 that the autophosphorylation-deficient Akt mutants have decreased enzymatic activity.

DISCUSSION

Over the past decade, the regulation of Akt by phosphorylation has been extensively studied. The consensus view is that Akt is regulated primarily by phosphorylation at two critical sites, Thr-308 in the activation segment and Ser-473 in the hydrophobic motif terminus. On the basis of our findings from in vitro kinase assays with serial cell-based biochemical assays through expression of transfected wild-type and point-mutated Akt constructs, we propose a novel mode of Akt activa-
Akt Autophosphorylation at Thr-72 and Ser-246

Akt-associated incorporation of experiments in which the pre-activated Akt recombinant protein was a contaminating component along with the recombinant Akt protein, such as phosphoinositide-dependent kinase 1 and MAPKAP2, which were used to pre-activate the recombinant Akt? Despite the purity assurance of analysis by the vendor, contamination is theoretically possible for the experiments in which the pre-activated Akt recombinant protein was used as the kinase. However, this is unlikely for the reason that if the Akt-associated incorporation of [γ-32P]ATP was indeed caused by a contaminating enzyme, such as phosphoinositide-dependent kinase 1 or MAPKAP2, no significant difference in protein phosphorylation should have been detected between wild-type Akt and Akt mutants (Akt/T72A, Akt/S246A, and Akt/T72A/S246A), because these enzymes do not phosphorylate Thr-72/Ser-246, and mutation of the two sites would not therefore alter the levels of phosphorylation of the protein. Furthermore, the autophosphorylation of Akt was independently verified by Western blot analysis using an anti-PAS antibody of the extracts of cell after IGF-1 stimulation; the process did not involve the use of the pre-activated recombinant Akt protein at all.

A critical study that needs to be performed is the evaluation and demonstration of the stoichiometry of phosphorylation on the Thr-72A and Ser-246A residues in vivo with respect to the kinase activity of Akt. The phosphorylation stoichiometry of Akt phosphorylation on Thr-308 and Ser-473 is already well established. Preparation of phosphorylation site-specific antibodies will be very useful in demonstrating the stoichiometry of phosphorylation on these two sites. These antibodies can be used to evaluate how these novel sites respond to stimulation of cells by growth factors and to treatment with phosphatidylinositol 3-kinase inhibitors such as wortmannin and LY-294002.

Use of tandem mass spectrometric analysis for identifying the specific amino acids phosphorylated in cells has become common in recent years. This approach may be used to further verify the phosphorylation sites (Thr-72 and Ser-246) of Akt in cells after expression of transfected wild-type Akt and Akt mutants as well as these sites in cells that express endogenous Akt after stimulation. The specificity and efficiency of Akt autophosphorylation on these two sites may also be verified by in vitro peptide competition assays. Synthetic peptides containing Thr-72 and Ser-246 could not only be tested in competition with the wild-type Akt when incubated with pre-activated Akt recombinant protein during in vitro kinase assays but may also be used to ascertain whether they serve effectively as substrates by comparing phosphorylation rates to the classic GSK3 cross-tide that we used in the experiment illustrated in Fig. 3. These approaches will also provide valuable information on the stoichiometry of phosphorylation on these two predicted sites and will allow comparison with the well established phosphorylation stoichiometry of Thr-308 and Ser-473. Lastly, additional studies may include investigation of the location of these two sites in the crystal structure of the PH domain and the kinase domain to establish the likelihood of these domains being available or exposed for phosphorylation.

In conclusion, the results from our pilot studies suggest that Akt regulates its kinase activity through autophosphorylation in trans. Our findings warrant further investigation of this potential important mechanism of Akt kinase regulation.

Acknowledgment—We thank Elizabeth L. Hess of the Department of Scientific Publication of M.D. Anderson Cancer Center for editorial assistance.

REFERENCES

1. Sun, M., Paciga, J. E., Feldman, R. L., Yuan, Z., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2001) Cancer Res. 61, 5985–5991
2. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Panici, P. B., Mancuso, S., Neri, G., and Testa, J. R. (1995) Int. J. Cancer 61, 280–285
3. Sun, M., Wang, G., Paciga, J. E., Feldman, R. L., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tischler, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) Am. J. Pathol. 159, 431–437
4. Brognard, J., Clark, A. S., Ni, Y., and Dennis, P. A. (2001) Cancer Res. 61, 3986–3997
5. Ringel, M. D., Hayre, N., Saito, J., Sanbier, B., Schuppert, F., Burch, H., Bernet, V., Burman, K. D., Kohl, L. D., and Saji, M. (2001) Cancer Res. 61, 6105–6111
6. Perugini, R. A., McCade, T. P., Vittimberga, F. J., Jr., and Callery, M. P. (2000) J. Surg. Res. 90, 39–44
7. Altomare, D. A., Tanno, S., De Bianco, A., Klein-Szanto, A. J., Tanno, S., Skele, K. L., Hoffman, J. P., and Testa, J. R. (2003) J. Cell. Biochem. 88, 470–476
8. Alkan, S., and Iban, K. F. (2002) Blood 99, 2278–2279
9. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tischler, P. N. (1995) Cell 81, 727–736
10. Coffey, P. J., Jin, J., and Woodgett, J. R. (1998) Biochem. J. 335, 1–13
11. Nicholson, K. M., and Anderson, N. G. (2002) Cell Signal. 14, 381–395
12. Alessi, D. R. (2001) Biochem. Soc. Trans. 29, 1–14
13. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reeve, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–269
14. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reeve, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
15. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reeve, C. B., McCormick, F., Tempst, P., Coldwell, J., and Hawkins, P. T. (1998) Science 279, 710–714
16. Alessi, D. R., Andjelkovic, M., Cadwallader, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) EMBO J. 15, 6541–6551
17. Rane, M. J., Coxon, P. Y., Powell, D. W., Webster, R., Klein, J. B., Pierce, W., Ping, P., and McLeish, K. R. (2001) J. Biol. Chem. 276, 3517–3523
18. Delcomenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11211–11216
19. Feng, J., Park, J., Cron, P., Hess, D., and Hemmings, B. A. (2004) J. Biol. Chem. 279, 41189–41196
20. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Science 307, 1098–1101
21. Chen, R., Kim, O., Yang, J., Sato, K., Eisenmann, K. M., McCarthy, J., Chen, H., and Qiu, Y. (2001) J. Biol. Chem. 276, 31838–31862
22. Smith, A. J., Francis, S. H., and Corbin, J. D. (1993) Mol. Cell. Biochem. 127–128, 51–70
23. Toker, A., and Newton, A. C. (2000) J. Biol. Chem. 275, 8271–8274
24. Alessi, D. R., Cadwallader, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) FEBS Lett. 399, 333–338
25. Knuefmann, C., Lu, Y., Liu, B., Jin, W., Liang, K., Wu, L., Schmidt, M., Mills, G. B., Mendelsohn, J., and Fan, Z. (2003) Oncogene 22, 3205–3212
26. Liu, B., Fang, M., Schmidt, M., Lu, Y., Mendelsohn, J., and Fan, Z. (2000) Br. J. Cancer 82, 1991–1999
27. Kane, S., Sano, H., Liu, S. C., Asara, J. M., Lane, W. S., Garner, C. C., and Lienhard, G. E. (2002) J. Biol. Chem. 277, 22115–22118
28. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tischler, P. (1998) Oncogene 17, 313–325
29. Andjelkovic, M., Maira, S. M., Cron, P., Parker, P. J., and Hemmings, B. A. (1999) Mol. Cell. Biol. 19, 5061–5072
30. Carter, M. D., Southwick, K., Lukov, G., Willardson, B. M., and Thulin, C. D. (2004) J. Biol. Chem. 15, 257–264

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 281 • NUMBER 19

MAY 12, 2006

13843